Effects of Short Term Genotropin (rhGH) Subcutaneous Injection on Active Ghrelin Concentration in Rat Plasma, Stomach, Kidney, and Pancreas

by

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ABSTRACT

Ghrelin is a 28-amino acid octanoylated peptide that has been isolated from rat stomach and stimulates pituitary growth hormone secretion. Growth hormone (GH) is a potent endocrine modulator of growth and metabolism. Genotropin is a recombinant human growth hormone (rhGH) that increases lean body mass, skeletal muscle mass, muscle force and strength, aerobic performance, and fat loss. A very important issue regarding ghrelin physiology is whether an axis or loop exists between pituitary GH on one hand and plasma, stomach, pancreas and kidney ghrelin on the other hand thus reflecting how GH elevations or reductions directly affect ghrelin homeostasis and secretion. The purpose of this experiment was to study the effects of a 5 day treatment of genotropin subcutaneous injection (2, 20, and 100 µg/rat/day) on active ghrelin concentration in rat plasma, stomach, kidney, and pancreas samples. The Active Ghrelin ELISA kit results showed a significant increase in active ghrelin concentration in the rat plasma samples and a significant decrease in active ghrelin concentration in the rat stomach, kidney, and pancreas supernatants. Moreover, protein results using Lowry assay for protein determination showed that genotropin injection led to an increase in protein concentration in stomach and kidney samples and to a drastic decrease in protein concentration in the pancreas samples. Calculations revealed that genotropin injection decreased active ghrelin concentration per µg of protein in stomach and kidney supernatant but had no significant effect on active ghrelin concentration per µg of protein in pancreas supernatant. In conclusion, these findings show that GH lowers active ghrelin concentration in the stomach, kidney, and pancreas samples thus suggesting a feedback loop between stomach, kidney and pancreas ghrelin on one hand, and pituitary GH on the other hand.
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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Ghrelin is a 28-amino acid octanoylated peptide that has been isolated from rat stomach as an endogenous ligand of the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima et al., 1999). Ghrelin is predominantly produced by the A-like cells found in the stomach mucosa, less quantities are produced by the gastric antrum, and still fewer in the small intestine (Date et al., 2000; Dornonville de la Cour et al., 2001). Lower amounts of ghrelin have been detected in the hypothalamus (Kojima et al., 1999), pituitary (Korbonits et al., 2001), kidneys (Mori et al., 2000), pancreas (Date et al., 2002; Volante et al., 2002a; Wierup et al., 2002; Colombo et al., 2003), testes (Tena-Sempere et al., 2002), placenta (Gualillo et al., 2001), and lungs (Volante et al., 2002b).

1.1. Historical background:

Ghrelin, the gastric hormone, was identified as an endogenous ligand for the receptor GHS-R1a (Smith et al., 1997; Kojima et al., 1999). Synthetic GHS are a family of ligands which include peptidyl and nonpeptidyl molecules (Bowers, 1993; Smith et al., 1997; Ghigo et al., 2001; Muccioli et al., 2002). The first synthesized molecules were GH-releasing peptides which are nonnatural peptides that were designed by Bowers and Momany in the late 1970’s (Momany et al., 1981; Momany et al., 1984; Bowers, 1998). Studies that focus on the distribution of GHS-Rs showed a concentration of these receptors in the hypothalamus-pituitary area; however, the expression of GHS-R and presence of specific binding sites has been found in many other parts of the brain and peripheral, endocrine, and nonendocrine human and animal tissues (Kojima et al., 1999; Date et al., 2000; Mori et al., 2000; Muccioli et al., 2000; Volante et al., 2001).
1.2. Functions of ghrelin and its GHS:

Ghrelin and its GHS have many functions:

- Stimulate GH, Prolactin (PRL), Adrenocorticotropic hormone (ACTH), Luteinizing hormone (LH) and cortisol secretion (Arvat et al., 2000; Arvat et al., 2001; Yoshihara et al., 2002; Iqbal et al., 2005).
- Stimulate appetite (orexigenic effect) and induce a positive energy balance leading to hyperphagia, obesity and adiposity (Tschop et al., 2000; Wren et al., 2000; Horvath et al., 2001; Wren et al., 2001a; Wren et al., 2001b; Muccioli et al., 2002; Yoshihara et al., 2002).
- Control gastric acid secretion and gastric motility (Masuda et al., 2000).
- Negatively Influence (Negative-feedback mechanism) the pituitary-gonadal axis (Korbonitis et al., 2001).
- Influence exocrine and endocrine pancreatic function therefore affecting glucose metabolism (Arvat et al., 2001; Zhang et al., 2001).
- Modulate the proliferation and survival of neoplastic cells (Cassoni et al., 2001).
- Act on the immune system by modulating the proliferation of B cells, T cells and neutrophils (Hattori et al., 2001).
- Induce anxiety-like behavior (Asakawa et al., 2001).
- Affect sleep-wake patterns in rats and are reported to be sleep-promoting factors in humans (Tolle et al., 2002; Weikel et al., 2003).
- Influence proliferation of cardiovascular cells (Muccioli et al., 2000).

1.3. The Biology of Ghrelin and its Receptor Family:

The hypothalamic growth hormone releasing hormone (GHRH) and somatostatin both regulate the pulsatile release of growth hormone (GH) from the anterior pituitary gland (Frohman et al., 1992; Smith et al., 1997). GHRH acts as a stimulant for GH synthesis, whereas somatostatin acts as an inhibitor for GH release. Moreover, the release of GH from the pituitary is stimulated by synthetic molecules called “GH secretagogues”. GHSs are non-natural synthetic peptidyl and nonpeptidyl molecules that directly stimulate GH secretion (Bowers et al., 1980; Bowers, 2001). GHSs act through a specific G-protein-
coupled receptor, the GHS-R that exerts an upgraded synergistic effect when administered in addition to GHRH (Guan et al., 1997; Gnanapavan et al., 2002).

Ghrelin exists in two major forms: the 28 amino acid ghrelin having n-octanoylated serine in position 3, and the 27 amino acid des-acyl ghrelin produced by alternative splicing of the ghrelin gene. The n-octanoyl group at serine 3 is very essential for the bioactivity of ghrelin hormone especially in terms of GH release. Non-acylated ghrelin is found in much greater quantities in blood circulation than the acylated form and the former does not possess any GH-releasing or other endocrine activities in humans and rats; however, it is able to exert some nonendocrine actions including cardiovascular and antiproliferative effects (Kojima et al., 1999; Date et al., 2000; Hosoda et al., 2000; Bowers, 2001; Broglio et al., 2003).

Figure 1. Unacylated and Acylated forms of ghrelin (Van der Lely et al., 2004)
1.4. Ghrelin in the plasma:
There are two major forms of ghrelin found in tissues and plasma: n-octanoyl-modified and des-acyl ghrelin (Hosoda et al., 2000). In humans, the normal ghrelin concentration of plasma samples is 10-20 fmol/ml for n-octanoyl ghrelin and 100-150 fmol/ml for total ghrelin. Plasma ghrelin concentration is elevated during fasting conditions and reduced after feeding (Cummings et al., 2001; Tschop et al., 2001). This suggests that ghrelin might either act as an initiation signal for food intake, or ghrelin secretion might be controlled by different nutritional factors in the blood. Moreover, the postprandial suppression of ghrelin levels in the plasma is directly proportional to food intake and caloric load (Callahan et al., 2004). This reinforces the hypothesis that ghrelin acts as a hunger signal in the blood. Several studies reported that ghrelin levels were lower in obese subjects than in age-matched lean controls (Tschop et al., 2001b; Hansen et al., 2002; Shiiya et al., 2002). Furthermore, other studies reported that plasma ghrelin concentrations were significantly lower in Pima Indians than in Caucasians. This was reinforced by the fact that Pima Indians are more prone to develop insulin resistance and obesity (Tschop et al., 2001b).

1.5. Ghrelin in the stomach:
In vertebrates, ghrelin is mainly produced in the stomach (Ariyasu et al., 2001). The ghrelin-containing cells are a distinct endocrine cell type found in the mucosal layer of the stomach and are more abundant in the fundus than in the pylorus (Date et al., 2000; Rindi et al., 2002; Yabuki et al., 2004). Four different types of endocrine cells have been identified in the stomach mucosa: ECL, D, enterochromaffin (EC), and X/A-like cells (Davis, 1954; Capella et al., 1969; Solcia et al., 1975). The X/A-like cells contain compact, round, electron-dense granules that are filled with ghrelin (Date et al., 2000; Dornonville de la Cour et al., 2001; Yabuki et al., 2004). In adult oxyntic glands, the X/A-like cells account for approximately 20% of the endocrine cell population. In fetal stomach, on the other hand, the number of X/A-like cells is very low and increases gradually after birth (Hayashida et al., 2002). The gastric X/A-like cells can be stained by an antibody which is specific to the NH₂ – terminal, acyl-modified portion of ghrelin. This indicates that ghrelin has already been acyl-modified in the secretory granules of the
gastric X/A-like cells. “Ghrelin concentration in rat stomach is 377.31 ± 55.83 fmol/ml (n-octanoyl ghrelin) and 1,779.8 ±533.9 fmol/ml (total ghrelin)” (Hosoda et al., 2000).

Ghrelin-immunoreactive cells are also found in the duodenum, jejunum, ileum, and colon. Therefore, ghrelin m-RNA expression as well as ghrelin peptide is found in decreasing quantities from the stomach to the colon (Hosoda et al., 2000; Date et al., 2002; Sakata et al., 2002). Gastrectomy in rats or removal of the acid-producing part of the stomach reduces serum ghrelin concentration to approximately 80% thus proving that the stomach is the primary source of GHS-R ligand (Date et al., 2000; Dornonville de la Cour et al., 2001). However, recent studies have proved that other body tissues can compensate for the loss of ghrelin production by gradually increasing the plasma ghrelin levels after total gastrectomy (Hosoda et al., 2003). The gastric acid secretion and the amplitude of gastric motility are both stimulated by intravenous injection of ghrelin (Masuda et al., 2000; Dornonville de la Cour et al., 2004). Ghrelin stimulates gastric emptying but has no effect on acid secretion and gastric endocrine cells (Dornonville de la Cour et al., 2004). Gastric ghrelin production is regulated by many hormonal and nutritional factors (Inui, 2001; Beck et al., 2002; Lee et al., 2002; Pinkney & Williams, 2002). Inhibitory signals include GH, interleukin 1β, somatostatin, high-fat diet, and vagal tone. On the other hand, fasting and a low-protein diet lead to increased expression and plasma concentrations of ghrelin. Moreover, gastric ghrelin shows distinctive hormone reactivity with respect to somatostatin D, histamine enterochromaffin-like, glucagon A, or serotonin enterochromaffin cells (Rindi et al., 2002). There are no reports to date that clearly show the stimulatory function of GH on gastric function. This proves that the increased gastric function after ghrelin injection is not due to increased GH release. One can conclude that ghrelin is a strong gastrokinetic agent that acts as a link between energy balance and growth on one hand and regulation of digestive function on the other hand (Masuda et al., 2000). GH lowers ghrelin expression and concentration thus suggesting the presence of a feedback loop between stomach ghrelin and pituitary GH (Lee et al., 2002). A high-fat diet administered after fasting increases plasma ghrelin expression (Asakawa et al., 2003). This proves that, unlike the majority of gut hormones whose secretion increases with food intake and decreases with fasting, stomach ghrelin secretion increases with fasting.
1.6. Ghrelin in the kidney:
There is a possible endocrine role for ghrelin in the kidney due to the fact that few experiments using reverse-phase high performance liquid chromatography coupled with radioimmunoassay have indicated the presence of ghrelin in the kidney, glomerulus and renal cells. These findings suggest that mouse kidney contain both ghrelin and desacyl peptide and that the desacyl peptide predominates ghrelin. Furthermore, ghrelin is more abundant in the kidney than in plasma (Mori et al., 2000).

1.7. Ghrelin in the pancreas:
Ghrelin is produced in small quantities in the human pancreas by insulin-producing H cells as confirmed by immunofluorescence studies. In this case ghrelin m-RNA expression and immunoreactivity has been localized in β-cells of human islets (Volante et al., 2002). Ghrelin and its receptor(s) are present in the human pancreatic islets α-cells and analyses combining HPLC and ghrelin-RIA reveal that ghrelin exists in rat pancreas and suggest that ghrelin stimulates insulin release and increased calcium ions in rat islets β-cells (Date et al., 2002; Lee et al., 2002). This proves that ghrelin plays an essential role in the regulation of energy homeostasis. On the other hand, many studies show that ghrelin inhibits insulin secretion in humans and in rodents. These findings suggest that there exists a negative association between ghrelin and insulin secretion from the pancreatic islets β-cells (Broglio et al., 2001; Toshinai, 2001; Egido et al., 2002; Pagotto, 2002; Colombo et al., 2003; Reimer et al., 2003). Some studies proved that pancreatic ghrelin profile changes dramatically during fetal development (Chanoine & Wong, 2004; Wise, et al., 2004). Moreover, ghrelin mRNA expression and total ghrelin concentration are elevated in fetal pancreas six to seven times greater than that in the fetal stomach and pancreatic ghrelin levels are not affected by fasting (Hayashida, et al., 2002).

1.8. Ghrelin in the brain and pituitary:
The ghrelin receptor GHS-R is mainly expressed in the hypothalamic regions and pituitary (Howard et al., 1996; Guan et al., 1997). This is supported by the finding that GHRH which is another GH-releasing peptide is produced in the hypothalamus and is
secreted into the hypophysial portal system to stimulate GH release from the pituitary somatotrophs. However, research has reported that the ghrelin content of the brain is found to be very low (Kojima et al., 1999; Hosoda et al., 2000). Ghrelin has also been detected in the hypothalamic arcuate nucleus which is an important region for controlling appetite (Kojima et al., 1999; Lu et al., 2002). Moreover, a recent study has reported the presence of ghrelin in hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (Cowley et al., 2003). These localization patterns of ghrelin suggest a role in controlling food intake. Actually, GH-releasing somatotrophs in the pituitary gland are the target cells of ghrelin. In an in vivo assay, ghrelin stimulated the primary pituitary cells and increased their intracellular Ca²⁺ concentration, thus indicating that GHS-R is expressed in the pituitary cells (Bennett et al., 1997; Guan et al., 1997; McKee et al., 1997). Additionally, ghrelin has been detected in the pituitary gland itself and it might influence the release of GH in an autocrine or paracrine manner (Korbonits et al., 2001). The ghrelin expression level in the pituitary is high after birth and declines with puberty.

1.9. Hypothalamic-pituitary actions of ghrelin:

1.9.1. GH-releasing activity:
Ghrelin along with its synthetic GHS possess a strong GH-releasing activity and thus stimulate GH release from somatotroph cells (Herrington & Hille, 1994; Smith et al., 1997; Kojima et al., 1999; Arvat et al., 2000; Peino et al., 2000; Seoane et al., 2000; Takaya et al., 2000; Ghigo et al., 2001; Hataya et al., 2001; Glavaski-Joksimovic et al., 2003). The stimulatory effect of GHS on GH secretion is greater in vivo than in vitro (Clark et al., 1989; Bowers et al., 1991). In animals with pituitary stalk lesions, the GH-releasing effect of GHS is reduced. This proves that ghrelin and synthetic GHS act via mediation of GHRH-secreting neurons at the hypothalamic level in order to release GH (Clark et al., 1989; Bowers et al., 1991; Mallo et al., 1993; Fletcher et al., 1994; Conley et al., 1995; Hickey et al., 1996).
1.9.2. **PRL and ACTH-releasing activities:**

Both ghrelin and its GHS have pronounced stimulatory effects on the lactotroph and corticotroph systems. The stimulatory effect of ghrelin and its analogs on PRL secretion in humans is less age and gender dependant than the effect on GH secretion. On the other hand, the effect of ghrelin on ACTH secretion is more pronounced than that elicited by synthetic GHS (Arvat et al., 1997; Bowers et al., 1990; Peino et al., 2000; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001; Riley et al., 2002).

1.10. **Effects of ghrelin and GHS on food intake:**

Ghrelin which is the endogeneous ligand of GHS-R is one of the most powerful orexigenic (appetite-stimulating) and adipogenic agents discovered in mammals (Tschop et al., 2000; Wren et. al., 2000; Bowers, 2001; Inui, 2001). Ghrelin is involved in energy balance regulation thus pointing out an additional endocrine role of the stomach (Kojima et al., 1999; Dornonville de la Cour et al., 2001). Ghrelin administration induces weight gain in rodents. Astonishingly, this weight gain is not reflected by longitudinal skeletal growth but rather by an increase in fat mass and a decrease in lean muscle mass; moreover, changes in body weight are significant after 48 hrs of ghrelin administration to rodents and are very evident at the end of two weeks (Tschop et al., 2000; Nakazoto et al., 2001; Wren et al., 2001; Tschop et al., 2002). In contrary to leptin, ghrelin administered to rodents influences energy intake and metabolism. Therefore, it is likely that the positive energy balance is mediated via leptin-responsive neurons in specific hypothalamic regions (Kamegai et al., 2000; Horvath et al., 2001; Nakazato et al., 2001; Shintani et al., 2001; Tung et al., 2001; Tschop et al., 2002). In brief, ghrelin generates a positive energy and leads to adiposity and obesity via several mechanisms including increased food intake, reduced fat oxidation and decreased locomotor activity. Ghrelin, therefore, plays an indispensable role in the process of adipogenesis in rats, by stimulating the differentiation of preadipocytes and antagonizing lipolysis (Choi et al., 2003).
1.11. Effects of ghrelin and GHS on sleep and behavior:
Age-related changes in neurotransmitters and neuropeptides lead to alternations in the sleep patterns. Moreover, GH and Insulin Growth Factor I (IGF-I) also have a potential influence on sleep patterns in aged subjects due to an aged-related decrease in the activity of the GH axis (Van Cauter et al., 1998). On the other hand, some studies reported that the acute administration of synthetic GHS can modify sleeping patterns in elderly subjects by increasing the length of rapid eye movement sleep phases while decreasing rapid eye movement latency (Moreno-Reyes et al., 1998; Frieboes at al., 1999). Other studies also support the idea that ghrelin affects sleep-wake patterns in rats and is reported to be a sleep-promoting factor in humans (Tolle et al., 2002; Weikel et al., 2003).
Besides regulating eating behavior and sleep, it has been recently reported that ghrelin affects anxiety-like behavior in mice through mechanisms involving the hypothalamic-pituitary-adrenal axis thus inducing anxiogenic effects. These studies suggest that ghrelin may have a role in mediating neuroendocrine and behavioral responses to stressors. It is also suggested that the stomach could not only play an endocrine role in stimulating appetite, but also in the induction of anxiety (Asakawa et al., 2001).

1.12. Control and Measurement of Ghrelin Secretion and its Importance in Biology:
Ghrelin expression and secretion are influenced by many factors, mainly changes in energy balance and glucose homeostasis, as well as increasing GH concentrations. Based on the data found in the literature till date, ghrelin seems to be an essential part of a molecular regulatory phase between energy homeostasis, glucose metabolism and several physiological mechanisms regulated by endocrine axes such as growth and reproduction (Van der Lely et al., 2004).

The Regulation of the magnitude of ghrelin action involves several mechanisms (Van der Lely et al., 2004):
- Transcription and translation of ghrelin gene and its regulation.
- Enzymatic activity of acyl transferase which is responsible for the octanoylation of the ghrelin molecule.
- Rates of bioactive ghrelin secretion.
- Enzymatic processes deactivating circulating ghrelin.
- Influence of ghrelin binding proteins on its bioactivity.
- Blood-brain barrier transport thus reflecting the variable accessibility of target tissue.
- Elimination of degraded ghrelin by kidney or liver.
- Concentration of other ligands or cross-reacting competitive hormones in the circulation.
- Expression of ghrelin receptor in target tissue.
- Signaling mechanisms sensitivity.

Ghrelin is very unstable; therefore, some technical difficulties arise when measuring ghrelin immunoreactivity. Most studies focus on measuring the changes in gastric ghrelin mRNA expression or the variation of ghrelin concentration by using immunoassay techniques; however, all results should be interpreted with much caution due to the high instability of the ghrelin molecule. A very sensitive tool used in the measurement of ghrelin immunoreactivity is the specific sandwich immunoassay based on two monoclonal antibodies (ELISA) that can detect octanoylated ghrelin in humans & rodents mainly rats and mice. However, even these sensitive & specific immunoassays that target the octanyl side chain of the molecule suffer from the interference of many other circulating octanoylated molecules. Therefore, plasma ghrelin levels vary among different experiments according to many factors:
- Antiserum used
- Additional extraction steps
- Storing time, freeze-thaw cycles
- pH changes
- addition of enzyme-blocking agents

(Van der Lely et al., 2004)
1.13. Potential applications of ghrelin:

- Ghrelin may be applied in the diagnosis and treatment of GH deficiency due to its potent GH-releasing activity and specificity (Thorner et al., 1997; Baldelli et al., 2001; Deghenghi et al., 2003). The most common GH stimulus used to diagnose GH deficiency is insulin-induced hypoglycemia where the blood glucose levels decrease to  < 40mg/dl. However, the hypoglycemic action of insulin might cause side effects. Nowadays, intravenous injection of ghrelin into humans doesn’t show any adverse side effects, therefore, presenting ghrelin as a useful tool for diagnosing GH deficiency.

- The GH-releasing activity of ghrelin is comparable to that of GHRH; thus ghrelin treatment may benefit adult and child GH deficiency (Peino et al., 2000; Takaya et al., 2000; Ariyasu et al., 2001). Moreover, combined administration of ghrelin and GHRH has the most potent synergistic effect on GH secretion (Hataya et al., 2001).

- Intravenous injection of ghrelin is a peripheral orexigenic signal (Drazen & Woods, 2003; Ukkola, 2004). Therefore, blocking ghrelin’s action may be a reasonable approach to reversing a state of chronic obesity. However, a ghrelin antagonist might have a limited effect on obesity, since appetite is regulated by many factors that may interact with each other (Bays, 2004).

- Ghrelin may be very useful as an orexigenic agent for the treatment of severe eating disorders such as Anorexia Nervosa (Muccioli et al., 2002). Therefore, ghrelin injection in Anorexia Nervosa patients can stimulate appetite and thus improve their nutritional state.

- Ghrelin might be used for the treatment of postoperative gastric ileus due to the fact that ghrelin stimulates gastric motility (Masuda et al., 2000; Dornonville De La Cour et al., 2004). Therefore, ghrelin speeds up gastric emptying and the small intestinal transit of liquid meals and reverses delayed gastric evacuation, thus counteracting gastric ileus (Trudel et al., 2002).

- *In vitro*, ghrelin inhibits apoptosis of cardiomyocytes and endothelial cells and therefore, has positive cardiovascular effects (Baldanzi et al., 2002). In humans, injection of ghrelin in patients with heart failure, increases cardiac output and
decreases systemic vascular resistance. Ghrelin infusion also improves cardiac structure and function, and inhibits the development or cardiac cachexia in rats with heart failure (Nagaya & Kangawa, 2003; Chang et al., 2004a; Chang et al., 2004b). Therefore, ghrelin may act as a new therapeutic agent for the treatment of severe chronic heart failure since ghrelin has cardiovascular protective effects and it regulates energy metabolism through GH-dependent and independent mechanisms (Kojima & Kangawa, 2005).

- Ghrelin might also be clinically used in the treatment of osteoporosis, aging, and catabolic states including AIDS and cancer associated wasting syndromes (Hanada et al., 2003; Hanada et al., 2004; Neary et al., 2004).

Ghrelin, therefore, is a very important gut hormone that shows a direct relationship between caloric restriction and refeeding, neuroendocrine regulation of GH secretion, glucose metabolism, and growth. Finally, the broad aspect of ghrelin functions makes this hormone an interesting candidate for more profound future studies and applications.

### 1.14. An overview on Growth Hormone:

Growth Hormone is a polypeptide hormone synthesized and secreted by the anterior pituitary gland and it is a potent endocrine modulator of growth and metabolism. It is a member of the GH gene family, which includes prolactin and the placental lactogens. GH stimulates food intake and has many anabolic and lipolytic effects thus increasing lean mass and reducing fat mass in normal and obese subjects. Moreover, GH has many other metabolic effects which could affect survival following shock or critical illness. (Moller et al., 1995; Malmlof et al., 2002). In the rat model, many of the GH physiological effects are influenced by the pattern of GH administration, such as growth, insulin-like-growth factor-I responses and lipolytic effects (Clark et al., 1995, 1996). GH has many direct and indirect effects on target tissues and these actions of GH are dependant on its pattern of secretion and administration (Clark & Robinson, 1996; Yakar et al., 1999). For example, the different patterns of endogenous GH secretion in female and male rats may contribute to the sexual dimorphism in several aspects of growth and metabolism (Clark & Robinson, 1996). Effects of GH on the immune system are also pattern dependant (Clark,
1997). It has been demonstrated that continuous GH infusion is more potent than GH injections at stimulating lymphoid tissue growth (Clark et al., 1995). GH may also act as an endocrine and autocrine modulator of inflammatory responses since both GH and GH receptors are expressed in peripheral blood mononuclear cells (Clark, 1997). GH also affects thermoregulation (Juul et al., 1995) since GH administration enhances hyperthermia duration heat stress (Elvinger et al., 1992) and reduces heat shock protein expression in the liver (Deane et al., 1999). GH therapy has been also approved for the treatment of protein-wasting disorder of AIDS (Jenkins & Ross, 1996).

GH plays an important role in metabolic regulation (Ho et al., 1996; Breier, 1999). Combined administration of GHRH and GHSs is the most effective and potent stimulus of GH release in humans with GH deficiency. GHS induces a more physiological role of GH levels in plasma; thus, administration of GHS is preferable to direct subcutaneous administration of recombinant GH in case of GH deficiency (Casanueva & Dieguez, 1999; Dieguez & Casanueva, 2000; Takaya et al., 2000; Casanueva & Dieguez, 2002).

1.15. Growth Hormone excess:

Growth Hormone excess might lead to several disorders summarized by the following:

- Formation of pituitary tumors comprised of somatotroph cells of the anterior pituitary. These somatotroph benign adenomas grow slowly and they gradually produce more GH. Eventually the adenoma may become large enough to cause headaches, impair vision by pressure on the optic nerves, or cause deficiency of other pituitary hormones by displacement.

- Thickening and heaviness of the jaw and increased thickness of digits which is referred to as acromegaly. Accompanying problems can include muscle weakness, pressure on nerves, insulin resistance or even a rare form of type 2 diabetes, and reduced sexual function.

- Excessive growth, traditionally referred to as pituitary gigantism, which results when GH-secreting tumors occur in childhood.

(Moller et al., 1995; Shalet et al., 1998; Carroll et al., 2000; Malmlof et al., 2002; Abs, 2003)
1.16. Growth Hormone deficiency:
Growth Hormone deficiency produces significantly different problems at various ages:

- In children: Growth failure and short stature.
- In adults: reduced skeletal muscle, reduced lean body mass, increased fat mass, altered metabolism, reduced exercise capacity, impaired quality of life, deficiencies of strength, energy, and bone mass, as well as increased cardiovascular risk.

GH deficiency might be due to mutations of specific genes, congenital malformations of the hypothalamus and/or pituitary gland, and damage to the pituitary from injury, surgery or disease.

Diagnosing GH deficiency in children is straightforward, as it is associated with growth retardation. In adults, GH deficiency is detected using a multiple step diagnostic process, which involves GH stimulation tests to see if the patient's pituitary gland will release a pulse of GH when provoked by various stimuli. Examples of such tests include insulin tolerance test, the combined arginine and GHRH stimulation test, and the combined GHRH and GH releasing peptide 6 stimulation test.

(Shalet et al., 1998; Carroll et al., 2000; Abs, 2003)

1.17. Growth Hormone replacement therapy:
GH replacement therapy has beneficial effects on body composition, lipid patterns, coagulation, glucose metabolism, bone turnover, cardiovascular risk factors, and quality of life. Studies investigating the effects of GH replacement on body composition have reported an increase of lean body mass by 2 to 5.5 kg, and a reduction of abdominal fat mass by 4 to 6 kg after six months. Moreover, long term GH treatment for 12 months or more led to increases in bone mineral density and biochemical evidence of bone remodeling. It was reported that GH treatment increases serum insulin-like growth factor-1 (IGF-1), fat free mass, and serum concentration of albumin. GH replacement therapy also benefits elderly patients with end stage renal disease, who often have protein and/or
caloric malnutrition that severely affects general wellbeing and mortality. (Johannsson et al., 1999; Carroll et al., 2000; Svensson et al., 2001; Colao et al., 2002; Kehely et al., 2002).

Furthermore, GH replacement therapy leads to improved cardiac performance and a significant increase in left ventricular mass and stroke volume (Colao et al., 2004).

GH therapy leads to an improvement in vascular risk due to changed nitric oxide availability, changes in lipid fractions and inflammation mediators. However, most studies to date do not show a beneficial effect on insulin resistance. (McCallum et al., 2002).

1.18. Risks and side effects of GH treatment:
The possible risks and side effects of GH treatment include:

- Edema: swelling of the hands and feet.
- Thickening of the bones especially the jaw.
- Tingling in the extremities.
- Numbness in hands and feet.
- Increased organ growth that might lead to irregular cancerous growth and tumor formation.
- Decreased insulin reception.
- Acromegaly.
- Decreased thyroid output.
(Moller et al., 1995; Shalet et al., 1998; Carroll et al., 2000; Malmlof et al., 2002; Abs, 2003)

1.19. Major functions of rhGH:
Recombinant human growth hormone (rhGH) is used for the treatment of GH deficiency and many pediatric growth disorders (Vance & Mauras, 1999). Studies have shown that rhGH increases lean body mass, skeletal muscle mass, muscle force and strength, aerobic performance, and fat loss (Cuneo et al., 1992; Moller et al., 1995). GH also increases
cardiac output during exercise, increases sweating rates and improved thermal homeostasis, increases lipolysis to provide fuel and energy, and possibly enhances ligamentous strength, and increases wound-healing rates. On the other hand, possible side effects of rhGH abuse include sodium and water retention, hypertension, cardiac failure, accelerated osteoarthritis, and increased incidence of cancerous malignant tumors (Wallace et al., 1999).

The major functions of rhGH can be summarized by the following:

- **Lipid metabolism**: rhGH reduces serum LDL, apolipoprotein B, and serum total cholesterol therefore resulting in lipid mobilization, reduction in body fat stores and adipose tissue, and increased plasma fatty acids.
- **Carbohydrate metabolism**: rhGH increases insulin secretion but usually keeps blood glucose level unchanged. Large doses of GH may impair glucose tolerance.
- **Protein metabolism**: rhGH increases cellular protein synthesis leading to linear growth and increases muscle mass through the creation of new muscle cells. It also leads to nitrogen retention due to decreased urinary nitrogen excretion and serum urea nitrogen.
- **Water and Mineral Metabolism**: rhGH induces sodium, potassium and phosphorous retention.
- **Bone metabolism**: rhGH stimulates skeletal bone turnover and increases the bone mineral content and density at weight-bearing sites. Moreover, it directly stimulates the division and multiplication of chondrocytes of cartilage and increases calcium retention and strengthens the mineralization of bone.
- **Physical capacity**: rhGH improves cardiac output, muscle strength, and physical exercise capacity.

(Cuneo et al., 1992; Moller et al., 1995; Vance & Mauras, 1999; Wallace et al., 1999)

### 1.20. Indications and usage of Genotropin (rhGH)

Genotropin lyophilized powder is indicated for:

- Long-term treatment of pediatric patients who suffer from growth failure due to an inadequate secretion of endogenous GH.
• Long-term treatment of pediatric patients who have growth failure due to Prader-Willi syndrome (PWS).
• Long-term treatment of growth failure in children who fail to manifest catch-up growth by age 2.
• Long-term treatment of growth failure associated with Turner Syndrome in patients who have open epiphyses.
• Long-term replacement therapy in adults who suffer from growth hormone deficiency (GHD) either during childhood or adult-onset etiology.

1.21. Purpose of study:
A very important issue regarding ghrelin physiology is whether an axis or loop exists between pituitary GH on one hand and plasma, stomach, pancreas and kidney ghrelin on the other hand thus reflecting how GH elevations or reductions directly affect ghrelin homeostasis and secretion. The aim of this experiment is to study the effects of a 5 day treatment of genotropin (rhGH) subcutaneous injection on active ghrelin concentration in rat plasma, stomach, kidney, and pancreas samples; and to compare the different ghrelin concentrations per microgram (µg) of protein in rat stomach, kidney and pancreas supernatant.
Chapter 2

MATERIALS AND METHODS

2.1. Animals:
Sprague-Dawley male rats n=28 (Lebanese American University stock) were randomly divided into four groups (7 rats per group) with an average body weight of 200 g. Animals were maintained at a constant ambient room temperature of 22 ± 2°C with a 12-h light, 12-h dark cycle and were given standard laboratory diet and water ad libitum. All animal procedures were performed in accordance with the National Institutes of Health guidelines for the human care of laboratory animals.

2.2. Recombinant human somatropin (rhGH):
The rhGH used for this experiment is Genotropin 5.3mg powder for injection with solvent and it was obtained from Pfizer New Zealand and supplied by Food & Drug Corporation (FDC) pharmaceutical company, Lebanon. Genotropin lyophilized powder contains somatropin [rDNA origin], which is a polypeptide hormone of recombinant DNA origin. It has 191 amino acid residues and a molecular weight of 22,124 daltons. The amino acid sequence of the product is identical to that of human growth hormone of pituitary origin (somatropin). Genotropin is synthesized in a strain of Escherichia coli that has been modified by the addition of the gene for human growth hormone. Genotropin 5.3mg is a sterile white lyophilized powder intended for subcutaneous use. It is made up of a two-chamber cartridge which contains 5.3mg somatropin glycine, sodium dihydrogen phosphate anhydrous, disodium phosphate anhydrous, water for injection, m-cresol, and mannitol.

2.3. Dosage and Administration:
The four groups of experimental animals were divided into one control group and three treatment groups. The 3 treatment groups (Group I, Group II, Group III) received a subcutaneous injections of Genotropin (2, 20, and 100 µg/rat/day) respectively. Injections were performed once per day in the morning (between 8am and 10am) for 5 consecutive
days. Genotropin 5.3mg powder was diluted using sterilized normal saline 0.9% NaCl in order to reach the respective injection dosages.

2.4. Sample preparation and storage:

Rats were sacrificed in the ad lib fed condition 24 hrs after the end of the 5-day experiment (Day 6 between 8am and mid-day).

Plasma samples: In order to prepare plasma samples, animals were sacrificed using Diethyl ether and 4ml of whole blood was directly drawn from the Inferior Vena Cava into a centrifuge tube that contains 500U of Aprotinin and 1.25mg of EDTA-2Na per 1 mL of whole blood. Tubes were gently shaken and then immediately the blood samples were centrifuged at 1,500 x g for 15 minutes at 4 °C. To each tube, 100 µL of 1 mol/L HC1 per mL of collected plasma was added. Plasma samples were then transferred and stored in separate tubes in deep freeze at < -80°C for later use.

Avoid freeze/thaw cycles. Samples should be processed as quickly as possible and kept on ice to prevent or minimize the breakdown of active ghrelin.

Tissue samples: Rat stomach, kidney, and pancreas were removed, cleaned and washed thoroughly with cold saline. Tissues were then blotted using filter paper and the weight of each tissue was recorded then tissues were dipped in liquid nitrogen and stored in deep freeze at < -80°C for later use. When samples were to be prepared, tissue was diced and boiled for 5 mins in a 10-fold volume of water (for example: 1.4gm of tissue + 14gm of distilled water). Solutions were adjusted to a final concentration of 1 M acetic acid & 20mM HCl after cooling. Tissues were then homogenized using a homogenizer Polytron mixer and then centrifuged at 15,000 rpm for 10mins at 4°C using a Sorvall Evolution RC Centrifuge. Supernatants were obtained as tissue samples (Sato et al., 2005).

2.5. Protein Determination:

Protein concentrations in stomach, kidney and pancreas supernatants were determined colorimetrically by using Lowry assay (Lowry et al., 1951). Bovine Serum Albumin (BSA) was used as a standard and absorbance was measured at a wavelength = 750nm.
Protein concentrations in the different samples were calculated in µg/ml using the BSA standard curve.

Solutions used in Lowry assay:

Solution A: 0.5g CuSO₄.5H₂O + 1g Na₃C₆H₅O₇.(2H₂O) Add distilled water to 100ml.

Solution B: 20g Na₂CO₃ + 4g NaOH Add distilled water to 1 liter.

Solution C: 1ml solution A + 50ml solution B

Solution D: 10ml Folin-Ciocalteu phenol reagent + 10ml distilled water

Assay: 1. Bring sample size to 0.5ml with distilled water.
   Note: In this experiment, the original sample size used was 50 µl. Therefore dilute samples 10 times and take 0.5ml for assay.
   2. Add 2.5ml Solution C.
   3. Vortex and let stand at room temperature for 5-10 minutes.
   4. Add 0.25ml Solution D and vortex.
   5. After 20-30 minutes, read absorbance at 750nm.
   6. Use BSA standard curve to record the respective protein concentrations. Then multiply by the dilution factor.

2.6. Quantification of plasma and tissue samples:

To quantify plasma, stomach, kidney and pancreas samples, we use Active Ghrelin ELISA Kits, according to the manufacturer’s protocol (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). The Active Ghrelin ELISA kit is used for the non-radioactive quantification of Active Ghrelin in EDTA plasma. This kit recognizes the octanoyl-modified portion of ghrelin based on the principle of 2 site Sandwich enzyme-linked immunosorbent assay (ELISA)

ASSAY PROCEDURE:
Pre-warm all reagents to room temperature prior to setting up the assay.
1. Dilute the 20X concentrated Wash Buffer 20 fold by mixing the entire content of
buffer with 760 mL deionized or distilled water.

2. Dilute only the required volume of HRP conjugated antibody with appropriate volume of HRP dilution buffer such that the conjugate is diluted 1:100 (example: 10 µL added to 990 µL). Unused portion of the HRP conjugated antibody should be stored at 4°C in the original concentrated form.

3. Remove the required number of strips of the microtiter plate from the foil pouch. Store unused strips in the pouch at 4°C

4. Add 150 µL Assay Buffer to all wells.

5. Add in duplicate 50 µL Assay Buffer to blank wells.

6. Add in duplicate 50 µL Active Ghrelin Standards in the order of ascending concentration to the appropriate wells. Add in duplicate 50 µL QC1 and 50 µL QC2 to the appropriate wells. Add sequentially 50 µL of the unknown samples in duplicate to the remaining wells.

7. Cover the plate with plate sealer and incubate at room temperature for 2 hours.

8. Remove plate sealer and decant solutions from the plate. Tap to remove residual solutions in the wells. Wash wells 3 times with diluted Wash buffer, 300 µL per well per wash, with 1 minute soak time with the wash buffer between each wash. Decant and tap firmly after each wash to remove residual buffer.

9. Add 200 µL diluted HRP conjugated antibody to all wells.

10. Cover the plate with plate sealer and incubate at room temperature for 1 hour.

11. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in the wells. Wash wells 4 times with diluted Wash buffer, 300 µL per well per wash, with 1 minute soak time with the wash buffer between each wash. Decant and tap firmly after each wash to remove residual buffer.

12. Add 200 µL Substrate Solution to each well. Cover plate with sealer and incubate in the dark at room temperature for 30 minutes.

13. Remove sealer and add 50 µL Stop Reagent [CAUTION: CORROSIVE SOLUTION] and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn to yellow after acidification. Read absorbance at 450 nm in a plate reader within a few minutes and ensure that there are no air bubbles in any well. Record the difference of absorbance units.
N.B: Range of assay is 2.5 fmol/ml – 160 fmol/ml. Therefore, any result greater than 160 fmol/ml in a 50µl sample should be diluted using assay buffer, and the assay repeated until the results fall within range.

Preliminary experiments were first conducted in order to determine the optimum dilution factor that needs to be used with each type of tissue samples. Data have shown that:

- All stomach samples need to be diluted 100 times using assay buffer.
- All kidney and pancreas samples need to be diluted 10 times using assay buffer.

Results of ghrelin final concentrations were then multiplied by their respective dilution factors.

2.7. Statistical analysis:

Results were presented as mean ± SEM for each group. Comparison between the control group versus treatment groups I, II, and III was analyzed using independent t test. P < 0.05 was accepted as statistically significant.
3.1. Protein Determination:

3.1.1. Protein Concentration in Stomach Supernatant:

Genotropin injection led to an increase in protein concentration in stomach samples that was very evident in Group III which received the highest dosage of Genotropin subcutaneous injection (100 µg/rat/day). Data are shown in Figure 3.1.

**Figure 3.1** Protein concentration in stomach supernatant measured in µg/ml.

* Significant difference with respect to the control (p<0.05)
Bars denote mean ± SEM (n=7)
3.1.2. Protein Concentration in Kidney Supernatant:

Genotropin injection led to an increase in protein concentration in kidney samples and this was evident in Groups II & III which received respectively 20 and 100 µg of genotropin injection/rat/day. Data are shown in Figure 3.2

![Protein Concentration in Kidney Supernatant](image)

**Figure 3.2** Protein concentration in kidney supernatant measured in µg/ml.
* Significant difference with respect to the control (p<0.05)
Bars denote mean ± SEM (n=7)

3.1.3. Protein Concentration in Pancreas Supernatant:

Genotropin injection led to a drastic decrease in protein concentration in the pancreas samples and this was clearly shown in all treatment Groups I, II, & III. Data are shown in Figure 3.3
**Figure 3.3**  Protein concentration in pancreas supernatant measured in µg/ml.

* Significant difference with respect to the control (p<0.05)

Bars denote mean ± SEM (n=7)

### 3.2. Active Ghrelin Concentration in plasma and tissue samples:

#### 3.2.1. Active Ghrelin Concentration in Plasma samples:

Genotropin injection led to a significant increase in active ghrelin concentration in the plasma. This increase was proportional to the concentration of genotropin being injected. Data are shown in Figure 3.4
3.2.2. Active Ghrelin Concentration in Stomach Supernatant:

Genotropin injection led to a significant decrease in active ghrelin concentration in the stomach supernatant which was inversely proportional to the concentration of genotropin being injected. Data are shown in Figure 3.5.

Figure 3.4  Active ghrelin concentration in plasma measured in fmol/ml.

* Significant difference with respect to the control (p<0.05)

Bars denote mean ± SEM (n=7)
**Figure 3.5** Active ghrelin concentration in stomach supernatant measured in fmol/ml.

* Significant difference with respect to the control (p<0.05)

Bars denote mean ± SEM (n=7)

### 3.2.3. Active Ghrelin Concentration in Kidney Supernatant:

Genotropin injection led to a decrease in active ghrelin concentration in the kidney. This was very evident in Groups II & III which received respectively 20 and 100 µg of genotropin injection/rat/day. Data are shown in Figure 3.6
3.2.4. Active Ghrelin Concentration in Pancreas Supernatant:
Genotropin injection led to a significant decrease in active ghrelin concentration in the pancreas supernatant which was inversely proportional to the concentration of genotropin being injected. Data are shown in Figure 3.7

**Figure 3.6**  Active ghrelin concentration in kidney supernatant measured in fmol/ml.
* Significant difference with respect to the control (p<0.05)
Bars denote mean ± SEM (n=7)
**Figure 3.7** Active ghrelin concentration in kidney supernatant measured in fmol/ml.

* Significant difference with respect to the control (p<0.05)

Bars denote mean ± SEM (n=7)

### 3.3. Average Active Ghrelin Concentration in fmol/µg of Protein in Tissue

**Supernatant:**

#### 3.3.1. Active Ghrelin Concentration in fmol/µg of Protein in Stomach Supernatant:

Genotropin injection led to a decrease in active ghrelin concentration found in 1 µg of protein in stomach supernatant. This was evident in Groups II & III which received respectively 20 and 100 µg of genotropin injection/rat/day. Data are shown in Figure 3.8.
Active Ghrelin Concentration in fmol per Microgram of protein in Stomach Supernatant

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>Concentration in fmol/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.80</td>
</tr>
<tr>
<td>Group I</td>
<td>5.95</td>
</tr>
<tr>
<td>Group II</td>
<td>5.22 *</td>
</tr>
<tr>
<td>Group III</td>
<td>2.20</td>
</tr>
</tbody>
</table>

Bars denote mean ± SEM (n=7)

* Significant difference with respect to the control (p<0.05)

**Figure 3.8** Active ghrelin concentration measured in fmol/µg of protein in stomach supernatant.

3.3.2. Active Ghrelin Concentration in fmol/µg of Protein in Kidney Supernatant:

Genotropin injection led to a significant decrease in active ghrelin concentration found in 1 µg of protein in kidney supernatant. This was evident in all treatment Groups I, II, & III. Data are shown in Figure 3.9
Figure 3.9  Active ghrelin concentration measured in fmol/µg of protein in kidney supernatant.

* Significant difference with respect to the control (p<0.05)

Bars denote mean ± SEM (n=7)

3.3.3. Active Ghrelin Concentration in fmol/µg of Protein in Pancreas Supernatant:
There was no significant difference in active ghrelin concentration found in 1 µg of protein in pancreas supernatant in all experimental groups. Data are shown in Figure 3.10
Figure 3.10  Active ghrelin concentration measured in fmol/µg of protein in pancreas supernatant.

No significant difference was recorded with respect to the control.
Bars denote mean ± SEM (n=7)
### 3.4. Summary of Independent $t$ test

Significant $P$ values ($P < 0.05$) between control Group and treatment Groups I, II, III.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$P$ Value between control group &amp; group I</th>
<th>$P$ Value between control group &amp; group II</th>
<th>$P$ Value between control group &amp; group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein Concentration in Stomach Supernatant</td>
<td>No significant difference</td>
<td>No significant difference</td>
<td>7.20128E-10</td>
</tr>
<tr>
<td>Protein Concentration in Kidney Supernatant</td>
<td>No significant difference</td>
<td>0.01284</td>
<td>6.26371E-08</td>
</tr>
<tr>
<td>Protein Concentration in Pancreas Supernatant</td>
<td>0.00208</td>
<td>2.64062E-06</td>
<td>5.3934E-12</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in Plasma samples</td>
<td>No significant difference</td>
<td>0.00019</td>
<td>7.46914E-06</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in Stomach Supernatant</td>
<td>0.04015</td>
<td>0.00129</td>
<td>8.18696E-06</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in Kidney Supernatant</td>
<td>No significant difference</td>
<td>2.41533E-07</td>
<td>2.32993E-08</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in Pancreas Supernatant</td>
<td>0.00746</td>
<td>2.17789E-06</td>
<td>1.21197E-08</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in fmol/µg of Protein in Stomach Supernatant</td>
<td>No significant difference</td>
<td>0.00975</td>
<td>2.67473E-08</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in fmol/µg of Protein in Kidney Supernatant</td>
<td>0.02090</td>
<td>2.74157E-06</td>
<td>6.343E-09</td>
</tr>
<tr>
<td>Active Ghrelin Concentration in fmol/µg of Protein in Pancreas Supernatant</td>
<td>No significant difference</td>
<td>No significant difference</td>
<td>No significant difference</td>
</tr>
</tbody>
</table>

**Table 3.1.** Independent $t$ test Significant $P$ values ($P < 0.05$) between control Group and treatment Groups I, II, III.
Chapter 4

DISCUSSION AND CONCLUSIONS

Ghrelin is the endogenous ligand for the growth hormone secretagogue (GHS) receptor and has potent growth hormone-releasing activity. Ghrelin exists in two major forms: the 28 amino acid ghrelin having n-octanoylated serine in position 3, and the 27 amino acid des-acyl ghrelin produced by alternative splicing of the ghrelin gene (Kojima et al., 1999; Hosoda et al., 2000; Bowers, 2001; Broglio et al., 2003). Many studies in the literature have focused on measuring the concentration of ghrelin in plasma and tissues based on radioactive quantification assays, RNA analysis, chromatography, and histochemistry etc… however, the present study measures active ghrelin concentration in rat plasma, stomach, kidney, and pancreas supernatants based solely on Active Ghrelin ELISA kit which is used for the non-radioactive quantification of Active Ghrelin in EDTA plasma. This kit recognizes the octanoyl-modified portion of ghrelin based on the principle of 2 site Sandwich enzyme-linked immunosorbent assay (ELISA). Moreover, the study focuses on reflecting the direct effect of subcutaneous injection of genotropin on active ghrelin concentration in plasma, stomach, kidney and pancreas samples in the rat model.

The acyl-modified ghrelin is the active form; therefore certain crucial steps should be followed during sample extraction due to the fact that this modification is easily cleaved and peptides are easily digested by many proteases found in cells (Kojima & Kangawa, 2005). In order to measure the ghrelin concentration in plasma samples, it is necessary to use EDTA and aprotonin (Hosoda et al., 2000; Hosoda et al., 2004). As for the tissue samples, it is sufficient to keep active ghrelin intact by boiling the tissue in water for 5-10 minutes in order to inactivate proteases (Kangawa & Matsuo, 1984; Sudoh et al., 1988).

In this study, if we compare the active ghrelin concentration in all control groups, we notice that the highest concentration of active ghrelin is detected in the stomach supernatant which is much greater than that found in the kidney and pancreas supernatants and the plasma samples. The active ghrelin concentration in the stomach
supernatant is approximately 96 times more than that found in the plasma samples (1326.1 ± 17.31 fmol/ml versus 13.81 ± 1.1 fmol/ml). This result reinforces all previous findings which state that ghrelin is mainly produced in the stomach and that gastrectomy in rats or removal of the acid-producing part of the stomach reduces serum ghrelin concentration by approximately 80% thus proving that the stomach is the primary source of GHS-R ligand (Date et al., 2000; Hosoda et al., 2000; Ariyasu et al., 2001; Dornonville de la Cour et al., 2001).

Few experiments have indicated the presence of ghrelin in the kidney, glomerulus and renal cells. It has been shown that mouse kidney contain both ghrelin and desacyl peptide, and ghrelin is present more abundantly in the kidney than in plasma due to the fact that ghrelin immunoreactivities in rats and mice kidneys are approximately 10 times more abundant than those in the plasma (Mori et al., 2000). This is reinforced in the present study which proves that the active ghrelin concentration in kidney supernatant is approximately 8.5 times more than that found in the plasma samples of the control groups (116.56 ± 3.42 fmol/ml versus 13.81 ± 1.1 fmol/ml).

Some studies have proved that the pancreas is a ghrelin producing organ. Analyses based on ghrelin-RIA, m-RNA expression, and chromatography reveal that ghrelin is found in rat pancreas (Date et al., 2002; Volante et al., 2002). The current study proves that active ghrelin is found in rat pancreas and is approximately 12 times more than that found in the rat plasma samples in the control groups (167.64 ± 8.39 fmol/ml versus 13.81 ± 1.1 fmol/ml).

Ghrelin is produced mainly in the gastrointestinal organs in response to hunger, and it is an orexigenic hormone that circulates in the blood serving as a signal to the central nervous system in order to stimulate feeding (Cummings et al., 2001; Tschop et al., 2001a). Ghrelin is, therefore, an initiation signal for food consumption and a hunger signal. This is reinforced by the fact that plasma ghrelin levels increase and rise approximately two-fold before each meal during intervals of food deprivation possibly due to increase of ghrelin secretion from the gastrointestinal tract into the bloodstream.
and plasma. Moreover, plasma ghrelin levels fall to their minimum levels within 1 hour after eating, most likely reflecting acutely reduced ghrelin secretion from the gastrointestinal tract. This postprandial suppression of plasma ghrelin level is directly proportional to the type of food ingested and to the caloric load (Cummings et al., 2001; Tschop et al., 2001a; Callahan et al., 2004). In this experiment, rats were sacrificed in the ad libitum fed state in compliance with the experimental procedures of most published papers (Mori et al., 2000; Date et al., 2002; Egido et al., 2002; Qi et al., 2003; Sato et al., 2005). This experimental procedure, however, might be a drawback or a disadvantage because the time before or after meal initiation or fasting was not monitored since all experimental animals had free access to food anytime through the day or night. The above mentioned reasons might lead to fluctuations in recorded plasma ghrelin concentrations which are different from those published in the literature. For more precise results, a good suggestion for further studies would be to fast the rats overnight prior to sacrificing them the following morning.

A very important issue regarding ghrelin physiology is whether an axis or loop exists between pituitary GH and stomach ghrelin thus reflecting how GH elevations or reductions directly affect stomach ghrelin homeostasis and secretion. Qi et al., in 2003, proved that a 3-day treatment with high doses of exogenous GH subcutaneous injections (400µg 3 times/day) decreases mRNA expression of stomach ghrelin and secretion in rats. This suggests that pituitary GH exhibits a feedback regulation on stomach ghrelin. However, in the same study, administration of exogenous GH did not affect ghrelin stores in the rat. This inability of increased systemic GH levels to lower stomach ghrelin levels may be attributed to the fact that caloric intake affects stomach homeostasis (Lee et al., 2002) which may be difficult to separate from the GH feedback effect.

Another study conducted by Lee et al. (2002) shows that there is a gradient of ghrelin expression in the rat gastrointestinal tract with the most abundant expression in the stomach fundus. Moreover, the same study reports that GH treatment lowers stomach ghrelin expression levels thus suggesting that a neuroendocrine feedback loop exists between stomach ghrelin and pituitary GH.
Furthermore, Tshop et al. (2002), shows that administration of GH reduces circulating ghrelin levels in normal rats and increases circulating ghrelin levels 3-fold in hypophysectomized rats thus suggesting a neuroendocrine feedback loop between pituitary GH and stomach ghrelin in the regulation of gastric ghrelin secretion. On the other hand, a study conducted by Van der Toorn et al. (2002), concludes that high GH concentrations do not lead to decreased ghrelin concentrations in human acromegaly. Moreover, Janssen et al. (2001), state that systemic ghrelin levels remain unchanged and are not affected after one year of growth hormone replacement therapy in subjects with GH deficiency. Whereas, another study conducted by Dall et al. (2002), records lower plasma ghrelin levels after exercise during GH replacement for a short term. Moreover, Engström et al. (2003), suggests that treatment with GH for 9 months leads to a decrease in systemic ghrelin levels.

In the current study, genotropin injection led to a significant decrease in active ghrelin concentration in the stomach supernatant in agreement with most published research papers; however, it led, unexpectedly, to an increase in plasma ghrelin concentration. This controversy in the results dealing with an increase in plasma ghrelin concentration rather than a decrease, as proved by Qi et al. (2003), might be due to the fact that ghrelin secretion is associated much more with food intake than with GH pulses which may be difficult to separate from the feedback effect of GH (Lee et al., 2002; Tolle et al., 2002).

However, GH stimulates food intake (Moller et al., 1995; Malmlof et al., 2002), which is directly related to an increase in appetite; hence, we suspect that the increase in plasma ghrelin concentration which was recorded in the current study might be attributed to the increase in appetite after rhGH injections basing our assumptions on the fact that ghrelin stimulates appetite and has an orexigenic effect (Tschop et al., 2000; Wren et al., 2000; Horvath et al., 2001; Wren et al., 2001a; Wren et al., 2001b; Muccioli et al., 2002; Yoshihara et al., 2002).

Therefore, stomach ghrelin homeostasis and secretion are greatly affected by food intake, caloric restriction and dietary manipulations. Furthermore, the high ghrelin
concentration in the stomach explains why small changes in its concentration lead to drastic changes in the plasma ghrelin.

The half-life of genotropin after subcutaneous administration is 2-3 hours due to slow absorption from the injection site following subcutaneous administration (Information for health professionals, Data Sheet, 2004). Moreover, several studies suggest that the half-life of circulating ghrelin is less than one hour (Cummings et al., 2001; Nagaya et al., 2001). In the current study, the experimental rats were sacrificed 24 hours after the last genotropin subcutaneous injection; therefore, the difference between the current results and other studies should not be attributed to fluctuations in plasma ghrelin levels due to the short half-life of ghrelin. However, the difference in experimental setups, GH dosage used, duration of GH treatment (short term versus long term), and assay procedures all play an important factor in the different recorded results. Therefore, further studies should be performed in order to accept or negate this hypothesis.

The results of this study showed that genotropin injection led to a decrease in active ghrelin concentration in the kidney and pancreas supernatant. This was very evident in Groups II & III which received respectively 20 and 100 µg of genotropin injection/rat/day. No published papers till date reflect the effect of subcutaneous GH injection on active ghrelin concentrations in kidney and pancreas samples. In our opinion, the kidney and pancreas both showed a similar trend of decrease in active ghrelin concentration as that detected in the stomach samples after GH administration. Since the results showed that GH lowers active ghrelin concentration in kidney and pancreas, we suspect that there might exist a feedback loop between kidney and pancreas ghrelin on one hand and pituitary GH on the other hand. This suggests that pituitary GH exhibits a negative feedback regulation on kidney and pancreas ghrelin similar to the case of stomach ghrelin. However, many further studies should be conducted on a larger number of subjects and using several assays for ghrelin determination in order to confirm or reject the results of the current study.
It was also noticed in this study that the changes in stomach ghrelin were much less than that observed in the kidney and pancreas by far. Moreover, there is a high inverse correlation between plasma ghrelin on one hand and the pancreas and kidney ghrelin on the other hand. It might be suspected here that ghrelin secreted by the stomach may not be taken up properly by other organs due to resistance to ghrelin. We also suspect that the stores of stomach ghrelin might be affected by the five-day treatment of GH. This may be due to either a decreased ghrelin expression or to an increased ghrelin secretion by the stomach in order to cope with the increased resistance of tissues to ghrelin. The present study supports the idea of increased ghrelin secretion. Another hypothesis might be that since ghrelin expression was reduced in all organs, this might have affected the ghrelin stores in the kidney and pancreas that have shut down their receptor mediated endocytosis of ghrelin. That’s why we noticed a sharp decrease in their ghrelin concentration. Further suggested studies would include subcutaneous injection of GH and radio-labeled ghrelin in order to monitor receptor mediated endocytosis of ghrelin by different organs.

As for protein determination using Lowry assay, it was noticed that genotropin had a direct proportional effect on the protein concentration of stomach and kidney samples. The protein content was significantly higher than the control especially in Groups II & III which received respectively 20 and 100 µg of genotropin injection/rat/day. Lee et al., in 2002, explained that protein deprivation increases ghrelin secretion by stimulating apetite and GH secretion which, in turn, stimulates protein synthesis. This might explain the direct effect of genotropin injection on the increased protein concentrations in the rat stomach and kidney samples.

On the other hand, a significant decrease in the protein concentration in the pancreas supernatant was detected after GH treatment. This result supports the concept of Zhang et al.(2001), who proved that ghrelin is a potent inhibitor of pancreatic protein secretion in rats via a mechanism involving the intrapancreatic nervous system. As discussed before, genotropin injection led to a significant decrease in active ghrelin concentration in the pancreas supernatant which was inversely proportional to the concentration of genotropin
being injected. Therefore, more genotropin leads to less ghrelin levels and consequently more protein secretion from the pancreas into the bloodstream thus resulting in lower pancreatic protein concentration.

The study concludes that the active ghrelin concentration in fmol/µg of protein in the stomach and kidney supernatant decreases after treatment with genotropin due to the fact that genotropin led to the decrease of active ghrelin concentration in both stomach and kidney samples measured in fmol/ml on one hand, and to an increase in protein concentration in these 2 tissue samples measured in µg/ml on the other hand. Therefore, if we calculate the active ghrelin concentration in fmol/µg of protein in stomach and kidney samples, we notice an overall decrease in this concentration.

However, there was no significant difference in the active ghrelin concentration in fmol/µg of protein in the pancreas supernatant after treatment with genotropin due to the fact that genotropin led to a decrease in the active ghrelin concentration in pancreas samples measured in fmol/ml, and a decrease in the protein concentration in the pancreas samples measured in µg/ml. Therefore, if we calculate the active ghrelin concentration in fmol/µg of protein in pancreas samples, we notice that there is no significant difference between the control group on one hand and the experimental groups on the other hand. Therefore, the present study supports the protocol of whole organ ghrelin concentration rather than ghrelin concentration in fmol/µg of protein in tissue samples because this might be misleading.

In conclusion, the results of the present study suggest that a short-term treatment of genotropin (rhGH) subcutaneous injection increases active ghrelin concentration plasma but decreases active ghrelin concentration in stomach, kidney, and pancreas samples in the rat model thus suggesting a feedback loop between stomach, kidney and pancreas ghrelin on one hand, and pituitary GH secretion on the other hand. Moreover, the study suggests that short-term treatment of genotropin (rhGH) subcutaneous injection increases protein concentration in both stomach and kidney but decreases protein concentration in the pancreas.
Chapter 5

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