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European Journal of Pharmacology 440 (2002) 235–254

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Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity

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Received 1 October 2001; accepted 12 November 2001

Abstract

Ghrelin, a 28-amino acid acylated peptide predominantly produced by the stomach, displays strong growth hormone (GH)-releasing activity mediated by the hypothalamus–pituitary GH secretagogue (GHS)-receptors specific for synthetic GHS. The discovery of ghrelin definitely changes our understanding of GH regulation but it is also already clear that ghrelin is much more than simply a natural GHS. Ghrelin acts also on other central and peripheral receptors and shows other actions including stimulation of lactotroph and corticotroph secretion, orexia, influence on gastro-entero-pancreatic functions, metabolic, cardiovascular and anti-proliferative effects. GHS were born more than 20 years ago as synthetic molecules suggesting the option that GH deficiency could be treated by orally active GHS as an alternative to recombinant human GH (rhGH). Up to now, this has not been the case and also their usefulness as anabolic anti-aging intervention restoring GH/insulin-like growth factor-I axis in somatopause is still unclear. We are now confronted with the theoretical possibility that GHS analogues could become candidate drugs for treatment of pathophysiological conditions in internal medicine totally unrelated to disorders of GH secretion. Particularly, GHS receptor agonists or antagonists acting on appetite could represent new drug intervention in eating disorders.

Keywords: Ghrelin; Growth hormone secretagogue; Pituitary hormone; Food intake, control of; Metabolic fuel; Obesity

1. Introduction

Ghrelin is a 28-amino acid peptide predominantly produced by the stomach, with substantially lower amounts derived from bowel, pancreas, kidney, placenta, pituitary and hypothalamus (Kojima et al., 1999; Date et al., 2000; Mori et al., 2000; Gualillo et al., 2001a; Korbonits et al., 2001a; Volante et al., 2002).

Ghrelin displays strong growth hormone (GH)-releasing activity mediated by the activation of the type 1a GH Secretagogue (GHS1a) receptor which had been shown specific for a family of synthetic, peptidyl and non-peptidyl, GH Secretagogues (GHS) (Smith et al., 1997, 2001; Kojima et al., 1999; Arvat et al., 2000b, 2001; Bednarek et al., 2000; Takaya et al., 2000; Deghenghi et al., 2001; Ghigo et al., 2001; Muccioli et al., 2001).

GHS receptors are concentrated in the hypothalamus–pituitary unit but also distributed in other central and peripheral tissues (Smith et al., 1997; Ghigo et al., 2001; Muccioli et al., 1998a,b, 2000; Papotti et al., 2000; Cassoni et al., 2001). Indeed, besides potent GH-releasing actions, ghrelin as well as synthetic GHS has other remarkable activities including: (a) stimulation of lactotroph and corticotroph secretion; (b) orexant activity coupled with control of energy expenditure; (c) influence on sleep; (d) control of gastric motility and acid secretion; (e) influence on the endocrine pancreatic function and glucose metabolism; (f) cardiovascular actions; (g) anti-proliferative effects in neoplastic cell lines (Smith et al., 1997; Kamegai et al., 2000, 2001; Masuda et al., 2000; Muccioli et al., 2000; Tschöp et al., 2000; Wren et al., 2000; Arvat et al.,

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0014-2999/02/$ - see front matter
PII: S0014-2999(02)01432-2

2001; Broglio et al., 2001; Cassoni et al., 2001; Date et al., 2001; Ghigo et al., 2001; Nagaya et al., 2001a; Nakazato et al., 2001; Wren et al., 2001).

Less than 2 years after the discovery of ghrelin, literature reports an impressive number of major contributions; among them, considerable focuses on the central orexant activity and the endocrine and non-endocrine, gastro-entero-pancreatic and metabolic actions. Indeed there is already clear evidence that ghrelin is an hormone signaling the metabolic balance and managing the neuroendocrine and metabolic response to starvation; to this goal ghrelin might be complementary to leptin in informing the central nervous system (CNS) about the status of energy balance.

Obesity and related disorders are among the leading causes of illness and mortality in the developed world (Flier and Foster, 1998). To better understand the pathophysiological mechanisms that underlie metabolic disorders, increasing attention has been paid to central regulatory elements in energy homeostasis, including food intake and energy expenditure (Flier and Foster, 1998). The past two decades have provided overwhelming evidence of the critical role that hypothalamic peptidergic systems play in the central regulation of appetite and metabolism (Flier and Foster, 1998). The discovery of ghrelin and its influence on appetite, fuel utilization, body weight and body composition adds yet another component to the complexity in the central regulation of energy balance.

2. Historical milestones of synthetic and natural GH secretagogues

Ghrelin, a gastric hormone, has been discovered as a natural ligand of the orphan GHS1a receptor which, in turn, had been shown specific for synthetic GHS (Smith et al., 1997, 2001; Kojima et al., 1999; Bednarek et al., 2000; Deghenghi et al., 2001; Ghigo et al., 2001; Muccioli et al., 2001). Thus, ghrelin discovery is an example of reversed pharmacology starting from synthetic analogues and arriving to the natural ligand via the discovery of the natural receptor.

Synthetic GHS are a family including many peptidyl and non-peptidyl molecules (Smith et al., 1997; Bowers, 1999; Ghigo et al., 2001). The first molecules were non-natural peptides (GH-releasing peptides, GHRP) which were invented rather than isolated by Bowers and Momany in late 1970s as met-enkephalin derivatives devoid of any opioid activity (Smith et al., 1997; Bowers, 1999).

GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2) was the first hexapeptide active to release GH in vivo, in humans even more than in animals; one most remarkable property was that GHRP-6 showed strong GH-releasing activity even after oral administration though with low bioavailability and short-lasting effect (Smith et al., 1997; Bowers, 1999; Ghigo et al., 2001). Aiming to select orally active molecules with better bioavailability and longer half lives, further research led to synthesis of other GHRP, and above all, to the discovery of orally active non-peptidyl molecules, the most representative of which is the spiroindoline L-163,191 (MK-0677) (Smith et al., 1997; Ghigo et al., 1998a, 2001; Bowers, 1999). MK-0677 possesses impressive bioavailability and is able to enhance 24 h GH secretion after single oral administration (Smith et al., 1997; Chapman et al., 1996; Ghigo et al., 1998a,c, 2001; Bowers, 1999; Arvat et al., 2000b). Such data explain why it became the candidate drug for treatment of GH deficiency in childhood and as orally active anabolic, anti-aging intervention in frail elderly subjects (Ghigo et al., 1998a,c; Arvat et al., 2000b).

Apart from clinical implications, MK-0677 allowed discovery and cloning of the GHS receptor, the existence of which had been indicated by binding studies (Smith et al., 1997; Bowers, 1999). Studies focusing on the GHS receptor distribution showed particular concentration of GHS receptors in the hypothalamus–pituitary area but remarkable presence of specific binding sites also in other brain areas and peripheral, endocrine and non-endocrine animal and human tissues (Smith et al., 1997; Muccioli et al., 1998a,b, 2000; Papotti et al., 2000; Bluet-Pajot et al., 2001; Ghigo et al., 2001). Indeed this GHS receptor distribution explained its GH-releasing effect but also other endocrine and non-endocrine biological activities (Ghigo et al., 1999, 2001; Muccioli et al., 2000; Cassoni et al., 2001).

Based on this knowledge, it is less than 2 years ago that ghrelin was discovered by Japanese scientists involved in the cardiovascular field and attracted by the increasing evidence that synthetic GHS had cardiovascular activities.

Ghrelin is a 28 residue peptide predominantly produced by the stomach, while substantially lower amounts derive from bowel, pancreas, kidney, placenta, thyroid, pituitary and hypothalamus (Kojima et al., 1999; Date et al., 2000; Mori et al., 2000; Ariyasu et al., 2001; Gualillo et al., 2001a,b; Kanamoto et al., 2001; Korbonits et al., 2001a; Volante et al., 2002). Within the stomach, ghrelin is produced by the enteroendocrine cells, probably the X/A-like cells representing a major endocrine population in the oxyntic mucosa, the hormonal product of which had not previously been clarified (Kojima et al., 1999; Date et al., 2000; Dornonville de la Cour et al., 2001). Notably, ghrelin production has also been reported in gastric and intestinal carcinoids (Papotti et al., 2001) and in medullary thyroid carcinomas (Kanamoto et al., 2001).

Ghrelin is the first peptide isolated from natural sources in which the hydroxyl group of one of its serine residues is acylated by *n*-octanoic acid (Kojima et al., 1999). The acylation of the peptide had been supposed critical to cross the blood–brain barrier but is also essential for binding the GHS1a receptor and for its GH-releasing and other endocrine actions (Kojima et al., 1999; Bednarek et al., 2000; Matsumoto et al., 2001b; Muccioli et al., 2001). However, non-acylated ghrelin which circulates in amount far greater than the acylated form is not biologically inactive; it is able to exert some non-endocrine actions including cardiovascular and anti-proliferative effects probably binding different

GHS-receptor subtypes or receptor families (Cassoni et al., 2001; Date et al., 2000, personal unpublished observations).

There is also another endogenous ligand for the GHS1a receptor isolated from the stomach. It has been named Des-Gln14-ghrelin, has the same acylation in Ser3 and is homologous to ghrelin except one glutamine missing; it is the result of an alternative splicing of the ghrelin gene and possesses the same activity of ghrelin (Hosoda et al., 2000a).

Notice that the GHS receptor is bound also by other molecules such as adenosine, which is not able to activate the receptor, and cortistatin, a neuropeptide homologous to somatostatin which in turn is unable to recognize GHS1a receptor (Tullin et al., 2000; Deghenghi et al., 2001; Smith et al., 2001). It has been suggested that different molecules are able to bind different pockets of the GHS receptor but not necessarily to activate it; however, further studies are required to clarify whether ghrelin is the sole ligand or one of a number of ligands activating the GHS receptor and whether the GHS receptor used for ghrelin isolation is the sole receptor or one of a group of receptors for such ligands.

The GHS story led to the discovery of the motilin receptor which is a member of the GHS receptor family having 52% identity (Smith et al., 2001). Human ghrelin and motilin have 36% identity and pre-pro-motilin related peptide produced by the enteroendocrine cells of the stomach is fully identical with human pre-pro-ghrelin except that Serine 26 that is not octanoylated in pre-pro-motilin related peptide (Kojima et al., 1999; Tomasetto et al., 2000; Asakawa et al., 2001; Folwaczny et al., 2001). It is impressive that ghrelin and motilin share not only structural similarities but also biological activities; however, though able to stimulate GH secretion and to exert orexigenic effect, motilin does not bind the GHS1a receptor (Asakawa et al., 2001; Folwaczny et al., 2001; Smith et al., 2001). Thus, ghrelin and motilin represent a novel family of gastrointestinal peptides contributing to the regulation of diverse functions of the gut–brain axis. (Folwaczny et al., 2001).

The regulation of ghrelin secretion is still largely unknown. However, it is already clear that in humans circulating ghrelin levels are decreased in chronic (obesity) and acute (overfeeding) (Ravussin et al., 2001; Tschöp et al., 2001a,b) states of positive energy balance, while plasma levels of ghrelin are increased by fasting and in patients with anorexia nervosa (Toshinai et al., 2001; Ariyasu et al., 2001). Pre-meal rise of circulating ghrelin levels suggests its role as a hunger signal triggering meal initiation and this signal could be mediated by GHS receptor subtypes (Cummings et al., 2001).

3. Ghrelin and GHS receptor(s) distribution

In addition to the physiological stimulation by hypothalamic growth hormone-releasing hormone (GHRH), the release of GH from the pituitary is stimulated by small synthetic peptidyl and non-peptidyl molecules called GHS (see for reviews Camanni et al., 1998; Bowers, 1999; Casanueva and Dieguez, 1999). They act through a specific G-protein coupled receptor (Howard et al., 1996), the GHS receptor, for which the ligand was unknown until a Japanese group of scientists (Kojima et al., 1999) isolated an endogenous ligand specific for GHS receptor, ghrelin, from the stomach. The discovery of this novel gastric hormone, ghrelin, which consists of 28 residues containing an *n*-octanoyl modification at Serine 3, has been recently reviewed by Bowers (2001), Kojima et al. (2001) and Inui (2001).

The GHS receptor is expressed by a single gene found at chromosomal location 3q26.2 (McKee et al., 1997). Two types of GHS receptor complementary DNAs (cDNA) that are presumably the result of alternate processing of a pre-mRNA have been identified and designated receptor 1a and 1b (see for reviews Smith et al., 1997, 1999 and references in Petersenn et al., 2001). Their sequences do not shown significant homology with other known receptors; the closest relatives are the neurotensin receptor and the motilin receptor type 1A, with 59% and 52% similarity, respectively. cDNA 1a encodes a receptor, named GHS1a receptor, of 366 aminoacids with seven-transmembrane regions and a molecular mass of approximately 41 kDa. The 1b cDNA encodes a shorter form, named GHS1b receptor, which consists of 289 aminoacids with only five-transmembrane regions. The human GHS1a receptor shares 96% and 93% identity with the rat and pig GHS1a receptor, respectively, and the existence of this receptor can apparently be extended to pre-Cambrian times as amino acid sequences strongly related to human GHS1a receptor have been identified in teleost fish (Smith et al., 2001). These observations strongly suggest that the GHS1a receptor is highly conserved across the species and plays a fundamental role.

The binding of ghrelin and synthetic GHS (such as the peptidyl, GHRP-6 and the non-peptidyl derivative, MK-0677) to the GHS1a receptor activates the phospholipase C signalling pathway, leading to increased inositol phosphate turnover and protein kinase C activation, followed by the release of Ca^{2+} from intracellular stores (Smith et al., 1997; Kojima et al., 2001). GHS receptor activation also leads to an inhibition of K^+ channels, allowing the entry of Ca^{2+} through voltage-gated L- and T-type channels (Chen et al., 1996; Casanueva and Dieguez, 1999). Differently from the GHS1a receptor, the GHS1b receptor failed to bind GHS and to respond to GHS (Howard et al., 1996) and its functional role remains to be defined. Synthetic GHS and ghrelin, as well as des-Gln^{14}-ghrelin, a natural isoform that has the same GH-releasing activity of ghrelin (Hosoda et al., 2000a), bind with high affinity to the GHS1a receptor; their efficacy in displacing [^{35}S]MK-0677 or [^{125}I][Tyr^4]ghrelin binding to pituitary membranes correlates well with concentrations required to stimulate GH release (Smith et al., 1997; Hosoda et al., 2000a; Muccioli et al., 2001). The *n*-octanoyl group at Serine 3 of the ghrelin molecule seems to

be essential for the hormone's binding and bioactivity at least in terms of GH release. In fact, the non-acylated ghrelin that circulates in amount far greater than the acylated form (Hosoda et al., 2000b) does not displace radiolabelled ghrelin from its hypothalamic or pituitary binding sites (Muccioli et al., 2001) and has no GH-releasing or other endocrine activities in rat (Kojima et al., 1999; Bowers, 2001) and in man (Ghigo et al., submitted for publication). Recent studies, dealing with the minimal sequence of ghrelin needed to activate the GHS1a receptor, have shown, in HEK-293 cells transfected with the human GHS1a receptor that short octanoylated peptides encompassing the first four to five residues of ghrelin were capable to increase intracellular Ca^{2+} almost as efficiently as the full-length ghrelin (Bednarek et al., 2000; Matsumoto et al., 2001a). Based on these in vitro results, it has been postulated that the ''active core'' required for the activation of the receptor is the Gly-Ser-Ser(*n*-octanoyl)-Phe sequence. However, the ability of the above ghrelin derivatives to activate the GHS1a receptor in transfected cells seems not indicative of their capability to stimulate GH secretion from somatotroph cells. In fact, we have recently demonstrated that octanoylated ghrelin-(1–4) or octanoylated ghrelin-(1–8) are unable to stimulate GH release in rats and neither of these two truncated molecular forms of ghrelin are effective in displacing [^{125}I][Tyr^4]ghrelin from its binding sites in membrane preparations from human hypothalamus or pituitary gland (Torsello et al., in press). Possibly, overexpression of the GHS1a receptor, or lack of the other receptor populations, physiologically present in pituitary cells, may be responsible for the reported activity of ghrelin analogs in HEK-293 cells. Other authors working on the same cells expressing human or pig GHS1a receptor have found that also adenosine activate the transfected receptor but, similarly to short ghrelin analogs, does not possess a biological counterpart being unable to stimulate GH secretion and amplify the GHRH effects on normal pituitary cell cultures (Tullin et al., 2000). It has been suggested that adenosine is a partial agonist of the GHS1a receptor and binds to a receptor site distinct from the binding pocket recognized by MK-0677 and GHRP-6 (Smith et al., 2000). More recently, we have reported (Deghenghi et al., 2001) that the GHS receptor is bound also by another endogenous molecule such as cortistatin, a neuropeptide homologous to somatostatin which in turn is unable to recognize GHS1a receptor. This finding supports the hypothesis that other natural ligands, other than ghrelin and adenosine, could modulate the activity of the GHS receptor.

Expression of the GHS1a receptor was shown in the hypothalamus and anterior pituitary gland (Howard et al., 1996; Yokote et al., 1998; Shuto et al., 2001) consistent with its role in regulating GH release. GHS1a receptor is largely confined in somatotroph pituitary cells and in the arcuate nucleus (Howard et al., 1996; Smith et al., 1997; Willesen et al., 1999), an hypothalamic area that is crucial for the neuroendocrine and the appetite-stimulating activities of ghrelin and synthetic GHS (Bluet-Pajot et al., 2001; Shintani et al., 2001). This is supported by the demonstration that ghrelin, as well as synthetic GHS, effectively stimulates the expression of some markers of neural activity (c-*fos* and early growth response factor-1) in the arcuate nucleus neurons (Dickson and Luckman, 1997; Hewson and Dickson, 2000). The activated hypothalamic cells include GHRH-containing neurons, but also cells containing the appetite stimulating neuropeptide Y (Willesen et al., 1999; Tannenbaum and Bowers, 2001) and an endogenous melanocortin receptor antagonist such as the Agouti-related protein (AGRP) (Kamegai et al., 2000). Less abundant but detectable levels of GHS1a receptor mRNA were also demonstrated in various extra-hypothalamic areas such as the dentate gyrus of the hippocampal formation, CA2 and CA3 regions of the hippocampus, the pars compacta of the substantia nigra, ventral tegmental area and dorsal and medial raphe nuclei and Edinger-Westphal nucleus, pons and medulla oblongata (Guan et al., 1997; Muccioli et al., 1998a; Katayama et al., 2000), possibly indicating its involvement in as yet undefined physiological extra-neuroendocrine actions. More recent localization studies have demonstrated that GHS1a receptor is also expressed in multiple peripheral organs. GHS1a receptor mRNA was shown in the stomach and intestine (Date et al., 2000), pancreas (Guan et al., 1997), kidney (Mori et al., 2000), heart and aorta (Nagaya et al., 2001a), as well as in different human pituitary adenomas (Korbonits et al., 2001a,b; Kim et al., 2001) and various endocrine neoplasms of lung (de Keyzer et al., 1997), stomach (Papotti et al., 2001) and pancreas (Korbonits et al., 1998, 2001b; Volante et al., 2002). These data are in keeping with the reported observations indicating, for ghrelin and synthetic GHS, broader functions beyond the control of GH release and food intake (see Section 5).

Ghrelin and all the GHS compounds developed so far seem to exhibit a high binding affinity to the cloned GHS1a receptor. However, there is strong evidence suggesting the existence of additional receptor subtypes which may exhibit different affinities for these compounds. In fact, specific binding sites for Tyr-Ala-hexarelin [Tyr-Ala-His-D-2Methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2] and other peptidyl GHS (GHRP-2 [D-Ala-D-βNal-Ala-Trp-D-Phe-Lys-NH_2], GHRP-6 and hexarelin [His-D-2Methyl-Trp-Ala-Trp-D-Phe-Lys-NH_2]), with a density more remarkable or at least overlapping to the pituitary, have been found in rat (Ong et al., 1998; Bodart et al., 1999) and human heart (Muccioli et al., 1998b, 2000), as well as in a wide range of other non-endocrine peripheral human tissues such as lung, arteries, skeletal muscle, kidney, and liver (Papotti et al., 2000; Ghigo et al., 2001). These binding sites are presumably different from the GHS1a receptor because they show a very low binding affinity for ghrelin and the non-peptidyl GHS MK-0677 (Papotti et al., 2000). Furthermore, as reported by Bodart et al. (1999), the cardiac GHS receptor has a molecular mass larger (84 kDa) than that of GHS1a

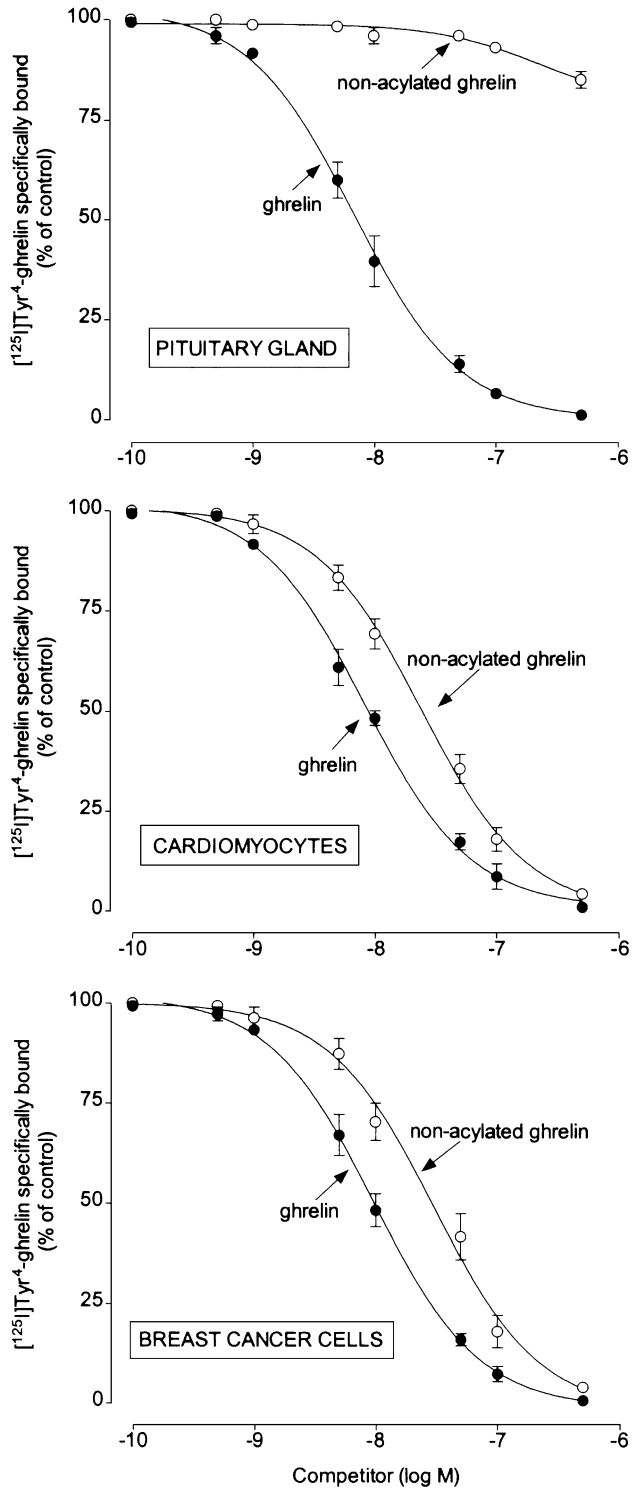

Fig. 1. Displacement of radiolabelled human ghrelin from membranes of human pituitary gland, rat H9C2 cardiomyocytes and human MCF7 mammary carcinoma cells by unlabelled ghrelin and non-acylated ghrelin. The ordinate represents binding as a percentage of control (specific binding in the absence of unlabelled competitor): values are mean ± S.E.M. of four separate experiments.

receptor and shows no homology with this receptor. The predicted amino acid sequence of the heart GHS receptor is similar to that of CD 36, a multifunctional receptor also known as glycoprotein IV (Bodart et al., personal communication). The functional significance of the peptidyl GHS receptor in peripheral non-endocrine tissues is still unknown. Some findings suggest, however, that at least in the cardiovascular system, these binding sites could mediate GH-independent cardioprotective activities of peptidyl GHS (see Section 5 below). Recently, we have demonstrated that specific GHS receptors, exhibiting a binding profile different from the GHS1a receptor, are present in human thyroid, breast and lung tumours and related cancer cell lines, in which synthetic GHS and analogs cause inhibition of cell proliferation (see Section 5). It is likely that further subtypes of GHS receptors will be cloned, since there are other studies indicating the possible existence of a wide spread of ghrelin/GHS receptor subtypes different from the already cloned one. In agreement with this assumption, there is the fact that not all synthetic peptidyl GHS (GHRP-6, hexarelin and many its analogs) show the same neuroendocrine and extra-neuroendocrine activities (Chen, 2000; Melis et al., 2001; Torsello et al., 2000; Toth et al., personal communication) and that the non-acylated ghrelin, although is unable to bind the hypothalamo-pituitary GHS1a receptor, exerts antiproliferative (Cassoni et al., 2001) and cardioprotective effects (Graziani et al., submitted for publication), interacting with a specific receptor, common for ghrelin and non-acylated ghrelin, in cardiac muscle and breast cancer cells. This is illustrated in the experiment of Fig. 1 that compares the ability of unlabelled ghrelin and non-acylated ghrelin to displace [^{125}I][Tyr^4]ghrelin binding to membranes from cultured pituitary explants, H9C2 cardiomyocytes and MCF-7 mammary carcinoma cells (Muccioli et al., unpublished observations).

4. Endocrine activities of synthetic and natural GHS

4.1. GH-releasing activity

Ghrelin as well as synthetic GHS possesses strong and dose-related GH-releasing activity which is more marked in humans than in animals (Smith et al., 1997; Kojima et al., 1999; Arvat et al., 2000a, 2001; Peino et al., 2000; Seoane et al., 2000; Takaya et al., 2000; Ghigo et al., 2001; Hataya et al., 2001).

GHS and GHRH have a synergistical effect indicating that they act, at least partially, via different mechanisms (Smith et al., 1997; Bluet-Pajot et al., 2001; Ghigo et al., 2001; Tannenbaum and Bowers, 2001). Nevertheless, GHS need GHRH activity to fully express their GH-releasing effect and probably act triggering GHRH-secreting neurons (Smith et al., 1997; Bluet-Pajot et al., 2001; Ghigo et al., 2001; Tannenbaum and Bowers, 2001). In humans, the GH response to GHS is strongly inhibited, though not abol-

ished, by a GHRH receptor antagonist as well as by hypothalamo-pituitary disconnection (Popovic et al., 1995; Pandya et al., 1998) in agreement with the assumption that the most important action of GHS takes place at the hypothalamic level (Smith et al., 1997; Bluet-Pajot et al., 2001; Ghigo et al., 2001). Moreover, patients with GHRH-receptor deficiency show no GH response to GHRP which maintain their stimulatory effect on PRL, adenocorticotropin hormone (ACTH) and cortisol secretion (Maheshwari et al., 1999).

GHS probably act also as functional somatostatin antagonists both at the pituitary and the hypothalamic level (Ghigo et al., 2001; Tannenbaum and Bowers, 2001). In humans, the GH response to GHS is not modified by substances acting via somatostatin inhibition (such as acetylcholine receptor agonists, arginine) which, in turn, truly potentiate the GHRH-induced GH rise (Ghigo et al., 2001). Moreover, the GH-releasing activity of GHS is partially refractory to the inhibitory effect of substances acting via stimulation of hypothalamic somatostatin (such as acetylcholine receptor antagonists, β-adrenoceptor agonists, glucose) which, in turn, almost abolish the somatotroph responsiveness to GHRH (Ghigo et al., 2001). Indeed GHS are partially refractory to the inhibition of substances acting on somatotroph cells such as free fatty acids and even to exogenous somatostatin (Ghigo et al., 2001). GHS are also partially refractory to the negative GH autofeedback (Ghigo et al., 2001) and show peculiar sensitivity to the negative Insulin-like Growth Factor I (IGF-I) feedback action (Ghigo et al., 2001).

The GH-releasing effect of GHS undergoes marked age-related variations increasing at puberty, persisting similar in adulthood and decreasing with age (Arvat et al., 2000b; Ghigo et al., 2001). The mechanisms underlying the age-related variations in the GH-releasing activity of GHS differ by age. For instance, the enhanced GH-releasing effect of GHS at puberty reflects positive influence of estrogen which could trigger an increase in GHS receptor expression (Arvat et al., 2000b; Ghigo et al., 2001). However, estrogen insufficiency does not explain the reduced GH response to GHS in postmenopausal women (Arvat et al., 2000b; Ghigo et al., 2001). In agreement with the reduction in hypothalamic GHRP receptors in human aging brain (Arvat et al., 2000b; Ghigo et al., 2001), the GH response to hexarelin in elderly subjects is further increased but not restored by supramaximal doses (Arvat et al., 2000b; Ghigo et al., 2001). The most important mechanism accounting for reduced GH-releasing activity of GHS in aging is probably represented by age-related variations in the neural control of somatotroph function including GHRH hypoactivity and somatostatinergic hyperactivity (Arvat et al., 2000b; Ghigo et al., 2001). On the other hand, it has also been hypothesised that declining GH secretion would reflect age-related decrease in the activity of the endogenous GHS ligand, i.e. ghrelin (Bowers, 2001). This hypothesis remains to be verified.

GHS could theoretically have diagnostic and therapeutic usefulness based on their strong and reproducible GH-releasing effect even after oral administration.

Particularly when combined with GHRH, GHS represent one of the most potent and reliable test to evaluate the pituitary GH releasable pool for the diagnosis of GH deficiency (Leal-Cerro et al., 1995; Ghigo et al., 1998c; Popovic et al., 2000). Testing with GHS is as sensitive and specific as insulin tolerance test and GHRH + arginine, the two golden standard tests for the diagnosis of GH deficiency, provided that appropriate cut-off limits are assumed (Ghigo et al., 1998c; Popovic et al., 2000).

The potential usefulness of GHS as growth promoting factor in children with GH Deficiency seems unlikely based on the results of some trials showing that their efficacy is not comparable with that of recombinant human GH (rhGH) (Yu et al., personal communication).

On the other hand, the GHS would represent anabolic treatment in frail elderly subjects with somatopause based on the following evidence: (a) the age-related reduction in the activity of GH/IGF-I axis probably accounts for changes in body composition, structure functions and metabolism in normal elderly subjects which are remarkably similar to (but of lesser extent than) those in GH deficiency adults (Corpas et al., 1993; Ghigo et al., 1996); (b) the pituitary GH releasable pool is still remarkable in aged subjects (Ghigo et al., 1996) and (c) GH-releasing substances would represent a more physiological approach to increase endogenous GH pulsatility (Corpas et al., 1993; Ghigo et al., 1996). GHRH needs to be administered parenterally while GHS are active even after oral administration (Ghigo et al., 1998a,c); among GHS, the non-peptidyl spiroindoline MK-0677 was the most promising candidate showing impressive bioavailability and long lasting effect after single oral daily administration (Smith et al., 1997).

So far, the following results have been obtained by trials testing the effects of chronic treatment with MK-0677: (a) in elderly subjects it restores IGF-I levels in the normal young range indicating successful enhancement of somatotroph secretion (Chapman et al., 1996); (b) in elderly subjects it increases rapid eye movements (REM) sleep while decreases REM latency, thus counteracting alterations in sleep pattern that are hallmarks of brain aging (Copinschi et al., 1997; Van Cauter et al., 1998); (c) it reverses diet-induced catabolism in young volunteers indicating anabolic effect (Murphy et al., 1998) while increases fat-free mass and energy expenditure in obese patients (Svensson et al., 1998); (d) in a large population of postmenopausal osteoporotic women, 1 year treatment with MK-0677 alone and in combination with alendronate, a bisphosphonate, attenuates the indirect suppressive effect of alendronate on bone formation but does not translate into significant increases in bone mass density at sites other than the femoral neck (Murphy et al., 2001). In all, at present, there is no definitive evidence showing the therapeutic efficacy of GHS as anabolic agents acting via rejuvenation of GH/IGF-I axis

in elderly subjects and further studies are needed to clarify this hypothesis.

4.2. PRL- and ACTH-releasing activity of GHS

The activity of both ghrelin and synthetic GHS is not fully specific for GH and includes stimulatory effect on both lactotroph and corticotroph secretion (Arvat et al., 2000b; Ghigo et al., 2001).

The stimulatory effect of GHS on PRL secretion in humans is slight, independent of both gender and age and probably comes from direct stimulation of somatomammotroph cells (Arvat et al., 2000b; Ghigo et al., 2001).

The stimulatory effect of GHS on the activity of hypothalamus–pituitary–adrenal axis in humans is remarkable and similar to that after naloxone, vasopressin and even corticotropin-releasing hormone (CRH) but is an acute neuroendocrine effect probably vanishing during prolonged treatment (Ghigo et al., 1998b, 2001; Bowers, 2001).

The ACTH-releasing activity of GHS is independent of gender but shows peculiar age-related variations. It increases at puberty, then shows a reduction in adulthood, and again, a trend toward increase in aging when the GH response to these molecules is clearly reduced (Ghigo et al., 1998b, 2001; Arvat et al., 2000b). Such evidence suggests that these molecules act at different levels and/or on different receptor subtypes (Smith et al., 1997; Ghigo et al., 1998b, 2001; Ong et al., 1998).

In physiological conditions, the ACTH-releasing activity of GHS totally depends on CNS-mediated mechanisms (Ghigo et al., 1998b, 2001). These mechanisms could include CRH- or vasopressin-mediated actions (Ghigo et al., 1998b, 2001) though the possibility that they act via neuropeptide-Y, γ-aminobutyric acid (GABA) or the putative endogenous ligand has also been hypothesized (Ghigo et al., 1998b, 2001). The ACTH response to GHS is generally sensitive to the negative cortisol feedback mechanism (Ghigo et al., 1998b, 2001). However, the stimulatory effect of GHS on corticotroph secretion is exaggerated and higher than that to human CRH in patients with pituitary ACTH-dependent Cushing's disease probably reflecting peculiar action directly on the pituitary ACTH-secreting tumor cells (Ghigo et al., 1998b, 2001; Korbonits et al., 1998).

5. Peripheral activities of synthetic and natural GHS

In agreement with the existence of specific receptors for natural (ghrelin) and synthetic GHS receptor in peripheral tissues (see above), peripheral endocrine and non-endocrine activities of these substances have been recently demonstrated. Besides potent GH-releasing effect, ghrelin, as well as synthetic GHS, has other remarkable effects including: control of gastric motility and acid secretion, influence on endocrine pancreatic function, glucose metabolism, cardiovascular actions and antiproliferative effects in neoplastic thyroid, mammary and lung cell lines (Fig. 2).

5.1. Gastro-entero-pancreatic actions

It is not surprising that a gastric hormone such as ghrelin acts at the gastro-entero-pancreatic level, where GHS1a receptor expression has been demonstrated (Guan et al., 1997; Date et al., 2000; Shuto et al., 2001; Volante et al., 2002). To be noted also is that there is a close structural relationship between motilin and ghrelin, as well as their respective precursor peptides (Asakawa et al., 2001) and that the gastrointestinal motilin receptor 1A and the GHS1a receptor show a high degree of structural homology (Smith et al., 2001); the similarity between activities of ghrelin and motilin has recently been emphasized (Del Rincon et al., 2001; Folwaczny et al., 2001). Ghrelin stimulates gastric acid secretion and motility in rats (Masuda et al., 2000) and circulating ghrelin levels are correlated with gastric emptying time in humans (Tschöp et al., 2001a). These actions are mediated by the cholinergic system as they are abolished by muscarinic blockade (Masuda et al., 2000); interestingly, the acetylcholine-mediated stimulatory effect of ghrelin on gastric acid secretion takes place, at least partially, at the central level (Date et al., 2001).

Ghrelin and GHS1a receptor mRNA are present also in normal (Guan et al., 1997) and neoplastic endocrine pancreas (Korbonits et al., 2001a,b; Papotti et al., 2001) and we have recently demonstrated that ghrelin induces significant hyperglycemia that is surprisingly followed by reduction in insulin secretion in humans (Broglio et al., 2001). Coupled with the observation that chronic treatment with GHS, particularly non-peptidyl derivatives, induced hyperglycemia in a considerable number of elderly subjects (Svensson et al., 1998), this evidence points to ghrelin as gastro-entero-pancreatic hormone exerting a significant role in the glucose metabolism and insulin secretion. These data suggest that ghrelin would integrate the hormonal and metabolic response to fasting which, at least in humans, is connoted by a clear-cut increase in GH secretion coupled with inhibition of insulin secretion and activation of mechanisms devoted to maintain glucose levels (Cryer and Polonsky, 1998).

5.2. Cardiovascular and hemodynamic effects

Specific binding sites for GHS are present in the cardiovascular system. The presence of GHS1a receptor mRNA has been demonstrated in rat aorta and heart (Nagaya et al., 2001a) and specific binding sites for ghrelin have been recently identified in rat heart and human arteries, where the density of ghrelin receptors is up-regulated with atherosclerosis (Katugampola et al., 2001). Furthermore, considerable specific binding of radiolabelled peptidyl GHS (such as [^{125}I]Tyr-Ala-hexarelin and [^{125}I]Tyr-benzoylphenylalanine-hexarelin) is well detectable in rat myocardium (Ong et

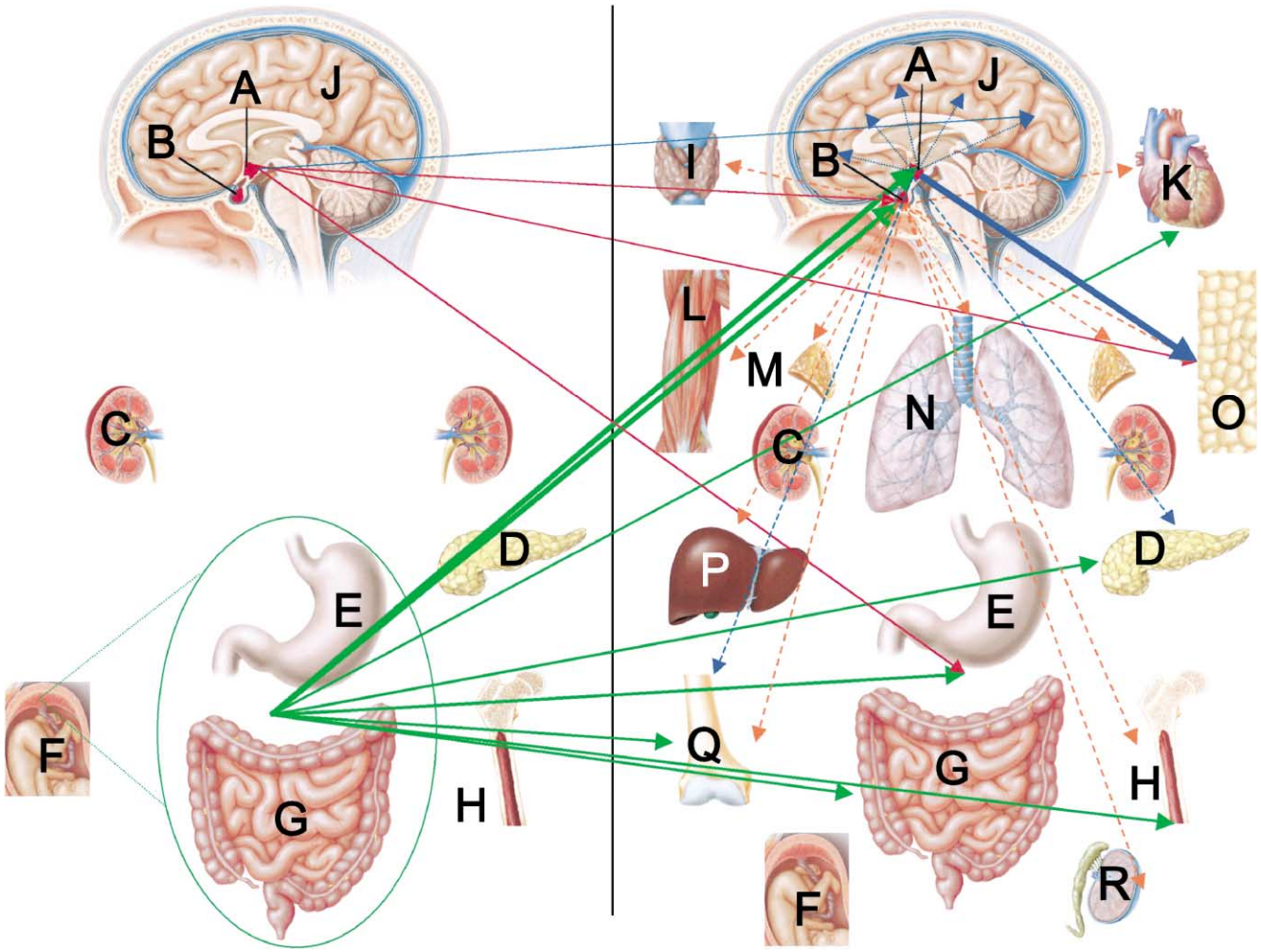

Fig. 2. This overview is a hypothetical model based on currently available data from rodent and clinical studies, extrapolating to human ghrelin physiology. In this schematic view, we attempt to emphasize the paradigm-shift from ghrelin as a gastric growth hormone secretagogue to ghrelin as a pleiotropic hormone that is generated by multiple different tissues. Ghrelin is evoking a broad variety of diverse, partially even antipodal, effects. Underlying physiological processes may include autocrine (in all organs presented in Fig. 2A and B) and paracrine signaling (not shown), as well as direct action on target cells and indirect effects mediated by the hypothalamus and the anterior pituitary. We did not include any ghrelin-related endocrine feedback-loops, since those are likely to exist but cannot yet be based on published evidence. Fig. 2A represents all tissues/organs that are known to express ghrelin mRNA and/or ghrelin-peptide (Date et al., 2000; Mori et al., 2000; Dornonville de la Cour et al., 2001; Gualillo et al., 2001a; Kojima et al., 1999; Horvath et al., 2001). Fig. 2B summarizes ghrelin targets that have been shown to either express the GHS receptor, which bind ghrelin peptide, or that are known to be affected by ghrelin actions (Papotti et al., 2000; Guan et al., 1997; Bowers, 2001; Horvath et al., 2001). Green arrows indicate direct effects from circulating ghrelin target tissue. Circulating plasma ghrelin is derived in its vast majority from the stomach and the small intestine (Kojima et al., 2001), however, the placenta might contribute additional ghrelin signals during pregnancy (Gualillo et al., 2001a). Dashed lines indicate indirect effects mediated via the hypothalamus (red) or via pituitary hormones (orange). Blue arrows indicate possible direct effects of hypothalamically expressed ghrelin. In (a): (A) hypothalamus, (B) pituitary, (C) kidney, (D) pancreas, (E) stomach, (F) placenta, (G) intestine, (H) bone marrow. In (b): (A) hypothalamus, (B) pituitary, (C) kidney, (D) pancreas, (E) stomach, (F) placenta, (G) intestine, (H) bone marrow, (I) thyroid, (J) brain, (K) heart, (L) muscle, (M) adrenals, (N) lungs, (O) adipose tissue, (P) liver, (Q) bone, (R) gonads.

al., 1998; Bodart et al., 1999) and in different human cardiovascular tissues (ventricles, atria, aorta, coronaries, carotid, endocardium and vena cava), with values often higher than those found in the pituitary gland (Muccioli et al., 1998b, 2000; Papotti et al., 2000). This binding is inhibited by unlabelled Tyr-Ala-hexarelin, hexarelin and other peptidyl GHS, but not by the non-peptidyl GHS MK-0677 and even by ghrelin (Papotti et al., 2000). Thus, these binding sites, which are not recognized by classical cardioactive substances, are probably GHS receptor subtypes distinct from the classical GHS1a receptor (see Section 3 above). Indeed, there is already evidence that cardiac GHS receptors mediate GH-independent cardioprotective effects of synthetic peptidyl GHS (Locatelli et al., 1999; Muccioli et al., 2000). The first report on the cardiovascular actions of GHS in rats appeared in 1997 (De Gennaro Colonna et al., 1997). These studies revealed that prolonged treatments with the peptidyl GHS hexarelin markedly protected against cardiovascular damage in GH-deficient rats with post-ischemic ventricular dysfunction. More recent reports revealed that hexarelin improves cardiac performances in rats after myocardial infarction (Tivesten et al., 2000), protects against

diastolic dysfunctions of myocardial stunning in isolated, perfused rabbit heart (Weekers et al., 2000) and enhances left ventricular contractility in pigs with dilated cardiomyopathy (King et al., 2001). On the other hand, acute administration of high dose peptidyl GHS was reported to induce clear though transient coronary vasoconstrition in perfused rat heart (Bodart et al., 1999). The protectant activity against ischemia seems peculiarly exerted by peptidyl GHS since this effect has never been confirmed by studies focusing on the effect of ghrelin.

In humans, the acute administration of hexarelin was found able to increase left ventricular ejection fraction in normal young volunteers (Bisi et al., 1999a), as well as in hypopituitaric patients with severe GH deficiency without any variations of mean blood pressure, heart rate and catecholamine levels (Bisi et al., 1999b). The same positive inotropic effect of hexarelin was found in patients with ischemic, but not in those with idiopathic dilated cardiomyopathy (Ghigo et al., 2001). Recently, it has been demonstrated that also ghrelin possesses cardiovascular activities. In fact, chronic subcutaneous ghrelin administration has been reported able to improve left ventricular dysfunction and to attenuate the development of left ventricular remodeling and cardiac cachexia in rats with chronic heart failure (Nagaya et al., 2001b). Moreover in humans, the administration of ghrelin in normal young volunteers is followed by reduction in cardiac afterload and increase in cardiac output without any change in heart rate (Nagaya et al., 2001a). This evidence indicates that the positive influence of GHS on cardiac contractility is probably mediated by the GHS1a receptor.

Ghrelin, as well as hexarelin, have been shown able to prevent cell death of cultured H9C2 cardiomyocytes and endothelial cells induced by either doxorubicin, serum withdrawal or activation of FAS (Filigheddu et al., 2001; Graziani et al., submitted for publication). Interestingly, the same cytoprotective effect of acylated ghrelin is shared by non-acylated ghrelin and both molecules stimulate in cultured cardiomyocytes survival intracellular signaling pathways, including tyrosine phosphorylation of intracellular proteins and activation of extracellular-signal-regulated kinase-1 and -2 and protein kinase B/AKT (Graziani et al., submitted for publication). As non-acylated ghrelin is generally unable to activate the GHS1a receptor (Bednarek et al., 2000) and stimulate GH release (Kojima et al., 1999), these data indicate that acylation of peptide is needed for endocrine actions only and that even the non-acylated ghrelin is a biologically active peptide. This evidence would imply the existence of another cardiac GHS receptor subtype, common for acylated and non-acylated ghrelin, whose activation mediates an antiapoptic effect in the cardiovascular system.

The cardiovascular activities of ghrelin and synthetic GHS suggest potential pharmaco-therapeutic implications. Theoretically, GHS analogs could be designed to protect from coronary ischemia and/or to prevent dilated cardiomyopathy improving cardiac performances and/or to reduce the progression of endothelial dysfunction and microangiopathy.

5.3. Antiproliferative activity

Specific binding sites for peptidyl and non-peptidyl GHS are present in normal and neoplastic human thyroid tissue. All follicular-derived thyroid carcinomas, but not parafollicular-derived neoplasms (medullary carcinomas) show GHS binding sites, even more than in non-tumoural thyroid. The presence of these binding sites was also demonstrated in different human thyroid tumour cell lines (follicular, papillary and anaplastic carcinoma cell lines). Synthetic peptidyl and non-peptidyl GHS inhibit the incorporation of [^{3}H]thymidine and cause inhibition of cell proliferation, which is evident at earliest time of treatment (24 h), in all the thyroid tumour cell lines. These findings were the first ones demonstrating that GHS inhibit tumour cell growth (Cassoni et al., 2000).

Interestingly, GHS receptors have been found even in tumoural tissues from organs which do not express these receptors in physiological conditions such as the breast (Cassoni et al., 2001). The presence of specific GHS receptors was shown in breast cancer, but not in fibroadenomas and normal mammary parenchyma. In breast tumours, the highest binding activity is present in well-differentiated invasive breast carcinomas and is progressively reduced in moderately to poorly differentiated tumours. GHS receptors are also present in both estrogen dependent (MCF7, T47D) and independent (MDA-MB231) breast cancer cell lines, in which ghrelin, synthetic GHS and EP-80317 (Haic-D-2Me-Trp-D-Lys-Trp-D-Phe-Lys-NH_2), an hexarelin analog devoid of any GH-releasing properties in vivo (Deghenghi, 2000; Locatelli et al., personal communication; Reismann et al., personal communication; Robinson et al., personal communication), cause inhibition of cell proliferation at concentrations close to their binding affinity. Like in the cardiovascular system, the same effect of acylated ghrelin is shared by the non-acylated molecule further indicating that even the non-acylated ghrelin is a biologically active peptide possessing also antiproliferative actions (Cassoni et al., 2001). As non-acylated ghrelin is generally unable to bind the GHS1a receptor, this evidence supports the possibility that the antiproliferative effects of acylated and non-acylated ghrelin on breast cancer cells seem to be mediated via a GHS receptor subtype distinct from the classical GHS1a receptor.

Our recent data indicate that neuroendocrine (carcinoid tumours) and non-endocrine (adenocarcinomas) lung tumours possess specific GHS binding sites which are also expressed by a non-endocrine human lung cancer cell line (CALU-1), the proliferation of which is inhibited only by synthetic peptidyl GHS and analogs such as EP-80317 (Ghè et al., 2002).

The antiproliferative effects of natural (ghrelin), synthetic GHS and analogs further show their multiple biological

activities and suggest the possibility that these substances would exert an antineoplastic action.

6. Ghrelin and the regulation of energy balance

Years before ghrelin was discovered, sporadic and greatly neglected reports on observations in rodents indicated that some growth hormone secretagogues might possess orexigenic activity (Locke et al., 1995; Okada et al., 1996; Torsello et al., 1998, 2000). Rumors about GHS-induced ravenous hunger attacks in children with idiopathic short stature occurring within the framework of clinical studies on GHS have never been officially confirmed. A research group led by S. Dickson had however gathered a substantial amount of very intriguing data during the last decade showing GHS-induced neuronal activity in hypothalamic areas that are currently considered the central processing unit controlling energy balance (Bailey et al., 1999; Dickson et al., 1993, 1994, 1995a,b, 1996, 1997; Dickson and Luckman, 1997; Honda et al., 1999; Luckman et al., 1999; Willesen et al., 1999). On those neurons, dense expression of the G-protein-coupled receptor has been shown, which is bound and activated by ghrelin as well as by other GHS and GHRP (Willesen et al., 1999; Howard et al., 1996; McKee et al., 1997). Still, it was a surprise to many, when ghrelin, the endogenous ligand of the GHS receptor (Kojima et al., 1999, 2001), emerged as one of the most powerful orexigenic and adipogenic agents known in mammalian physiology (Tschöp et al., 2000; Wren et al., 2000; Bowers, 2001; Inui, 2001). At first, it was puzzling to link a lipogenic hormone which had originally been discovered as a potent secretagogue of lipolytic hormone, GH (Tschöp et al., 2000; Salomon et al., 1989; Vernon, 1996). However, a collection of data started to make sense as an evolving mosaic drawn together by a new hormone called ghrelin, which derives from the stomach which is the first stop for digestion (Dornonville de la Cour et al., 2001; Kojima et al., 1999).

Ghrelin administration in rodents causes weight gain (Nakazato et al., 2001; Tschöp et al., 2000, 2002; Wren et al., 2001). This effect would not be as astonishing if it was reflected by longitudinal growth or at least by an increase in lean mass, effects that one would expect to occur after stimulation of GH-secretion (Lissett and Shalet, 2000). However, a still growing amount of data generated in rodents clearly showed that ghrelin-induced weight gain is based on accretion of fat mass without changes in longitudinal skeletal growth and without an increase in lean (muscle) mass (Tschöp et al., 2000). These findings have not only been confirmed by several groups but have also been repeated using synthetic ghrelin receptor (GHS receptor) agonists NNC 26-0161 (ipamorelin), GHRP-2, and GHRP-6 (Tschöp et al., 2002; Lall et al., 2001). Changes in body weight induced by ghrelin administration become significant in rodents after no more than 48 h and are self-evident at the end of 2 weeks (Tschöp et al., 2000, 2002). Changes in fat mass have been quantified using dual energy X-ray absorptiometry measurements specifically adapted for analysis of rodent body composition (Tschöp et al., 2000, 2002; Lall et al., 2001) as well as by measuring the weight of omental and retroperitoneal fat pads (Lall et al., 2001). Currently, it seems likely that the effects that are causing a positive energy balance are mediated via leptin-responsive neurons in specific regions of the hypothalamus (Nakazato et al., 2001; Shintani et al., 2001; Horvath et al., 2001; Kamegai et al., 2000; Spiegelman and Flier, 2001; Luckman et al., 1999; Tung et al., 2001; Tschöp et al., 2002). However, the possibility of direct effects of ghrelin on adipose tissue (where GHS receptor mRNA expression has been shown by polymerase chain reaction (PCR) (Kojima et al., 1999)) as well as effects on the hypothalamus–pituitary–adrenal axis (Ghigo et al., 2001; Wren et al., 2000); i.e. a ghrelin-induced Cushing's syndrome still have to be ruled out as possible phenomenon contributing to ghrelin-induced adiposity.

To find the physiological mechanism behind these observations, the rapidly evolving field of obesity research focusing on energy homeostasis (Spiegelman and Flier, 2001; Ahima and Osei, 2001; Kalra et al., 1999; Schwartz et al., 2000; Elmquist, 2000) has to be integrated with existing knowledge about GHS and their actions (Bowers, 1998, 2001; Giustina and Veldhuis, 1998). Energy balance is achieved when energy intake is equal to energy expenditure (Spiegelman and Flier, 2001). A positive energy balance, leading to weight gain, occurs when calories ingested, digested and re-absorbed exceeds calories expended (Spiegelman and Flier, 2001). Like leptin, but in an opposite manner, ghrelin administered in rodents influences both energy intake and metabolism (Horvath et al., 2001).

The earliest published data on orexigenic effects of GHS are from Locke et al. (1995) showing an increase in food intake after icv-administration in rats without affecting plasma GH response. Similar effects have been shown, by several other groups (Okada et al., 1996; Lall et al., 2001; Torsello et al., 1998, 2000). These effects were described as most likely being independent of GH and unpreventable by blockade of the GHRH pathway (Torsello et al., 1998).

Once the first endogenous ligand of the GHS receptor had been found, we observed that ghrelin stimulates food intake in rodents (Tschöp et al., 2000). This effect is dose-dependent and occurs powerfully after central than after peripheral administration (Tschöp et al., 2000).

The increase in food intake after ghrelin injection in rodents occurs rapidly, less than 60 min, (Wren et al., 2000) which causes this effect to be easily missed by traditional methods of daily food intake measurements (measurement of food weight every 24 h). Ghrelin's orexigenic action (when administered centrally) is comparable to that of neuropeptide Y and therefore more potent than that of any other orexant (Wren et al., 2000). While peripherally

injected GHS or ghrelin does have less impressive (Tschöp et al., 2002) and only acute and maybe solely temporary orexigenic effects, on the other hand, continuously administered ghrelin into the third ventricle causes potent and constant stimulation of appetite in rats (Tschöp et al., 2000). However, future studies (i.e. involving mice with tissue-specific disruption of the ghrelin gene) will have to prove the existence of an endogenous ghrelin-tone that supports ghrelin's putative relevance for physiological appetite regulation and metabolic control. Some experiments involving central administration of ghrelin antiserum or GHS receptor antagonists (Nakazato et al., 2001) already provide some framework for this concept. Synthetic GHS (ghrelin receptor agonists) that have been shown to have orexigenic activity so far include GHRP-2, Ipamorelin, GHRP-6, Hexarelin and several of its analogues (Wren et al., 2000; Torsello et al., 1998, 2000; Lall et al., 2001; Tschöp et al., 2002).

Ghrelin is the first orexigenic signal that is derived from the stomach (Inui, 2001). Unlike other comparably potent orexigenic agents (neuropeptide Y, AGRP, melanin-concentrating hormone) which are solely active when injected in the brain (Kalra et al., 1999; Gehlert, 1999; Tritos and Maratos-Flier, 1999), peripherally administered synthetic ghrelin and ghrelin receptor analogs still exhibit orexigenic and adipogenic effects (Bowers, 2001). This does not necessarily mean that an acylated peptide molecule such as ghrelin is capable of crossing, or being transported, across the blood–brain barrier. Even if future studies show that unlike leptin, ghrelin cannot cross the blood brain barrier (Banks, 2001), ghrelin can still have hypothalamic actions since those hypothalamic areas which are crucial for the regulation of energy homeostasis are not completely protected by the blood–brain barrier (Merchenthaler, 1991). Some of these areas (i.e. the ventromedial arcuate nucleus) are therefore accessible by molecules in circulation (Horvath et al., 2001). The exact same hypothalamic nuclei intriguingly contain neurons that express the GHS receptor (Willesen et al., 1999) and might therefore be essential for the mediation of effects triggered by gastric or peripherally injected synthetic ghrelin (Horvath et al., 2001; Bowers, 2001). This concept has been shown by Dickson et al. who demonstrated that peripherally injected ghrelin induces increased expression of the early gene products c-Fos and EGR-1 in neuropeptide Y-, AGRP- and GHS receptor-co-expressing neurons in the arcuate nucleus (Hewson and Dickson, 2000).

The network of neurons, neuropeptides and receptors controlling energy balance is an extremely complex, multicentered system (Kalra et al., 1999). Based on early surgical and chemical deletion studies in rodents, the hypothalamus has long been recognized as a crucial interphase between afferent peripheral signals, CNS wiring and efferent neuroendocrine axes regulating energy balance in concert (Spiegelman and Flier, 2001). Several recent reviews give excellent overviews on the players and principles in this fascinating and rapidly advancing field (Ahima and Osei, 2001; Schwartz et al., 2000; Spiegelman and Flier, 2001; Kalra et al., 1999).

According to current knowledge, it seems that two major hypothalamic pathways are the predominant mediators of ghrelin's influence on energy balance (Shintani et al., 2001; Nakazato et al., 2001; Tschöp et al., 2002). One involves the neuropeptide Y neurons (Gehlert, 1999; Kamegai et al., 2001), the other one involves the melanocortin receptors and their agonistic and antagonistic ligands, the anorexigenic pro-opiomelanocortin-derived α melanocyte-stimulating hormone (αMSH) and the orexigenic AGRP, which is expressed in neuropeptide Y-neurons (Marks and Cone, 2001). Ghrelin, increases AGRP and neuropeptide Y after acute and chronic administration, and hypothalamic AGRP-mRNA expression levels are found to be up-regulated after chronic activation of the GHS receptor for several weeks (Kamegai et al., 2000, 2001; Nakazato et al., 2001; Tschöp et al., 2002). Complete absence of neuropeptide Y in neuropeptide Y-gene disrupted mice does not influence ghrelin action, while some studies show a prevention of orexigenic effects when co-administering a neuropeptide Y receptor antagonist with ghrelin (Tschöp et al., 2002). This virtual contradiction can possibly be explained by adaptive processes during the early development of the neurpeptide Y-knock out mice (Palmiter et al., 1998). We assume that the two pathways described above co-mediate ghrelin's effects on energy balance. We furthermore speculate that neuropeptide Y might be more important for acute effects while AGRP might be involved in both chronic and acute ghrelin action in the hypothalamus (Horvath et al., 2001).

This view is oversimplified and the most recent developments contributing to the current neuroendocrine model of energy homeostasis regarding a possible involvement in ghrelin action have not been examined. The hypothalamically localized cell surface heparan sulfate proteoglycan (HSPG) syndecan-3 locks the melanocortin receptors in a complex with AGRP and consecutively exerts anabolism (Reizes et al., 2001). A possible influence of ghrelin binding in the hypothalamus on the oscillation of Syndecan-3 levels has not yet been examined. Other agents possibly mediating ghrelin signals in the hypothalamus are POMC, cocain and amphetamine-regulated transcript (CART), melanin-concentrating hormone, orexin (hypocretin) a/b, ciliary neurotrophic growth factor (CNTF), GABA and galanin (Spiegelman and Flier, 2001; Ahima and Osei, 2001; Horvath et al., 2001). Regulation of expression levels of these hypothalamic agents will have to be completed by synaptological studies using electron microscopy and electrophysiology (Horvath et al., 2001). Utilization of these methods together with genetically engineered rodent models might provide more insight into the interplay between the aforementioned hypothalamic modulators of energy homeostasis and peripheral factors such as leptin, glucose, cholecystokinin, adiponectin and ghrelin. A specific question that will have to be answered in this context is to determine

if extra-gastrointestinal tract, especially putative hypothalamic ghrelin, contributes to energy balance regulation.

Apart from an increase of food intake, an increase in fat mass can also be caused by a decrease in energy expenditure and/or reduced fat oxidation (Spiegelman and Flier, 2001). No significant changes of 24-h energy expenditure have been observed in rodents after ghrelin administration. However, during the first 3 h after ghrelin injection we observed an acute decrease in energy expenditure that if extrapolated over several weeks, may account for the increased fat mass observed in these experiments (Tschöp et al., unpublished observation). Another effect that can be detected by indirect calorimetry, is an impressive increase of the respiratory quotient after ghrelin administration in rodents independent of an increase in food intake (Tschöp et al., 2000). This phenomenon is interpreted as a shift from fat utilization to carbohydrate oxidation and has been referred to as ''nutrition partitioning'' (Friedman, 1998).

Furthermore, there is some published evidence that ghrelin action might be mediated in part not only by efferent but also by afferent activity of the vagus nerve (Asakawa et al., 2001). These data were generated by electrophysiological studies, where intravenously administered ghrelin has been shown to decrease the afferent activity of the gastric vagal nerve at low doses (Asakawa et al., 2001). The described effects are in opposition to those of gastrointestinal ''satiety'' peptides such as cholecystokinin (Wei and Wang, 2000) and may add further pathways to the growing number of signaling routes ghrelin is connected with (see also Fig. 2A and B) (Inui, 2001).

In summary, administration of ghrelin and of at least some of its receptor agonists, generates a positive energy balance and consecutively increases adiposity in rodents via increased food intake and reduced fat oxidation.

In spite of the rapidly increasing amount of data on the influence of ghrelin and other GHS on energy homeostasis, several unresolved but exigent questions still await clarification.

(1) Several studies provide evidence that suggest the existence of several types or subtypes of ghrelin receptors with distinct effects on either energy balance or growth hormone secretion (Torsello et al., 2000; Chen, 2000; Horvath et al., 2001). We speculate that it might principally be possible to activate or antagonize one specific group or subgroup of these (so far unknown) receptors and consecutively only trigger distinct effects.

(2) The biological turnover of ghrelin and the clearance rates of synthetic injectable or orally active ghrelin receptor agonists appear to exhibit major differences (Bowers, 1998, 2001). We hypothesize that the differential effects of these compounds might not only be determined by the activated receptor type but also by the pharmacodynamic and pharmacokinetic properties of the respective agent.

(3) Not much is known about the time course of the observed changes in body mass and body composition. Even though these effects are very impressive at first, the dynamic of the process seems to slow down and is not noticeable progressing during a third week of chronic daily peripheral administration (Tschöp et al., 2002). Future studies will have to show the significance of repetitive vs. continuous vs. pulsatile administration of ghrelin or its receptor agonists for the generation of a positive energy balance.

(4) Possibly the most pressing question concerns the transferability and validity of the above described findings in rodents to human. Although GHS have been in use in clinical studies for many years, adipogenic effects as observed in rodents have not been described. However, the paradigm of GHS and ghrelin primarily being involved in the control of the somatotropic axis has been seriously challenged by the observed effects of these compounds on energy homeostasis in rodents. Furthermore, induction of insulin resistance by GHRP-6 after blockade of GH-action by the new GH-receptor antagonist Pegvisomant in humans and reports of significantly increased plasma insulin levels as side-effects of clinical studies have been published (Svensson et al., 1998). In addition, several recent clinical studies on the effects of ghrelin on GH-secretion in humans have reported hunger sensations as the only noticeable side effect in up to 80% of the treated individuals (Arvat et al., 2000a, 2001; Broglio et al., 2001). Prospective clinical studies focusing on all aspects of energy balance using cutting edge methods for the analysis of body composition, energy expenditure, metabolic and endocrine changes can help to clarify these issues.

7. Control of ghrelin secretion

Ghrelin is expressed in a variety of tissues which include the stomach, the small intestine, the pituitary, the placenta, the kidney, the pancreas and most likely the hypothalamus (Date et al., 2000; Mori et al., 2000; Ariyasu et al., 2001; Dornonville de la Cour et al., 2001; Gualillo et al., 2001a; Kojima et al., 1999; Horvath et al., 2001). The majority of ghrelin is originated from the stomach followed by the small bowel (Dornonville de la Cour et al., 2001; Date et al., 2000). From there, and possibly in addition from the placenta during pregnancy, (Gualillo et al., 2001a) ghrelin is secreted into the circulation and signals target tissues through the blood stream as a classical endocrine signal (see Fig. 2A and B). This peripheral ghrelin signal is most likely reflecting acute but also chronic challenges of energy balance and relays this information to the CNS. Within the gastrointestinal tract, ghrelin mRNA-expression as well as ghrelin peptide have been localized in X/A-like cells within the acid-producing oxyntic glands of humans and rats. X/A-like cells account for 20–25% of all endocrine cells in the oxyntic mucosa. Other endocrine cells in oxyntic cells, such as histamine-rich enterochromaffin-like (ECL) cells (ca. 70%) and D-(somatostatin) cells (10%) are not ghrelin-positive (Dornonville de la Cour et al., 2001; Date et al., 2000). Ghrelin-immunoreactive X/A-like cells are not

strictly confined to oxyntic mucosa. In fact, ghrelin is found from the stomach to the colon with caudally decreasing density of expression (Dornonville de la Cour et al., 2001; Date et al., 2000). Ghrelin containing entero-endocrine X/A-like cells have no continuity with the lumen, most likely respond to physical and/or chemical stimuli from the basolateral side and are closely associated with the capillary network running through the lamina propria (Dornonville de la Cour et al., 2001; Date et al., 2000). These observations make a classical endocrine role for ghrelin as a secreted peptide hormone very likely, even though local, paracrine activities of ghrelin within the intestinal tract have been suggested to possibly play an additional role (Dornonville de la Cour et al., 2001; Date et al., 2000). Removal of the stomach or the acid-producing part of the stomach in rats reduces serum ghrelin concentration by ca. 80%, further supporting the view that the stomach is the main source of the endogenous GHS receptor ligand (Dornonville de la Cour et al., 2001; Date et al., 2000).

Small amounts of ghrelin are found in the pancreas, where ghrelin immunoreactivity has been localized in a pancreatic group of endocrine cells that are also immuno-positive for pancreostatin. However, only a subpopulation of pancreostatin-immuno-positive cells was immuno-positive for ghrelin. Neither a co-localization between ghrelin and ECL-cells nor a co-localization between ghrelin and D-cells was found in pancreas. Therefore it was concluded that the ghrelin positive cells must belong to the third subpopulation, the A-cells (Dornonville de la Cour et al., 2001). Our group has recently shown that ghrelin is produced by a fraction endocrine pancreatic cells namely the insulin-producing β cells as confirmed by double immunofluorecence studies (Volante et al., 2002).

Ghrelin mRNA and ghrelin peptide have also been detected in rat and human placenta. Ghrelin is expressed predominantly in cytotrophoblast cells and very sporadic in syncytiotrophoblast cells. A pregnancy related time-course, represented by an early rise of ghrelin expression in the third week and decreasing stages in the latest stages of gestation, as well as still detectable presence of ghrelin at term was found in rats. In human placenta, ghrelin is mainly expressed in the first half of the pregnancy and is not detectable at term (Gualillo et al., 2001a). The involvement of ghrelin in fetal-maternal interaction via autocrine, paracrine, or endocrine mechanisms has been discussed (Gualillo et al., 2001a).

In addition to the known presence of GHS receptor (which has originally been cloned from the pituitary) ghrelin mRNA expression and ghrelin immunopositive cells have been detected in normal pituitary cells as well as in pituitary tumors (Korbonits et al., 2001a,b). This suggests a possible autocrine or paracrine role for hypophyseal ghrelin, although only ca. 5% of the detected ghrelin peptide has been found to be octanoylated (Korbonits et al., 2001a,b). Ghrelin synthesis has been shown by the use of real time-PCR and direct sequencing of the PCR-product in corticotroph, thyrotroph, lactotroph and somatotroph cells of the pituitary (Korbonits et al., 2001a,b). In human pituitary adenomas, the highest levels of ghrelin expression have been found in non-functioning adenomas, moderate ghrelin levels in GH-producing adenomas and gonadotropine-producing adenomas and the lowest level in prolactinomas (Korbonits et al., 2001a,b).

By combinatory use of reverse-phase high performance liquid chromatography and radioimmunoassay of purified aliquots, production of ghrelin in mouse kidney is shown in greater abundance (6.8 ± 0.5 fmol/mg) than in mouse plasma (0.3 ± 0.03 fmol/μl). In addition, prepro-ghrelin production is shown in rat mesangial cells and mouse podocytes, indicating the production of ghrelin in kidney, glomerulus and renal cells and suggesting possible paracrine roles of ghrelin in the kidney (Mori et al., 2000).

In several regions of the brain ghrelin was detected by means of immunohistochemistry. However, depending on the recognized epitope of the ghrelin antiserum used, slightly differently located as well as very few ghrelin-positive neurons have been identified (Horvath et al., 2001). Evidence for ghrelin mRNA expression (in-situ hybridisation) in location identical with peptide detection is yet to be shown and will have to proof hypothalamic ghrelin production. Until then, ghrelin found in the hypothalamus has to be considered as possibly derived from the periphery and a participation of hypothalamic ghrelin in neuropeptidergic energy balance control mechanisms remains questionable (Horvath et al., 2001).

Human ghrelin as well as GHS receptor mRNA-expression was shown by real time-PCR and confirmed by DNA-sequencing in human T-lymphocytes, B-lymphocytes and neutrophils from venous blood of healthy volunteers. Impressive inter-individual differences in ghrelin mRNA expression levels were described, however, cell type and maturity of the cells did not seem to have an influence on ghrelin production in immune cells. Interestingly, a different study group has shown recently that small molecule GHS have a considerable immune enhancing effect, particularly in the old mice (Hattori et al., 2001; Koo et al., 2001).

In summary, ghrelin is expressed in its majority by the stomach, followed by lower parts of the gastrointestinal tract. Expression levels in the hypothalamus, pituitary, kidney, placenta, pancreas and in immune cells are relatively low and their physiological significance remains to be shown. Other organs such as the heart, the spleen, the adrenals, the gall bladder and the testes might eventually contain very small amounts of ghrelin as well since ghrelin traces in those organs have very recently been detected in bullfrogs (Kaiya et al., 2001).

Published studies on the regulation of ghrelin expression did primarily focus on gastric ghrelin since the majority of circulating ghrelin derives from here by far strongest expression of ghrelin compared to any other tissue can be found in the stomach. Again, a separation between rodent and clinical data seems necessary for the interpretation of

data on ghrelin secretion to avoid premature assumptions for human ghrelin physiology. Plasma ghrelin levels as described by several research groups vary according to the antiserum used and are influenced by the use of an additional extraction step. While absolute plasma ghrelin levels and ghrelin reference standards still have to be determined, it appears reasonable to investigate ghrelin regulation and physiology via measurement of relative differences of circulating ghrelin levels in humans and rodents using available radio-immunoassays. In several species, including mice, rats, cows and humans circulating ghrelin levels and/or ghrelin mRNA-expression levels have been shown now to be decreased by food intake and to be increased by food deprivation (Tschöp et al., 2000, 2001a; Asakawa et al., 2001; Toshinai et al., 2001; Hayashida et al., 2001; Cummings et al., 2001). This phenomenon, which has been confirmed by several study groups in the recent past, further supports the emerging concept of ghrelin as a potentially important player in the regulation of energy homeostasis. In addition to fasting, ghrelin expression can be stimulated in rats by insulin-induced hypoglycemia and leptin administration (Toshinai et al., 2001). Ingestion of sugar appears to powerfully suppress ghrelin secretion in rats (Tschöp et al., 2000). Increasing concentrations of glucose in medium of rat stomaches decreases ghrelin concentrations in the supernatant fluid (Heiman et al., unpublished observations). This in vitro phenomenon indicates a direct inhibitory effect of glucose/caloric intake on ghrelin containing X/A-like cells in the oxyntic mucosa of the rat stomach rather than an insulin-mediated effect. Other (patho-)physiological situations that cause an increase of circulating ghrelin levels in rats are surgical interventions such as vagotomy and hypophysectomy (Tschöp et al., submitted for publication). Human GH-deficiency however does not seem to exhibit increased plasma ghrelin levels. This difference could be due to species-specific differences between rodents and humans or it could indicate that the loss of pituitary hormones other than GH influences gastric ghrelin secretion. An increase of circulating ghrelin levels in rats with age, up to 90 days (Gualillo et al., 2001b), has not been confirmed yet for human populations.

We hypothesize that the observed feeding-induced changes in plasma ghrelin concentration reflect differences in gastrointestinal ghrelin secretion. If ghrelin production in other tissues is affected by the same regulatory mechanisms that influence gastric ghrelin production/secretion remains for the moment as unclear as the physiological relevance of these sources.

A recent, very intriguing clinical study by Cummings et al. (2001) indicates that each daily meal is followed by decreases of circulating ghrelin levels most likely reflecting acutely reduced ghrelin secretion from the gastrointestinal tract. The authors speculate in addition that an observed pre-meal raise of circulating human ghrelin levels might reveal a role for ghrelin in meal initiation. This theory does fit well with the observation that ghrelin administration in healthy volunteers causes hunger sensations (Arvat et al., 2000a, 2001; Broglio et al., 2001). Ghrelin, if it is a ''hunger hormone'', might also reflect the acute state of energy balance, signaling the CNS in times of food deprivation that increased energy intake and an energy preserving metabolic state are desirable (Tschöp et al., 2000). In addition, we believe that one biological purpose of these multiple roles of ghrelin is to ensure the provision of calories that GH requires for growth and repair.

It is intriguing that several rodent studies have shown a GHS-induced increase in bone mass and/or bone density (Svensson et al., 2000, 2001; Tschöp et al., 2002). On the other hand it is known that gastrectomy not only decreases endogenous ghrelin levels by 60–80%, but also causes loss of bone mass and decreased bone density (Efstathiadou et al., 1999; Heiskanen et al., 2001; Tovey et al., 1992). This evidence suggests the possibility that ghrelin deficiency in gastrectomized patients contributes to metabolic bone disease and future clinical studies should clarify this hypothesis.

8. Implications of ghrelin in metabolism and obesity

Ghrelin administration in rodents generates a positive energy balance and consequently causes adiposity via appetite stimulation and reduced fat oxidation (Inui, 2001). It was therefore a surprise to many that plasma ghrelin levels in obese individuals were found to be lower, than in lean controls (Tschöp et al., 2001a). In general, human plasma ghrelin levels are negatively correlated with body mass index, body fat mass, adipocyte size, plasma insulin levels, plasma glucose levels and plasma leptin levels (Ravussin et al., 2001; Tschöp et al., 2001a). Pima Indians, which are prone to type 2 diabetes and obesity, also have lower circulating ghrelin levels than matched controls, independently from their body mass index (Tschöp et al., 2001a). On the contrary, patients with anorexia nervosa (Becker et al., 1999) exhibit high plasma ghrelin levels when compared to age- and sex-matched controls.

One explanation for these seemingly surprising findings might be that ghrelin secretion is triggered to counter further energy deficit and to prevent starvation in cachexia. To assess a possible role for ghrelin in the etiology of obesity, several interventional studies were performed. In overfeeding and negative energy balance studies in twins changes in fat mass failed to cause significant changes of plasma ghrelin levels. However, tendencies to increased ghrelin levels were observed after a 93-day period of negative energy balance (53.000 kcal) and a 100-day period of overfeeding (84.000 kcal) tended to result in decreased plasma ghrelin levels. Interestingly, the intraclass coefficient for the twin resemblance in this study indicated that plasma ghrelin concentration might be a familial trait (Ravussin et al., 2001). However, this ''clamped energy balance'' study failed to provide evidence that ghrelin is involved in the etiology of human obesity. A more severe change in body

mass index, this time due to an increase in body weight in patients with anorexia nervosa after treatment in a psychosomatic hospital, did cause a significant decrease in ghrelin levels (Otto et al., 2002). It will be important to perform further prospective studies to better understand the physiological role of ghrelin for the regulation of body weight in health and disease.

Possible ghrelin-gene mutations might be involved in impaired regulation of body weight in mammals. A first study on the association between mutations in the prepro-ghrelin/ghrelin gene and obesity has recently been published by Ukkola et al. (2001). In a population of 96 obese subjects and an equal number of lean controls, the authors report that a mutation at amino acid position 51 (Arg51Gln) of the prepro-ghrelin sequence was identified in six obese subjects but not in controls. Another mutation at codon 72 of the prepro-ghrelin gene (Leu72Met) was associated with lower age of onset of obesity (Ukkola et al., 2001). Genetic variation at the ghrelin locus could be one of the genetic causes of obesity and should be further investigated in prospective studies with larger cohorts.

It is ostentatious that the biological effects of ghrelin as well as the regulation of plasma ghrelin levels during different states of acute and chronic energy balance appear to mirror effects and regulation of leptin. Ghrelin and leptin not only seem to have opposite effects at the exact same targets, such as the hypothalamic neuroendocrine network encircling the melanocortin receptors and neuropeptide Y-neurons. They are also primarily secreted at opposite ends of the energy balance spectrum (Horvath et al., 2001). Although ghrelin might seem to be the counterpart of leptin (Friedman, 2000) in light of the current data situation, we propose that these two hormones rather represent the ''yin-yang'' of one regulatory system that has developed to inform the brain about the current energy balance state. It is the beauty of the ''reverse pharmacology'' history of the GHS field, that (even orally active) ghrelin receptor agonists have been available long before the respective receptor was cloned or an endogenous ligand known (Bowers, 1998, 2001). Under the perspectives summarized above, these agonists might offer therapeutic opportunities for any disease associated with decreased appetite or body weight. Clinical studies will have to show in the future if patients with anorexia nervosa, cancer cachexia, or aquired immunodeficiency syndrome-wasting syndrome might profit from a treatment with GHS. Just as in patients with type 2 diabetes mellitus (where hyperinsulinemic patients are treated with additional synthetic insulin), cachectic patients with relatively high levels of endogenous ghrelin might benefit from treatment with additional GHS. The disappointing outcome of clinical trials on treatment of human obesity with recombinant leptin (Heymsfield et al., 1999) has however already shown once that rodents are not small humans. Therefore, expectations on the efficacy of treatment of cachexia with GHS should not be too high. The generation of potent ghrelin receptor antagonists may or may not turn out useful for the treatment of the epidemic human obesity. We are however confident that the findings described above mean one more step forward towards a better understanding of the network regulating energy homeostasis, which may catalyze the development of new therapeutic options for diseases involving impaired body composition.

9. Conclusions and potential clinical implications

Ghrelin, a 28-residue acylated peptide predominantly produced by the stomach, displays strong GH-releasing activity mediated by the hypothalamus–pituitary GHS receptors which are specific for a family of synthetic, orally active GHS. GHS could represent a reliable provocative test for the diagnosis of GH deficiency but as orally active growth-promoting agents are not comparable with rhGH in term of efficacy. The usefulness of GHS as anabolic, anti-aging drug intervention in somatopause is also still unclear. GHS act on other central and peripheral receptors and show actions including orexigenia, influence on gastro-entero-pancreatic functions, cardiovascular and anti-proliferative effects. Regarding potential pharmaco-therapeutic implications, GHS analogs could be designed to protect from coronaric ischemia and/or to prevent dilated cardiomyopathy improving cardiac performances and/or to reduce the progression of endothelial dysfunction and microangiopathy. GHS analogs could exert an antineoplastic action devoid of stimulatory effect on the activity of GH/IGF-I axis because of the well known positive influence of growth factors on tumor cell proliferation. Above all, taking into account that ghrelin manages the neuroendocrine and metabolic response to starvation and exerts orexigenic effect, it is receiving major attention the possibility that GHS analogues acting as agonists or antagonists on appetite would represent new drug intervention for eating disorders.

Acknowledgements

The authors wish to thank Drs. Andrea Benso, Fabio Broglio, Filomena Catapano, Corrado Ghè and Cristina Gottero.

Personal studies reported in this review have been performed with financial support from the Italian Ministry of University and Research, Rome, Italy (''ex-60%'' 1997 and 2000, and MM06318538), the National Council for the Research, CNR, Rome, Italy (98.03040.CT04), from Eureka Project (Peptido project 1923), SMEM Foundation and Europeptides, France.

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