Insights from growth hormone receptor blockade

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Inzichten door groeihormoon receptor blokkade

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Contents

Chapter I - Introduction

The role of the pituitary gland as being responsible for "the hormone that accelerates skeletal growth", or the "hormone of growth" was first acknowledged by Harvey Cushing (1;2). Acromegaly is a rare disease due to prolonged hypersecretion of growth hormone (GH). In virtually all cases of acromegaly the cause is a benign GH-secreting pituitary adenoma. In 1909 Cushing reported that the clinical symptoms of acromegaly remitted after partial hypophysectomy thus pointing to the pituitary as playing a central role in the cause of acromegaly (3;4). He also recognized the general role that the pituitary gland has in the regulation of the endocrine system and postulated that certain diseases could be explained by pituitary hypo- or hyperfunction (1). Evans and Long confirmed the pituitary source of acromegaly when intraperitoneal injection of anterior pituitary extracts in rats resulted in acromegaly (5). Later it was demonstrated that these pituitary extracts increased tibial epiphyseal width, which then could be used as a bioassay (6;7). After Raben succeeded in isolating GH (8), and a radioimmunoassay for GH was developed it turned out that GH concentrations were indeed elevated in patients with acromegaly (9-11).

In 1953 the growth promoting effect of pituitary extracts was found to be related to sulphate incorporation into cartilage (12). However, in subsequent experiments it became clear that in vitro GH could not stimulate the incorporation of labeled sulphate into cartilage from hypophysectomized rats; whereas, serum from rats could (13). Apparently the growth promoting effects of GH were mediated through a "sulphation factor" present in serum. Interestingly, sera from patients with acromegaly contained elevated levels of this so called "sulphation factor" (14).

In 1963 Froesch et al. described that only a fraction of the insulin-like activity of normal serum could be blocked by the addition of anti-insulin antibodies (15). The term "non-suppressible insulin-like activity" was coined (15). Dulak and Temin described another factor called "multiplication-stimulating activity" which has mitogenic activity (16). In 1972 "sulphation factor", "non-suppressible insulin-like activity" and "multiplication-stimulating activity" were grouped together and named somatomedin (17). Later Rinderknecht and Humble isolated two active somatomedins from human plasma and demonstrated a striking resemblance to proinsulin (18;19). Accordingly the somatomedins were renamed Insulin-like growth factors.

Growth hormone regulation

Two hypothalamic hormones, GH-releasing hormone (GHRH), and somatostatin (SRIF) are key regulators of GH secretion. GHRH stimulates GH secretion whereas somatostatin inhibits GH secretion (20-22) (Figure 1).
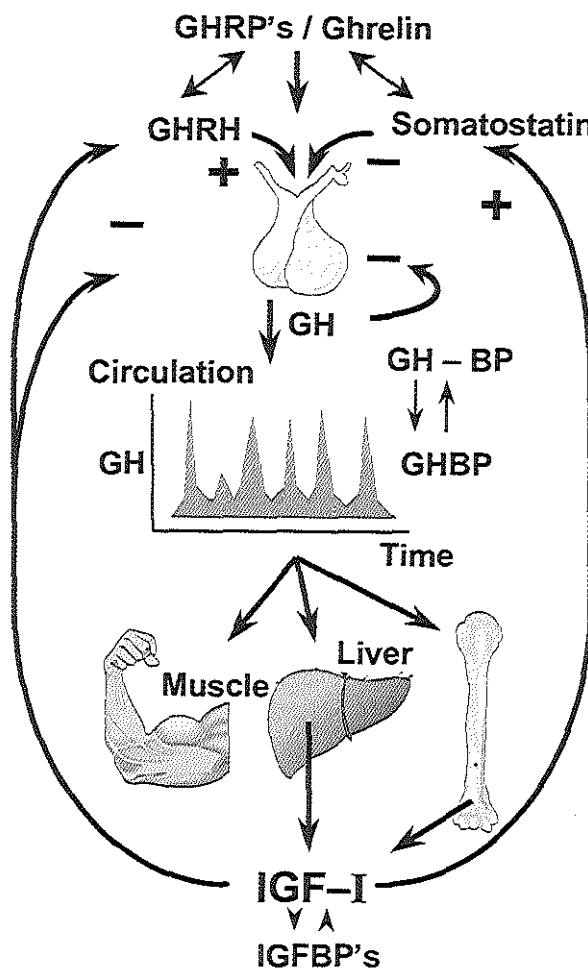
Figure 1: *Overview of the regulation of the GH-IGF-I axis.*

Only very recently a third player in the field of GH regulation has been identified: the growth hormone secretagogue (GHS) system and especially ghrelin (23;24). Considering the fact that in several of the studies described in this thesis ghrelin and one of its synthetic analogs (Growth Hormone Releasing Peptide-6) play an important role these compounds will be discussed in a separate section (vide infra).

GH stimulates the production of insulin-like growth factor-I (IGF-I) in the liver and local tissues (25). In addition to the stimulation of IGF-I synthesis GH also promotes the formation of the ternary IGF binding complex, including IGF binding protein3 (IGFBP3) and acid-labile-subunit (ALS) (vide infra), which stabilizes IGF-I in serum (25).

A role for IGF-I in the regulation of GH was suggested by intracerebroventricular injection of IGF-preparations in rats, which markedly diminished the amplitude of the observed GH pulses (26). However, these early preparations might have contained both IGF-I and IGF-II as in more recent experiments a combination of both recombinant human IGF-I and IGF-II was required to reproduce these observations (27;28). Further evidence for a role of IGF-I in the negative feedback regulation of GH secretion was gathered from several in vitro experiments in which IGF-I decreases GH secretion and mRNA levels in cultured rat pituitary cells (29;30). Moreover, even GH-secreting pituitary adenomas appear sensitive to the negative feedback exerted by IGF-I. Lamberts et al. demonstrated that during a 24-hour incubation IGF-I directly inhibits GH release in four of seven primary tumor cell cultures prepared from somatotroph adenomas (31).

IGF-I in serum is bound to several binding proteins: the so-called Insulin-like growth-factor binding proteins (IGFBP's). To date six IGFBP's have been identified (32;33). All IGFBP's have a high degree of sequence homology and are termed IGFBP 1 to 6 in the order in which their primary structure was unraveled (32;34). These circulating IGFBP's act as carrier proteins, transporting IGF to target tissues and prolonging the half-life of IGF by protecting them from proteolytic degradation (25). In addition to their major roles in circulation, most target tissues also express IGFBP's. These IGFBP's further regulate actions of IGF at the tissue level (25;34).

IGFBP-3 is the predominant binding protein in human serum (35). After binding IGF-I, these two couple with ALS and thus a ternary complex is formed that is the main storage form of IGF-I in circulation. In humans both IGFBP3 and ALS are GH dependent (35). Approximately 75-95% of total IGF-I is present in the ternary complex (35;36).

IGFBP1 was first identified and purified from amniotic fluid (37). IGFBP1 has been proposed as an acute regulator of IGF-I activity and insulin seems to be a major regulator of IGFBP1 production (33;38;39). Most studies are consistent with an inhibitory action of IGFBP1 on IGF-I production in certain cell systems. However, IGFBP1 potentiates IGF-I effects possibly through binding of IGFBP1 itself to the cell membrane (32;39-41). The role of

the other IGFBP's in IGF-I action is less well established and will not be discussed here.

Growth Hormone Releasing Peptides and ghrelin

Since 1976 Bowers et al. and other groups have developed a series of peptides derived from met-enkephaline with powerful GH-releasing capacities. These peptides are called GH-Releasing Peptides (GHRP's) (42-44). Moreover, in order to develop an orally active GH-secretagogue (GHS) several groups have developed non-peptidyl analogues of these GHRP's of which MK-0677 is probably the most studied compound to date (45;46). The GHRP's probably exert their GH-releasing action through a GHS-receptor (GHS-R) expressed in hypothalamus and pituitary (45).

All GHRP's exert a direct GH releasing effect on the pituitary, as they are capable to stimulate GH release from somatotrophs cultured in vitro (45). However, in vivo GH release - is exerted via a hypothalamic pathway. After administration of a specific antagonist to GHRH in healthy men, GH response to GHRP-6 is eliminated (47). Moreover, in patients with a hypothalamo-pituitary disconnection GHRP's do not induce GH release (48). Finally, the combined administration of GHRH and a GHS has profound synergy (43;49).

Only very recently a natural ligand for the GHS-R1a has been identified and called ghrelin (23). Surprisingly the highest concentration of ghrelin was found in the mucosa of the stomach and not in the hypothalamus or pituitary gland (23). Unique is the posttranslational addition of a n-octanoyl group covalently linked to the hydroxyl group of serine on position three via an ester linkage (50;51). The n-octanoyl group adds a hydrophobic property to the N terminus that may facilitate entry and distribution in the brain (50). Non-octanoylated (des-acyl) ghrelin is biologically inactive and has no GH releasing capacity (23;50).

The GHS-R was characterized using a radiolabeled synthetic GHS (MK-0677). Subsequently it was shown that GHRP-6 competitively inhibits binding of MK-0677 to the GHS-R (45). Also, the signal transduction pathways for MK-0677 and GHRP-6 both involve phospholipase C, resulting in a rise in inositol triphosphate and intracellular calcium (45). After identification of this GHS-R, a cell line expressing this receptor was developed and used to identify tissue extracts that could stimulate the GHS-R as monitored by increases in intracellular Ca^{2+} (24). By adding different tissue extracts to this cell line and monitor intracellular Ca^{2+} changes, Kojima et al. isolated

ghrelin from rat and human stomach (23). Therefore we can conclude that GHRP-6 and ghrelin bind to the same GHS-R that is responsible for GH release.

Intravenous ghrelin administration stimulates GH secretion (52-57). Interestingly, apart from this effect on GH secretion, a metabolic action of ghrelin can also be recognized. In animal studies peripheral ghrelin administration stimulates food intake, gastric secretion, gastric motility and obesity (58-65). Tschöp et al. have recently described that ghrelin induces obesity in rodents (58). During peripheral (subcutaneous) ghrelin administration once a day to mice for 2 weeks, body weight and fat mass increased without changes in food intake, locomotor activity, lean body mass, or bone mass (58). Because ghrelin releases GH, and GH is lipolytic rather than lipogenic (vide infra), these findings indicate that other mechanisms that lead to obesity are stimulated as well.

Relationships between GH and fat mass

Observations in acromegalic patients as well as studies on body composition in GH deficient (GHD) patients before and during GH replacement indicate that GHD affects body composition in man (66-68). Already in 1934 it was reported that administration of GH to experimental animals led to a reduction in fat mass (69). In subsequent years indirect evidence – by measuring free fatty acids (FFA) – became available that in man GH also mobilizes fat stores (70-72). Finally, these observations were confirmed by measuring body compositions in patients with disturbances in the GH-IGF-I axis (67;68). In acromegalic patients body composition is characterized by an increase in lean body cell mass, a reduction in fat mass and "overhydration" of the body due to an increase in extracellular water (68;73-75). Prolonged untreated GHD is associated with an increase in fat mass as well as an abnormal fat distribution (67;68;76;77). In GHD patients body fat mass was approximately 6-8 kg higher compared to controls (67;68;78). Moreover, GH replacement therapy leads to a decrease in fat mass in GHD patients (66;68). Based on these data it can be concluded that GH is an active player in the regulation of body composition. However, the opposite is also true: GH levels are also influenced by body composition as well as by nutritional status.

In fasting and other catabolic states IGF-I levels are decreased despite elevated GH concentrations (22). Apparently in these situations subjects lack the ability to produce an adequate IGF-I response to GH. Recent studies suggest that portal vein insulinopenia contributes to the observed "GH

resistance" in catabolism. In such a mechanism down-regulation of hepatic GH receptor expression secondary to portal vein insulin deficiency could explain the apparent GH resistance observed in this disease (22;79). The observed decrease in circulating concentrations of GH Binding Protein (GHBP), a putative index of GH receptor number, in patients with type I diabetes mellitus (characterized by impaired insulin secretion) is consistent with this hypothesis (80).

In obesity both basal and stimulated GH release is attenuated (22;81-86). The mediators of the regulation exerted by the adipose tissue on the GH/IGF-I axis are not fully understood yet, but in the last few years two relevant factors have emerged -FFA and the hormone leptin produced by adipocytes (22;87). FFA and GH integrate in a classical feedback loop whereby a rise in FFA ~ as a result of the previously discussed lipolytic effect of GH – blocks GH secretion (87). This action is rapid, dose-related and exerted at the pituitary level with no evident hypothalamic participation (87). A pharmacological reduction in FFA enhances GH secretion and eliminates the attenuation of GH release that is observed in obesity and Cushing's syndrome (87-89). Another mechanism by which adipose tissue participates in the regulation of GH secretion is leptin (90). Obesity is associated with elevated levels of serum leptin while malnutrition and fasting are associated with decreased leptin levels (87). Indeed, visceral fat mass as estimated by computed tomography scanning is the primary (negative) statistical determinant of GH secretion in middle-aged men and women and accounts for both gender and age dependent differences in GH secretion (84). In fasted rats, the pattern of GH pulsatility is eliminated with a near absence of spontaneous peaks, but the intracerebroventricular administration of leptin restores the altered pattern. On the other hand in fed rats injected intracerebroventricular with antileptin antibodies the normal GH secretory pattern is reversed to an absence of pulses, similar to the fasting state (87). Leptin seems to have no direct pituitary action and its action at the hypothalamic level appears to be mediated by neuropeptide Y, being an important modulator of the somatostatinergic tonus (91).

In conclusion, relevant data indicate that GH and adipose tissue have cross-talk mechanisms. However, at present it is unclear how to conceptualize the role of GH in the regulation and distribution of fat mass in man. Fortunately, recent research in this field – most notably the discovery and description of ghrelin and its physiological role – has generated the potential for new insights in the relationship between GH and fat mass (58;65).

Pegvisomant

In all studies described in this thesis the GH receptor (GHR) antagonist pegvisomant played an important role. In the next section the mode of action and some clinical data will therefore be presented.

Pegvisomant is a genetically manipulated GH molecule that disables signal transduction through the GHR and thus functions as a GHR antagonist (92). The GHR is a transmembrane receptor and consists of an intracellular and an extracellular domain linked by a transmembrane domain of 20 – 30 amino acids (93). The extracellular domain of the GHR is also present in serum where it acts as a GHBP (94). Binding of GH to the GHR is necessary for signal transduction. It has been shown that dimerization of GH with two GHR's is critical for signal transduction to occur (95-97). GH has two distinct binding domains that bind to two identical GHR's at the cell surface. Following the initial – high affinity – binding of the so called GH binding site I at the GHR, sequential binding at GH site II produces functional receptor dimerization and signal transduction for example leading to IGF-I
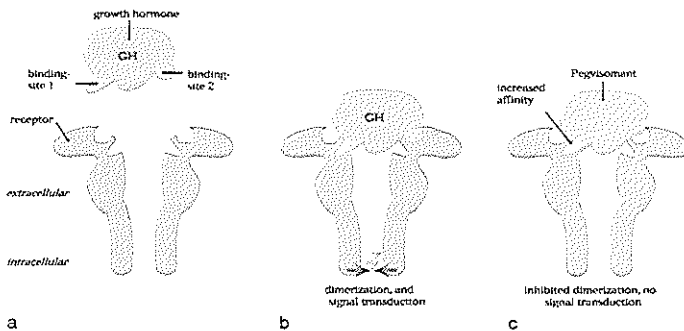
Figure 2: Mode of action of pegvisomant: (a) normally GH binds to the first GHR with binding site I; (b) the subsequent binding of binding site II to the second GHR results in receptor dimerization and signal transduction; (c) 8 mutations at binding site I increase the affinity of pegvisomant for the GHR and 1 mutation at binding site II inhibits receptor dimerization and thus signal transduction. Adapted from Muller AF, Janssen JAMJL, de Herder WW, van der Lely AJ. Growth hormone receptor antagonists: potential indications. Ned Tijdschr Geneeskd 2001; 145 (2):69-73.

generation (Figure 2a & b). The importance of receptor dimerization in signal transduction is indicated by a number of experiments. High concentrations of GH, which favor the monomeric GH-GHR complex, inhibit the GH signal (98). Truncated receptors lacking the cytoplasmic domain act as dominant

negative inhibitors of signaling by heterodimerization with the full-length receptor (99). Mutations in the interreceptor dimerization domain inhibit signaling without influencing GH binding (100). Finally, the strongest evidence comes from work with a GH molecule mutated at site 2 to prevent receptor dimerization (98;101-104). These GH mutants block GH-stimulated cell proliferation, the conformational change associated with receptor dimerization, and Jak-Stat signaling (105;106).

The understanding of the mechanism by which GH interacts with its receptor has facilitated the development of pegvisomant (Figure 2c). Through a single mutation at site II of the GH molecule functional GHR dimerization is inhibited. Moreover, to increase the binding affinity of the GHR antagonist and thus provide the GHR antagonist with a (pharmaco) kinetic advantage compared to endogenous GH, 8 amino acids at binding site I were also mutated (98;101;107). Pegvisomant is pegylated to increase serum half-life time and to reduce the likelihood of antibody formation. Although the unpegylated GHR antagonist does have a higher affinity at site I of the GH, compared with endogenous GH, one must conclude that the pegylation process has major influences on this affinity, because in normal subjects high concentrations of pegvisomant (up to 2000-fold that of endogenous GH) are required to suppress IGF-I production (108). In acromegaly daily doses that are 20 – 40 times the daily dose for replacement purposes of GH are required to control the disease. Indeed, recently it has been shown that pegylation reduces affinity of unpegylated pegvisomant for the GHR, an effect that is far greater at the cell surface than for GHBP (100). These results provide an explanation for the high blood concentration of pegvisomant required to suppress IGF-I levels in normal subjects and in acromegaly.

In a phase I, placebo-controlled, single rising-dose study in 36 normal young men it was shown that a single injection of pegvisomant 1.0 mg/kg, reduced insulin-like growth factor-I by $49 \pm 6\%$ after 5 days of "treatment" ($P < 0.001$ vs. placebo) (109). The efficacy of pegvisomant in the treatment of acromegaly has been studied in a 12-week, double blind, placebo-controlled study. In this study 112 patients where treated with pegvisomant or placebo. Compared to baseline serum IGF-I levels were reduced significantly in a dose dependent fashion in all patients assigned to active treatment (110). Taken together it can be concluded that pegvisomant effectively blocks the GHR.

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Chapter 2 – Aims and Outline of the thesis

Aims of the thesis

The aim of the studies presented in this thesis was:

a) to investigate the effects of GHR blockade - by using the GHR antagonist pegvisomant – and fasting either alone or in combination on determinants of GH release;

b) to investigate the effect of GHR blockade on cardiovascular risk markers in healthy non-obese males.

Outline of the thesis

This thesis consists of three parts:

In part I (chapter 3) we have investigated the effects of GHR blockade and fasting either alone or in combination on some known determinants of GH release. We have specifically investigated the putative role of ghrelin in the mechanism whereby fasting leads to an increase in GH release.

Then we describe and discuss the results of a study investigating the effects of GHR blockade and fasting either alone or in combination on GHRH and GHRP-6 stimulated GH release.

Finally, in the last study of part I we describe some GH independent metabolic effects of GHRP-6.

In part II (chapter 4) we studied whether GHR blockade – as a model of GH deficiency but without the typical alterations in body composition – influences insulin sensitivity, hemostasis parameters and serum lipid concentrations in healthy non-obese males.

Furthermore, we describe the case of an acromegalic subject that benefited from co-treatment with octreotide and the GHR antagonist pegvisomant.

In part III (chapter 5) we discuss the main findings and hypothesize on possible clinical significance and potential future developments.

Finally, the summary – both in English and in Dutch - is presented in chapter 6.

Chapter 3.1 - Ghrelin Drives Growth Hormone Secretion during Fasting In Man

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Abstract

In humans, fasting leads to elevated serum growth hormone (GH) concentrations. Traditionally, changes in hypothalamic Growth Hormone Releasing Hormone and Somatostatin release are considered as the main mechanisms that induce this elevated GH secretion during fasting. Ghrelin is an endogenous ligand of the Growth Hormone Secretagogue Receptor and is synthesized in the stomach. As ghrelin administration in man stimulates GH release, while serum ghrelin concentrations are elevated during fasting in man, this increase in ghrelin levels might be another mechanism whereby fasting results in stimulation of GH release. Here we show that while ghrelin levels do not vary considerably in the fed state, fasting rapidly induces a diurnal rhythm in ghrelin concentrations. These changes in serum ghrelin concentrations during fasting were followed by similar, profound changes in serum GH levels. The rapid development of a diurnal ghrelin rhythm could not be explained by changes in insulin, glucose, or free fatty acid levels. These data indicate that ghrelin is the main driving force behind the enhanced GH secretion during fasting.

Introduction

In humans fasting enhances growth hormone (GH) secretion and amplifies GH rhythm (1). Traditionally hypothalamic Growth Hormone Releasing Hormone and somatostatin release are considered as the mechanisms

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whereby somatotroph output is regulated (2). Ghrelin, is an endogenous ligand of the Growth Hormone Secretagogue receptor (GHS-R) and was recently isolated from rat and human stomach, where it is synthesized in a distinct endocrine cell type (3;4). The gene for ghrelin encodes a 117 aminoacid prepro-ghrelin and is expressed not only in the stomach but also in the hypothalamic arcuate nucleus (4). Ghrelin exists in at least two forms, an octanoylated and a non-octanoylated form. At present only octanoylated ghrelin is thought to be biologically active (5-7). Intravenous ghrelin administration stimulates GH secretion through the centrally located GHS-R (8-10). Besides this effect on GH secretion a metabolic action of ghrelin is also recognized. In animal studies peripheral ghrelin administration also stimulates food intake, gastric secretion, gastric motility and adiposity (11-13). As ghrelin administration in man strongly stimulates GH release and – at least in animal studies - ghrelin concentrations are elevated during fasting an increase in ghrelin concentration is another potential mechanism whereby fasting can stimulate GH release (8;10;12;14) The GHS-R was characterized using a radiolabeled synthetic GHS called MK-0677, and subsequently it was shown that the binding of MK-0677 to the GHS-R could be competitively inhibited by Growth Hormone Releasing Peptide-6 (GHRP-6) (15). Also, the signal transduction pathways for MK-0677 and GHRP-6 both involve phospholipase C resulting in a rise in inositol triphosphate and intracellular calcium (15). After identification of the GHS-R a cell line expressing the GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R as monitored by increases in intracellular Ca^{2+} (7). By adding different tissue extracts to such a cell line and monitor intracellular Ca^{2+} changes Kojima et al. isolated ghrelin from rat and human stomach (4). Taken together we can conclude from these data that GHRP-6 and ghrelin bind to the same receptor.

Pegvisomant is a mutated GH molecule that prevents functional dimerization and subsequent activation of the GH receptor (16;17). In normal subjects and patients with acromegaly pegvisomant effectively blocks GH action and induces a decrease in (free) Insulin-like growth factor-I (IGF-I) concentrations (18;19).

In the present study we have investigated the possible role of ghrelin in the mechanism whereby fasting leads to somatotroph hyperactivity. Firstly, we investigated the effects of fasting on GH and ghrelin concentrations. Secondly, in order to investigate the possible role of some well-known metabolic substrates in the generation of the hypothesized diurnal ghrelin

rhythm, we also assessed insulin, glucose and free fatty acid concentrations in serum. Thirdly, to determine the role of GH and its effects via the GH receptor in the regulation of ghrelin secretion during fasting we investigated if pretreatment with the GH receptor antagonist pegvisomant influenced the effect of fasting on GH and ghrelin levels. Finally, to determine whether an auto feedback system for ghrelin is operative, and if so to investigate the influence of fasting hereon we investigated the effect of an intravenous bolus of GHRP-6 (that does not cross-react with the ghrelin assay) on ghrelin levels.

Subjects and Methods

Ten healthy male subjects (mean ± SD age, 23.4 ± 2.7 year; range, 20 – 28) with a normal body weight (mean ± SD BMI, 21.8 ± 1.8 kg/m2; range, 19.7-25.8) were asked to participate. None of the subjects had a relevant medical history or used medication. The local ethical committee approved the study and all subjects gave written informed consent.

We performed a double blind placebo controlled crossover study comparing fasting with and fasting without GH receptor blockade. After an overnight fast subjects were admitted to the Clinical Research Unit on day 1 at 7.30 AM. On day 1 and day 4 a GHRP-6 test was performed. At 6 PM on day 1 either pegvisomant - 80 mg as a single subcutaneous injection - or placebo was administered. A dose of 80 mg was chosen, as this dose is capable to reduce the GH concentration-dependent serum IGF-I levels significantly (18), even in acromegalic patients. From 12 PM on day 1 until the end of the study - on day 4 at 8 PM - subjects fasted. Blood was drawn daily at 8 AM, 4 PM and 12 PM. Ghrelin levels were determined from all samples, GH, and free IGF-I levels were determined from the 8 AM samples. Between the study periods there was a wash out period of 3 to 7 weeks. GHRP-6 was administered intravenously as a bolus injection of 1 μg/kg. Blood samples were drawn 15 minutes and immediately before, and 15, 30, 45, 60, 75, 90, 105 and 120 minutes after GHRP-6 injection. GH levels were determined in all samples. Ghrelin levels were determined from the samples drawn immediately before and 15, 30, 60 and 90 minutes after GHRP-6 injection. Identical looking vials with GH receptor-antagonist (Pegvisomant (Somavert™), and placebo were supplied by Sensus Drug Development Corporation, Austin, Texas 78701, USA.

GH samples were measured in a two-site immunoassay that does not cross-react with pegvisomant (18). The assay exhibits a lower detection limit

of 0.02 µg/L GH, an upper end of the working range of 50 µg/L for 25 µg/L serum samples, and no cross-reaction with pegvisomant up to a concentration of 50,000 µg/L

It should be noted that the GH values obtained with this method are approximately 50% of those obtained by conventional RIA method. The inter-assay coefficients of variation (CV) are 4.1% at 4.0 µg/L and 3.8% at 20 µg/L. The intra-assay CVs are 3.4% at 0.25 µg/L, 1.9% at 2.5 µg/L, and 4.5% at 25 µg/L. Serum free IGF-I was determined with a commercially available immunoradiometric assay (Diagnostic System Laboratories, Webster, TX; intra- and interassay CV 10.3 and 10.7% respectively). Ghrelin was detected with a commercial radioimmunoassay (Phoenix Pharmaceuticals, Belmont, CA; intra- and interassay CV 4.5 – 5.3% and 9.0 – 13.6% respectively) that uses 125-I labeled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full-length octanoylated human ghrelin. This assay has no cross-reactivity with other relevant molecules. Insulin was assessed with a radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium; intra- and interassay CV 13.7 and 8.0% respectively). Glucose was assessed with an automatic hexokinase method (Roche, Almere, The Netherlands). Free fatty acids were determined with an enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany; intra- and interassay CV 1.1 and 4.1% respectively).

Means were compared with the Wilcoxon signed ranks test. All P-values are two-sided, P-values < 0.05 were considered significant. Area under the curve was calculated using the trapezoid rule.

▨▨▨▨ Results

Fasting rapidly induced a diurnal ghrelin and GH rhythm (Figure 1a), that was not seen in the fed state (data not shown). We also assessed insulin, glucose and free fatty acids concentrations in serum. Figure 1b shows that that the gradual changes in serum insulin, glucose and free fatty acid levels are not related in time to the acute changes in systemic ghrelin and GH levels during fasting.

Compared to fasting without pegvisomant, fasting with pegvisomant did not change the ghrelin rhythm (Figure 2a). However, compared to fasting without the presence of pegvisomant, fasting in combination with pegvisomant resulted in higher GH concentrations on day 3 (from 8AM on day 3 to 8AM on day 4; p<0.05 for difference in area under the curve) (Figure 2b). In the fasting state, both in the absence and in the presence of

Figure 1:

Ghrelin, GH, insulin, glucose and free fatty acid concentrations during fasting and after a bolus injection of GHRP-6. A. Solid line, ghrelin levels; dotted line, GH levels. B. Solid line, insulin levels; small dots, glucose levels; large dots, free fatty acid levels. Symbols represent mean ± S.E.M.
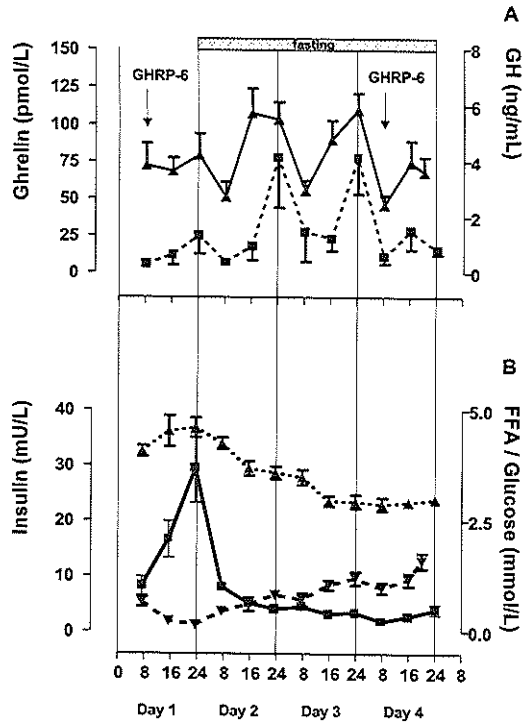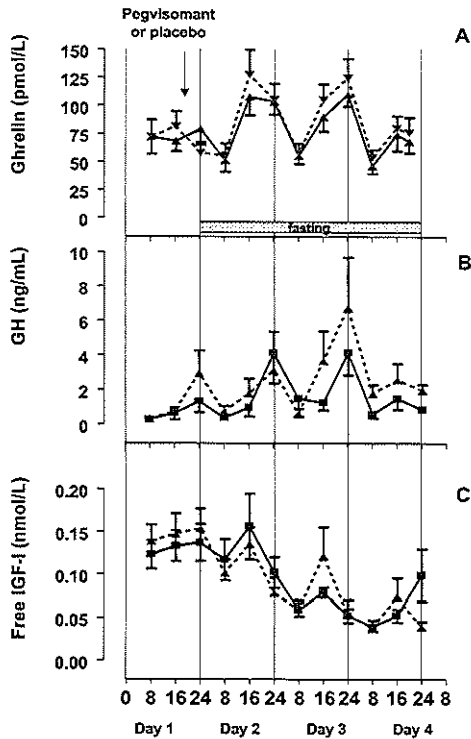

Figure 2:

Ghrelin, GH and free IGF-I concentrations during fasting and during fasting in the presence of the GH receptor antagonist pegvisomant. Symbols represent mean ± S.E.M. Solid line, fasting; dotted line, fasting in the presence of pegvisomant.

pegvisomant, serum free IGF-I levels decreased significantly. However, no additional effect of the presence of a GH receptor antagonist was observed (Figure 2c).

In all subjects and under all conditions GHRP-6 administration resulted in a powerful GH release (Figure 3a). GHRP-6 administration had no acute modifying effects on ghrelin levels (Figure 3b). However, on the third day of fasting early morning GHRP-6 administration attenuated peak ghrelin levels in the afternoon (Figure 1a).

Figure 3:
Acute GH and ghrelin response after administration of 1μg/kg GHRP-6 intravenously. A. Solid line, GH response on the 3rd day of fasting; dotted line, GH response at baseline. B. Dotted line, Ghrelin response at baseline; solid line, Ghrelin response on the 3rd day of fasting. Symbols represent mean ± S.E.M.
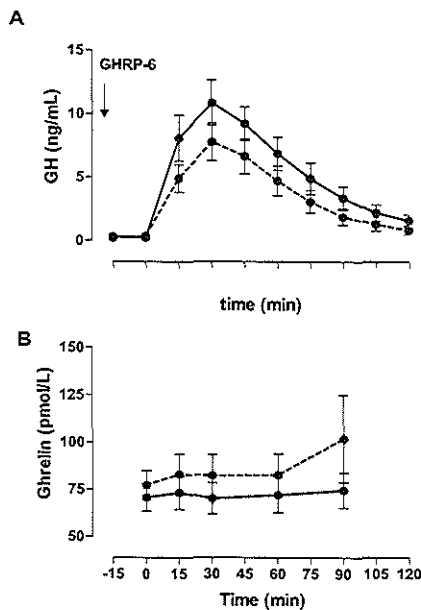
Discussion

In the present study we show that fasting rapidly induces an acute and distinct diurnal rhythm in systemic ghrelin concentrations that is not present in the fed state. These changes in serum ghrelin levels during fasting are followed by similar changes in serum GH concentrations, indicating that ghrelin is the driving force of increased GH secretion during fasting. As ghrelin is mainly produced in a distinct endocrine cell of the stomach this implies that the stomach can exert a direct control over the anterior pituitary (3).

In order to investigate the possible role of some well-known metabolic substrates in the generation of this diurnal ghrelin rhythm, we also assessed

insulin, glucose and free fatty acid concentrations in serum. Figure 1b clearly shows that that the gradual changes in these metabolic substrates is not related in time to the acute changes in systemic ghrelin and GH levels during fasting. Therefore, the rapid appearance of the observed ghrelin rhythm cannot be explained by acute alterations in insulin, glucose or free fatty acid levels. Interestingly, during fasting pancreatic polypeptide shows a similar diurnal rhythm as ghrelin does (20). This opens the possibility that pancreatic polypeptide is responsible for the appearance of a ghrelin rhythm. Since basal pancreatic polypeptide levels are low in human obesity this could also explain why circulating ghrelin levels are decreased in human obesity (21;22).

Compared to fasting without pegvisomant, pretreatment with pegvisomant resulted in a higher GH output on day 3 (from 8AM on day 3 to 8AM on day 4). Apparently, the blockade of the GH receptor induced higher GH levels. Serum free IGF-I levels decreased significantly in the fasting state, this decline was, however, not influenced by the presence or absence of pegvisomant. Thus, disabling the GH-GH receptor signaling system leads to an increase in GH output while in this same period free IGF-I levels did not change. These data indicate that other factors than free IGF-I – that is considered the most important GH dependent feedback factor on GH release (23;24) – are responsible for the additional increase in serum GH levels during fasting in the presence of pegvisomant. These data can be taken to indicate an ultra-short feedback loop of GH on its own secretion. Moreover, from these same data we can also conclude that GH does not exert feedback on the production of ghrelin.

An intravenous bolus injection of GHRP-6 had no acute – i.e. within 90 minutes - effect on ghrelin levels. However, on study day four - the third day of fasting - early morning GHRP-6 administration attenuated peak ghrelin levels in the afternoon. Therefore, we postulate that ghrelin is – at least partially – regulated by a long loop negative auto feedback system.

In conclusion, we have shown that fasting leads to a diurnal ghrelin rhythm that cannot be explained by changes in insulin, glucose, or free fatty acid levels. These changes in serum ghrelin levels during fasting are followed by similar changes in serum GH concentrations, indicating that ghrelin is the driving force of increased GH secretion during fasting. By using the GH receptor antagonist pegvisomant we also provide indirect evidence that these changes in serum ghrelin levels are not regulated by the GH receptor. Finally, we found that the administration of the synthetic GH secretagogue

GHRP-6 was followed by a decrease of peak ghrelin levels, but this effect could only be observed after several hours, suggesting that ghrelin concentrations are – at least partially – regulated by a long-loop negative auto feedback control.

Acknowledgements

▨▨▨▨ **Reference List**

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Chapter 3.2 – Effects of Fasting and Pegvisomant on the Growth-Hormone-Releasing-Hormone and Growth-Hormone-Releasing-Peptide-6 Stimulated Growth Hormone Secretion

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▨▨▨▨▨ **Summary**

Pegvisomant is a mutated GH molecule which prevents functional dimerization and subsequent activation of the growth hormone receptor. Pegvisomant and fasting both lead to GH resistance. We performed a double blind placebo controlled cross-over study comparing the effects of pegvisomant and fasting on the growth hormone releasing hormone (GHRH) and growth hormone releasing peptide-6 (GHRP-6) stimulated GH-release before and after three days of fasting in ten healthy lean male subjects. We also performed a single arm open label study under non-fasting conditions in five of these subjects. On day one, in random order, at 0800 hrs a GHRP-6 or GHRH test was performed. At 1600 hrs, a GHRH (if the first test was a GHRP-6 test) or GHRP-6 test (if the first test was a GHRH test) was done. After the second test either pegvisomant (80 mg as a single subcutaneous injection) or placebo was administered. On day four, GHRP-6 and GHRH tests were performed in the same order as on day one. During the cross-over study subjects fasted from 2400 hrs on day one until the end of the study. During the GH stimulation tests blood samples were drawn every 15 minutes from -15 to 120 minutes. GH was determined in all samples. Total IGF-I and free IGF-I were determined from the samples at 0 minutes only. Three days of fasting alone and pegvisomant alone as well as in combination increased GH concentrations whereas a decrease in serum free, but not total, IGF-I concentrations was observed. On day four, fasting and pegvisomant, either alone or in combination, significantly increased GH

concentrations after GHRH compared to baseline. Pegvisomant alone did not increase GH concentrations after GHRP-6 administration. Fasting alone increased GH levels after GHRP-6 administration. The combination of fasting and pegvisomant had a synergistic effect on GH release after GHRP-6. These human in-vivo data suggest that: 1. Circulating free IGF-I, and not total IGF-I, is the major component in the negative feedback on GH secretion; 2. Increased pituitary GHRH receptor expression plays a role in the mechanism whereby fasting leads to increased GH concentrations; 3. In-vivo, GHRP-6 sensitivity seems to be primarily regulated by metabolic factors and not by changes in GH-IGF-I axis.

Introduction

Pegvisomant is a mutated growth hormone (GH) molecule which prevents functional dimerization and subsequent activation of the GH receptor (1), and is therefore a potential new treatment for acromegaly (2;3). In normal subjects and patients with acromegaly pegvisomant effectively blocks GH action and induces a decrease in Insulin-like Growth Factor-I (IGF-I) concentrations. This decline in IGF-I levels is associated with a concomitant rise in GH levels (4); in other words pegvisomant induces GH resistance. Fasting also leads to GH resistance which – at least in part – can be explained by low portal vein levels of insulin insufficient to stimulate normal hepatic IGF-I synthesis (5;6).

Growth Hormone Releasing Peptide-6 (GHRP-6) is a synthetic hexapeptide that activates the GH secretagogue receptors in the hypothalamus and pituitary (7). An endogenous ligand for the GH secretagogue receptor, called ghrelin has recently been purified from human stomach extract (8). Besides Growth Hormone Releasing Hormone (GHRH) and somatostatin ghrelin could be another important factor in the regulation of GH secretion (9;10). Furthermore, relevant data indicate that GH secretagogues have an important – GH independent – role in metabolism (11-13).

In order to gain more insight in the stimulatory regulation of GH secretion under different metabolic conditions and the role of the GH receptor herein we performed a double blind placebo controlled cross-over study comparing the effects of placebo with pegvisomant on GHRH and GHRP-6 stimulated GH-release both before and after fasting for three days. Additionally, we investigated the effects of pegvisomant on GHRH and GHRP-6 stimulated GH-release under non-fasting conditions.

Subjects and methods

Study subjects

Ten healthy male subjects (mean ± SD age, 23.4 ± 2.7 year; range, 20 – 28) with a normal body weight (mean ± SD BMI, 21.8 ± 1.8 kg/m^2; range, 19.7–25.8) were asked to participate. None of the subjects had a relevant medical history or used medication. All ten subjects participated in the cross-over study and five participated in the single-arm study. The study was approved by the local ethical committee and all subjects gave written informed consent.

Experimental design

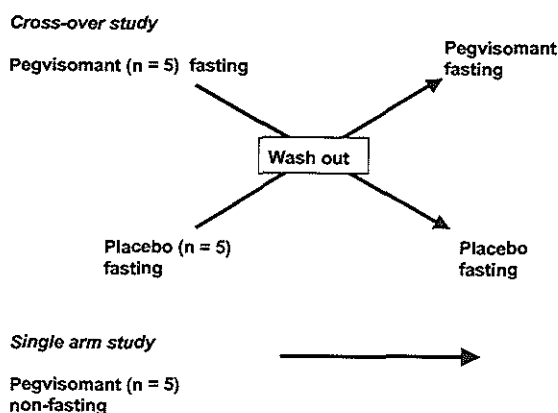Two studies were performed (Fig. 1):

Cross-over study

Pegvisomant (n = 5) fasting → Pegvisomant fasting

Wash out

Placebo (n = 5) fasting → Placebo fasting

Single arm study

Pegvisomant (n = 5) non-fasting →

Figure 1
General design of the study.

1) A double blind placebo controlled cross-over study comparing pegvisomant with placebo before and after three days of fasting.
2) A single arm open label study in which the effect of pegvisomant under non-fasting conditions was investigated.

Both studies lasted four days. All subjects were admitted to the Clinical Research Unit after an overnight fast. At admission two indwelling intravenous catheters were inserted. On day one, in random order, at 0800 hrs a GHRP-6 or GHRH test was performed. At 1600 hrs, a GHRH test (if a GHRP-6 test was performed in the morning) or a GHRP-6 test (if a GHRH test was performed in the morning) was done. Between the tests a standard light meal was served. After the second test either pegvisomant (80 mg as a

single subcutaneous injection) or placebo was administered. This pegvisomant dose has been shown to result in an effective blockade of the GH receptor. During the cross-over study subjects fasted from 2400 hrs on day one until the end of the study. On day four, GHRP-6 and GHRH tests were performed in the same order as on day one. In the single arm study all GHRP-6 tests were performed at 0800 hrs and all GHRH tests at 1600 hrs.

GH-stimulation tests
GHRH (Ferring, Hoofddorp, The Netherlands) and GHRP-6 (Clinalfa AG, Läufelingen, Switzerland) were administered intravenously as a bolus injection of 1 μg/kg. Blood samples were drawn every 15 minutes from - 15 to 120 minutes. GH was determined in all samples. Total IGF-I and free IGF-I were determined from samples at 0 minutes (T = 0) only.

Study medication
Pegvisomant and placebo were supplied by Sensus Drug Development Corporation, Austin, Texas, USA.

Assays
All assays were done in duplicate. Serum GH was determined with a previously described, non-commercially available two site immunoassay that does not cross react with pegvisomant (intra-assay coefficients of variation (CV) 3.4% at 0.25μg/L, and 4.5% at 25μg/L; interassay CV 4.1% at 4.0μg/L, and 3.8% at 20μg/L) (4). The absolute values for GH concentrations obtained by this assay are about 50% of those provided by commercially available assays. Serum IGF-I was determined with a commercially available radioimmunoassay (Biosource Europe S.A., Nivelles, Belgium; intra- and interassay CV 5.0 and 9.6%, respectively) and free IGF-I was determined with a commercially available immunoradiometric assay (Diagnostic System Laboratories, Webster, Texas, USA; intra- and interassay CV 10.3 and 10.7%, respectively).

Statistical analysis
All results are reported as mean ± SEM. Because all study periods were exactly the same until 1800 hrs on day 1 and in order to minimize intra-individual differences we used the means of the baseline values of the cross-over study as baseline values. Means were compared with the Wilcoxon signed rank test. Area under the curve (AUC) was calculated by the

trapezoidal rule. Correlations were calculated with pearson correlation coefficient. All p-values are two-sided, p-values <0.05 were considered significant.

Results

GH stimulation tests

Cross-over study

<u>GHRH:</u> Compared to baseline fasting alone and fasting in combination with pegvisomant significantly increased AUC after GHRH (Fig. 2, upper panel). The effect of fasting alone was not significantly different compared to the effect of fasting in combination with pegvisomant.

<u>GHRP-6</u>: Fasting alone increased GH release after GHRP-6 administration (Fig. 2, lower panel). Fasting in combination with pegvisomant had a synergistic effect on GH release after GHRP-6. Compared to fasting alone fasting in combination with pegvisomant had a significantly larger effect on AUC after GHRP-6.

Single arm study

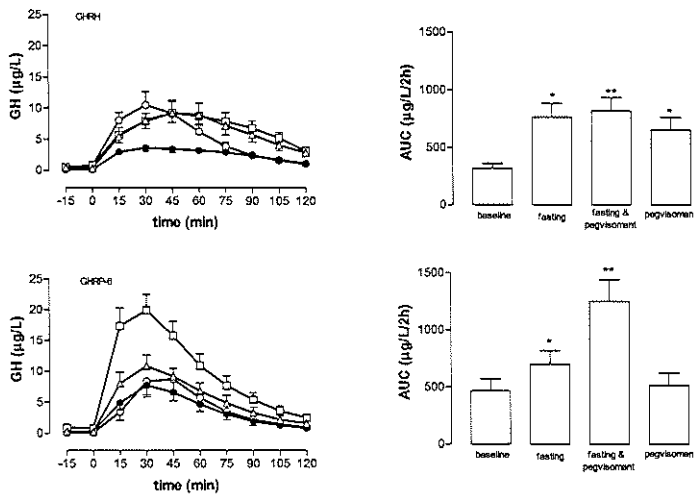<u>GHRH & GHRP-6</u>: Pegvisomant alone increased AUC after GHRH but not after GHRP-6 administration (Fig. 2).

Figure 2

*Mean (± SEM) GHRH (upper panel) and GHRP-6 (lower panel) stimulated GH release (µg/L) and area under the curve (AUC; µg/L/2h) at baseline (•), after pegvisomant (o), after fasting (Δ) or after pegvisomant in combination with fasting (□). *p<0.05, **p<0.01 compared to baseline (Wilcoxon signed rank test).*

Cross over study vs single arm study

GHRH: Compared to pegvisomant alone fasting alone and fasting in combination with pegvisomant had similar effects on AUC after GHRH. GHRP-6: The effect of fasting in combination with pegvisomant on AUC after GHRP-6 was significantly larger than the effect of pegvisomant alone on AUC after GHRP-6.

Non-stimulated early morning GH and IGF-I levels

Cross over study: Fasting alone non significantly increased GH levels (Fig. 3a). Three days of fasting combined with pegvisomant resulted in a significant increase in GH levels. Serum total IGF-I levels did not change (Fig. 3b).
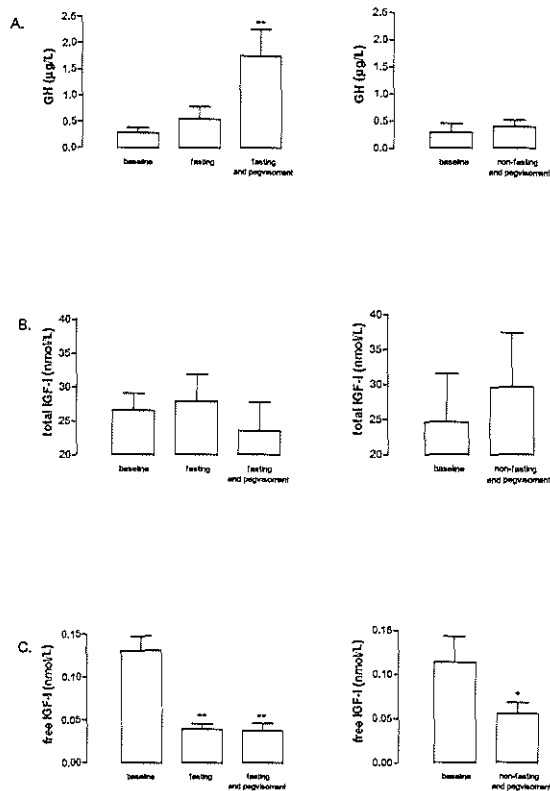

Figure 3

Mean (±SEM) values of GH (3A), total IGF-I (3B) and free IGF-I (3C). Effects of fasting alone, fasting with pegvisomant and pegvisomant alone.
*$p<0.05$, **$p<0.01$ compared to baseline (Wilcoxon signed rank test).

Fasting alone resulted in significantly smaller changes in GH and total IGF-I concentrations than fasting combined with pegvisomant. Three days of fasting alone as well as fasting in combination with pegvisomant decreased serum free IGF-I levels (Fig. 3c). The effects of fasting alone and fasting in combination with pegvisomant on free IGF-I levels were similar.

During none of the GH stimulation tests was the free IGF-I level at T = 0 minutes correlated with the AUC or maximum GH concentration after GHRH or GHRP-6.

Single arm study: Pegvisomant alone non significantly increased GH levels (Fig. 3a). Serum total IGF-I levels did not change (Fig. 3b), whereas serum free IGF-I levels decreased significantly (Fig. 3c). During none of the GH stimulation tests was the free IGF-I level at T = 0 minutes correlated with the AUC or maximum GH concentration after GHRH or GHRP-6.

Cross over study vs single arm study: Compared to pegvisomant fasting alone and fasting in combination with pegvisomant had similar effects on GH, total IGF-I and free IGF-I.

Discussion

Three days of fasting with or without pegvisomant as well as pegvisomant alone resulted in a decrease in serum free IGF-I levels but not in total IGF-I levels. This decline in free IGF-I can be explained by several mechanisms. Firstly, fasting leads to a lowering in portal vein insulin concentration and insulin is a known stimulator of hepatic IGF-I production (5;14). Secondly, IGF-binding protein-1 (IGFBP1) levels are inversely regulated by insulin (5), indeed in the cross-over (fasting) study we observed a significant increase in IGFBP1 levels (data not shown). Finally, pegvisomant is an effective blocker of the GH receptor and can thus significantly decrease hepatic IGF-I production (2;4). Interestingly, IGFBP1 and 3 remained unchanged in the single arm study (data not shown). As we did not measure other IGFBP's a rise in one of these binding proteins cannot be excluded.

Our data are in accordance with Frystyk and co-workers, they too observed that three days of fasting led to a reduction in serum free – but not total – IGF-I levels with a concomitant rise in serum GH levels (15). Also, Chapman and co-workers observed a close temporal negative correlation between GH and free – but not total – IGF-I concentrations after IGF-I infusion (16). Taken together, these and other data (17) suggest that circulating free IGF-I, but not total IGF-I, is the major component in the negative feedback on GH secretion. In this respect it should be noted that the IRMA we used to

determine free IGF-I measures the 'true' free IGF-I as well as the fraction of IGF-I which can be easily dissociated from the binding proteins. Thus measuring the concentration of IGF-I that is available to the tissues (17). Our data suggest that pegvisomant is able to mimic the effects of three days of fasting on GHRH mediated GH release. The mechanisms by which fasting increases GH levels is thought to be a decrease in somatostatinergic tonus as well as an increase in GHRH activity (18-20). A relevant animal model in this respect is the transgenic growth retarded rat in which GH is only expressed in the GHRH producing hypothalamic neurones. In these animals, high hypo-thalamic GH concentrations induce a decrease in hypothalamic GHRH out-put with a subsequent increase in pituitary GHRH receptor expression (21). These animals are therefore highly sensitive to GHRH administration (22). We observed that pegvisomant alone increased the sensitivity to exogenous GHRH. Apparently pegvisomant is unable to cross the blood-brain barrier and block the hypothalamic GH receptors. And thus a situation comparable to the one in the transgenic growth retarded rat ensues, whereby the increased serum GH concentration induced by pegvisomant decreases hypothalamic GHRH output and subsequently increases pituitary GHRH receptor expression. On the basis of molecular size Thorner et al. already postulated that pegvisomant does not cross the blood-brain barrier, our in vivo experimental data support this notion (4). Furthermore, considering the peculiar analogy that fasting alone and pegvisomant alone have on the GHRH stimulated GH release our data suggest an important role for increased pituitary GHRH receptor expression in the mechanism whereby fasting leads to an increased GHRH activity.

Because we did not observe an additional effect of the combination of fasting with pegvisomant on GHRH mediated GH secretion we conclude that the GHRH mediated GH secretion is maximally stimulated by fasting alone and GH receptor blockade alone.

Fasting alone, but not pegvisomant alone, increased GHRP-6 mediated GH secretion. This observation supports previous animal data showing that the GHRP-6 induced activation of cells in the hypothalamic arcuate nucleus is much greater in animals that have fasted for 48 hours (23). An additional explanation for the effect of fasting on the GHRP-6 induced GH release might be the decrease in somatostatinergic tone associated with fasting. Indeed, both in vitro and in vivo the GH releasing effect of GHRP-6 can be attenuated by the administration of the somatostatin analogue octreotide (24;25). In non-fasting conditions pegvisomant did not result in an

additional increase in GHRP-6 mediated GH secretion suggesting that the metabolic consequences of fasting and not the changes in GH-IGF-I axis induced by pegvisomant are of primary importance in increasing the in-vivo sensitivity to GHRP-6.

The combination of three days of fasting with pegvisomant had a synergistic effect on the GHRP-6 mediated GH secretion. In order to stimulate GH release GH secretagogues need an intact hypothalamopituitary system and GHRH antibodies virtually diminish the GH releasing capacity of GH secretagogues (26;27). However, most interestingly are data suggesting that the mechanism by which GH secretagogues lead to GH release is in part mediated via an increase in GHRH release (28). Such an increase in GHRH release as a result of exogenous GHRP-6 administration in the context of the decrease in somatostatinergic tonus associated with fasting together with the putative upregulation of pituitary GHRH receptor expression by pegvisomant could – at least in part – explain the observed synergism that fasting and pegvisomant have on GHRP-6 mediated GH secretion.

In conclusion, a period of three days of fasting as well as blockade of the GH receptor by pegvisomant results in a significant decrease in serum free IGF-I – but not in total IGF-I concentrations. This decrease in free IGF-I is accompanied by an increase in GH concentrations. Pegvisomant administration to non-fasting subjects mimics the effects of fasting on GHRH, but not GHRP-6 – mediated GH release. Fasting, in contrast to pegvisomant, is able to increase GHRP-6 mediated GH secretion. The combination of three days of fasting and pegvisomant has a synergistic effect on the GHRP-6, but not on the GHRH mediated GH secretion. These data suggest that: 1) Circulating free IGF-I, and not total IGF-I, is the major component in the negative feedback on GH secretion; 2) Increased pituitary GHRH receptor expression plays a role in the mechanism whereby fasting leads to increased GH concentrations; 3) In-vivo, GHRP-6 sensitivity seems to be primarily regulated by metabolic factors and not by changes in GH-IGF-I axis.

Acknowledgements

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Chapter 3.3 - Blockade of the Growth Hormone Receptor Unmasks Rapid Growth-Hormone-Releasing-Peptide-6 Mediated Tissue Specific Insulin Resistance.

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Abstract

The roles of growth hormone (GH) and its receptor (GHR) in metabolic control are not yet fully understood. We studied the role of GH and the GHR using the GHR antagonist pegvisomant for metabolic control in healthy non-obese men, in fasting and non-fasting conditions. Ten healthy subjects were enrolled in a double-blind, placebo controlled study on the effects of pegvisomant on GHRH and GH Releasing Peptide-6 (GHRP-6) induced GH secretion, before and after three days of fasting and under non-fasting conditions (n=5). Under the condition of GHR blockade by pegvisomant in the non-fasting state, GHRP-6 (1 µg/kg) caused a increase in serum insulin (10.3 ± 2.1 versus 81.3 ± 25.4 mU/L; $p < 0.001$) and glucose (4.2 ± 0.3 versus 6.0 ± 0.6 mmol/L; $p < 0.05$) concentrations. In this group, a rapid decrease in serum free fatty acids levels was observed. These changes were not observed under GHR blockade during fasting, or in the absence of pegvisomant. We conclude that although these results were obtained from an acute study, and long term administration of pegvisomant could render different results, that blockade of the GHR in the non-fasting state induces tissue specific changes in insulin sensitivity, resulting in an increase in glucose- and insulin levels (indicating insulin resistance of liver/muscle), but probably also in an increase in lipogenesis (indicating normal insulin sensitivity of adipose tissue). These GHRP-6 mediated changes indicate that low GH bioactivity on the tissue level can induce changes in metabolic control, which are characterized by an increase in fat mass and a decrease in lean body mass. As a mechanism of these GHRP-6 mediated metabolic changes in the

non-fasting state, direct non-pituitary mediated GHRP-6 effects on the "gastroentero–hepatic axis" seem probable.

Introduction

The role of growth hormone (GH) and its receptor (GHR) in the metabolic control of man are not fully understood yet. During fasting serum GH levels increase significantly, whereas serum total, and free insulin-like growth factor-I (IGF-I), as well as insulin levels decline (1-3). Also, GH deficient (GHD) patients show, in parallel with acromegalic patients with high GH concentrations, a reduction in insulin sensitivity (4-6). In contrast to acromegalics, GHD patients have an increase in fat mass (FM) (4;5;7;8). Pegvisomant is a genetically manipulated GH molecule, which disables functional dimerization of the two GH receptor (GHR) molecules involved in signal transduction, due to a single mutation at the site II of the GH molecule. Pegvisomant is pegylated to increase serum half-life time. Currently, the compound is used in Phase II and III trials in the treatment of acromegaly (9-12). Pegvisomant potentially can also be used in studies in normal individuals in order to obtain more insights in the role of GH, its receptor, and its secretagogues in metabolic control. We performed a double-blind, placebo controlled, cross-over study, comparing the effects of placebo versus a single subcutaneous injection of 80 mg of pegvisomant on Growth Hormone Releasing Hormone (GHRH) and Growth Hormone Releasing Peptide-6 (GHRP-6) induced GH secretion, both before and after a three days period of fasting in ten healthy young male subjects. We also repeated the same study in five of the ten subjects, but under non-fasting conditions. During this open-label study period, all subjects received pegvisomant.

Subjects and Methods

Ten healthy male subjects 20 – 30 years of age (mean ± SD age, 23.4 ± 2.7 year; range, 20 – 28) with a normal body weight (mean ± SD BMI, 21.8 ± 1.8 kg/m^2; range 19.7-25.8) were asked to participate. None of the subjects had a relevant medical history or used medication. All ten subjects participated in the cross-study and five of these ten subsequently participated in the single-arm study (see below). The study was approved by the local ethical committee and all subjects gave written informed consent before enrolment in the study.

The study consisted of two parts: a double blind placebo controlled cross-over study comparing GHR blockade with placebo before and after

3 days of fasting (with free access to non-caloric fluids), and a single arm
open label study in which the effect of GHR blockade under non-fasted
conditions (continuation of subjects normal daily diet) was investigated. All
study periods in both protocols lasted four days, during which the subjects
were admitted to the Clinical Research Unit (CRU). Except for fasting study
periods in both protocols were identical. In the fasting study, all subjects had
to fast from 2400 h on the day before admission until after the first test.
They were admitted to the CRU at 0730 h at which time two indwelling
intravenous catheters were inserted in both forearms. On days 1 and 4, a
GHRP-6, or GHRH-test was performed between 0800 h and 0900 h. A second
test (GHRH, or GHRP-6 test) was performed between 1600 h and 1700 h
(exactly eight hours after the first test). Between the two tests, a standard
light meal was served to all subjects on day 1, but not to subjects in the
fasting group on day 4. This light meal consisted out of two slices of bread,
with one unit of butter, one slice of cheese, one unit of fruit jelly, and one
unit of milk (caloric content of 1764 kJ). Immediately after the second GH
stimulatory test either 80 mg of pegvisomant or placebo was administered
subcutaneously. All subjects fasted from midnight day one until the end of
the study.

In the non-fasting arms of the study, all subjects also had to fast from 2400 h
on the day before admission until after the first test. Again, they were
admitted to the CRU at 0730 h at which time two indwelling intravenous
catheters were inserted in both forearms. On days 1 and 4, a GHRP-6 was
performed between 0800 - 0900 h. A GHRH test was performed exactly eight
hours after the GHRP-6 test. Between the two tests, a standard light meal as
described above was served to all subjects on day 1, and on day 4.
Immediately after the GHRH test on day one, 80 mg of pegvisomant was
administered subcutaneously. A wash-out period of at least three weeks was
chosen in between each of the admission periods for each subject. To detect
changes in concentrations over the day, blood samples for endocrine and
metabolic parameters were taken daily at intervals of 4 hours throughout
every study period of 4 days. These samples were used to calculate mean GH
concentrations over the day.

GH-stimulation tests
Either GHRH (Ferring, Hoofddorp, The Netherlands) or GHRP-6 (Clinalfa
AG, Läufelingen, Switzerland) was administered as an intravenous bolus
injection of 1 µg/kg body weight. Blood samples were drawn every

15 minutes from -15 to 120 minutes after injection. In the crossover study, tests were performed in random order.

Study medication

GHR-antagonist (Pegvisomant (Somavert™), and placebo were supplied by Sensus Drug Development Corporation, Austin, Texas 78701, USA.

Assays

Samples were measured for endogenous GH in a two-site immunoassay that does not cross-react with pegvisomant. The inter-assay CVs are 4.1% at 4.0 µg/L and 3.8% at 20 µg/L. The intra-assay CVs are 3.4% at 0.25 µg/L, 1.9% at 2.5 µg/L, and 4.5% at 25 µg/L (Med Klinik Innenstadt; LMU; Munich; Germany) (9). Serum IGF-I was determined, using a commercially available radio-immunoassay (Biosource Europe S.A., Nivelles, Belgium; intra- and interassay CV 5.0 and 9.6% respectively). This IGF-I assay measures IGF-I in acid ethanol extracts. Free IGF-I concentrations were assessed, using a commercially available immunoradiometric assay (Diagnostic System Laboratories, Webster, TX; intra- and interassay CV 10.3 and 10.7% respectively). Glucose was determined with an automatic hexokinase method (Roche, Almere, The Netherlands). Insulin was assessed, by RIA (Medgenix Diagnostics, Brussels, Belgium; intra- and interassay CV 13.7 and 8.0% respectively). Free fatty acids were determined with an enzymatic colorimetric method (Wako chemicals GmbH, Neuss, Germany; intra- and interassay CV 1.1 and 4.1% respectively).

Statistical analysis

Means were compared with the Wilcoxons matched pairs test. All P-values are two-sided, p-values <0.05 was considered significant. Unless otherwise noted all results are reported as mean ± SEM. Analyses were performed, using Prism version 3.00 for Windows (GraphPad Software, Inc., San Diego, CA). The area under the curve was calculated by the trapezoidal rule.

Results

Growth hormone

No significant differences were observed in serum GH concentrations on day 1, expressed as area under the curve for all GH data obtained in 24 hours, excluding GH concentrations that were obtained during the two stimulatory tests. On day 4, however, serum GH concentrations increased in all fasting

subjects, while only in the fasting, pegvisomant pretreated subjects this
increase in GH was significant (0.4 ± 0.4 ng/mL on day 1 versus 2.1 ± 0.7
ng/mL on day 4).

Insulin

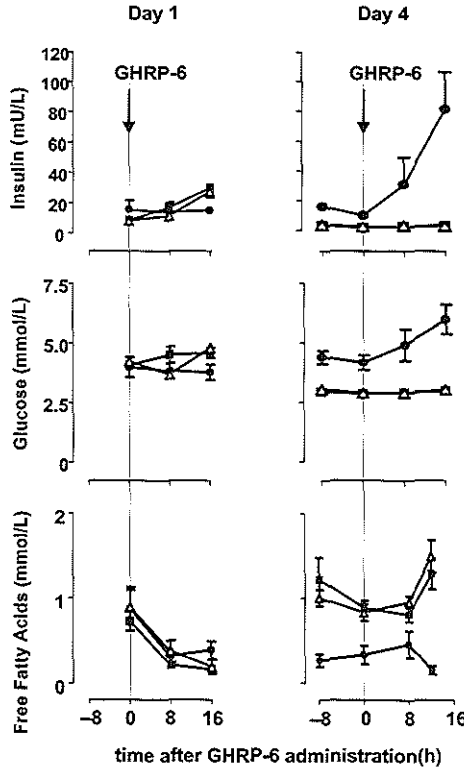In figure 1a, serum insulin concentrations are shown on days 1 and day 4

Figure 1:

Serum insulin (mU/L; figure 1a), serum glucose (mmol/L; figure 1b), and serum Free
Fatty Acids (mol/L; figure 1c) concentrations after subcutaneous administration of
1 µg/kg GHRP-6 in normal healthy subjects, with or without fasting and with or wit-
hout the presence of a GH blocker. j fasting; placebo (n=10); n fasting; pegvisomant
(n=10); d non-fasting; pegvisomant (n=5). All GHRP-6 tests were performed in all sub-
jects of all study groups (including the non-fasting group) after an overnight fasting
period. All subjects in the non-fasting group continued their normal daily diet throug-
hout the whole study. Although different symbols were used on day 1 for each study
group, study conditions regarding fasting were identical for all subjects on this day;
also pegvisomant was administered only after the two stimulatory tests were
finished.

of each of the study periods. No significant changes in serum insulin concentrations were observed after the administration of 1 μg/kg of GHRP-6. However, only in the presence of pegvisomant and in the non-fasting state was a rapid and significant increase in insulin levels found (day 4; Insulin – 0800 h versus 2400 h: 10.3 ± 2.1 versus 81.3 ± 25.4 mU/L; $p<0.001$).

Glucose
No significant changes were observed in glucose after GHRP-6 administration. Again, only in the presence of pegvisomant and in the non-fasting state, was a significant increase in serum glucose levels observed (day 4; Glucose – 0800 h versus 2400 h: 4.2 ± 0.3 versus 6.0 ± 0.6 mmol/L; $p<0.05$; Figure 1b).

Free fatty acids
Finally, only in the five non-fasting subjects, and only in the presence of pegvisomant was an acute and significant decrease in serum FFA levels observed (day 4; FFA – 0800 h versus 1600 h: 0.33 ± 0.1 versus 0.15 ± 0.03 mmol/L; $p<0.05$). Interestingly, on day 4, FFA levels did increase after GHRP-6 administration in the fasting state, regardless the presence of a GHR blockade (0800 h versus 1600 h: 0.89 ± 0.01 versus 1.30 ± 0.18 mmol/L; $p<0.05$; Figure 1c). A decrease in serum FFA concentrations in all groups was observed after GHRP-6 administration on day 1.

IGF-I
Serum total IGF-I levels did not change during the fasting period. However, we observed an increase in IGF-I levels on day 2, which was probably induced by the two stimulatory tests on day 1 (mean IGF-I at baseline 26.6 ± 2.6 nmol/L versus 31.9 ± 3.3 nmo/L at 1600 h on day 2). After day 2, total IGF-I levels began to decrease, but this decrease did not reach the level of significance on day 4. There was a slight, but significant decrease during fasting in the presence of pegvisomant compared with the IGF-I levels in controls at day 4 (controls 0800 h at day 4 versus pegvisomant 0800 h at day 4: 27.9 ± 4.0 versus 23.6 ± 4.2 nmol/L; $p<0.05$). Furthermore, blockade of the GHR did significantly reduce serum free IGF-I levels. This decrease was not further influenced during fasting (day 1 0800 h versus day 4 0800 h: 0.12 ± 0.02 versus 0.04 ± 0.00 nmol/L).
None of these parameters did change after the administration of 1 μg/kg of GHRH (data not shown).

▨▨▨▨▨ **Discussion**

In this study pegvisomant was used to study the roles of GH and its receptor in the metabolic control of the fasting status. To our surprise, we found that the administration of a standard dose of 1 µg/kg of GHRP-6 induced an acute and significant increase in serum insulin and glucose and a significant decrease in FFA levels in normal individuals pretreated with pegvisomant when not fasted. Moreover, GHRP-6 administration caused a significant increase in FFA in the fasting state regardless of the presence of GHR blockade. Why these changes were not observed after the administration of GHRH is not clear. Possibly, the effects of GHRH on metabolic processes are either not important or are mainly mediated by GH action, which in this study was reduced by pegvisomant and/or fasting. All of these GHRP-6 mediated changes in insulin, glucose and FFA concentrations in the pegvisomant pretreated, non-fasting group returned to baseline values the next day (data not shown). The decrease in serum FFA levels down to normal non-fasting levels on day 1 in all groups is probably caused by an exaggerated food intake the night before admission, as all subjects were aware of the coming fasting period, which started on day 1 at 2400 hr As they were only found in the presence of a GHR blocker, one must conclude that these GHRP-6-induced changes in insulin, glucose and FFA levels are not modulated by the GH system. These observations suggest that a certain degree of GH action in peripheral target tissues, such as the pancreas, adipose tissue, and liver, control these GHRP-6-dependent changes in serum insulin, glucose and FFA levels. Apparently there is a delicate balance between the GH – GHR system, and the GH secretagogue (GHS) – GHS receptor (GHS-R) system in the control of the insulin – glucose – FFA system in healthy men, whereas blockade of the GHR unmasks tissue specific insulin sensitivity. This tissue specific sensitivity results in an increased lipogenesis (and therefore potentially in an increase in fat mass), whereas insulin resistance of carbohydrate metabolism might eventually lead to insulin resistance–related changes, as observed in type 2 diabetes mellitus (13-17). Strikingly, the metabolic changes observed in this study parallel those that occur during physiological aging (18-22). Interestingly, such changes have also been observed to a certain degree in some studies with some other GHS (23-26). The fact that GHRP-6 administration induces these metabolic effects could hypothetically be explained by an upregulation of the GHS-R in the presence of pegvisomant (27;28). Our data also provide some insight in the mechanisms that might underlie the observed reduced

insulin sensitivity in both acromegalics and in GH deficient patients, with respect to carbohydrate metabolism, and opposite changes in lipogenesis and lipolysis in GHD versus acromegaly (29-31). Apparently the high GH concentrations in acromegaly augment glycolysis and lipolysis, and as a consequence, hyperinsulinism develops. In conditions in which GH signaling is lacking in peripheral tissue (e.g. GHD), however, GHS-mediated glycolysis increases, and hyperinsulinism develops, which in turn induces lipogenesis. Unpublished observations (A.J. van der Lely) support this delicate balance between insulin and GH action, as a number of acromegalic patients, who were treated weekly with high dosages of 80 mg of pegvisomant, sc, developed hypertrophy of sc adipose tissue at the injection site. This was a reversible phenomenon, as during the follow-up period off medication these changes in adipose tissue quickly resolved.

The mechanisms responsible for the observed GHRP-6-mediated increase in serum glucose and insulin concentrations during GHR blockade in the non-fasting state, are not known. Strikingly, on day 1, when all subjects were also in the non-fasting state, but without the presence of GHR blockade, no changes in serum glucose and/or insulin concentrations were observed after GHRP-6 administration. Possible candidates for the observed metabolic changes, as cortisol, glucagon, and glucagon-like peptide-1, or somatostatin, all lack at least one of the characteristic metabolic reactions observed in this study (increases in glucose and insulin, as well in lipogenesis). It is unlikely that glucocorticoids are responsible for the increase in serum glucose and insulin concentrations, because on day 4, serum cortisol and ACTH levels decreased rapidly during the first eight hours after GHRP-6 administration, as expected on the basis of a normal diurnal pattern (data not shown). Although the metabolic effects of glucagon on glucose and insulin levels are the same as those observed after GHRP-6 administration, it has strong lipolytic activities as well (32-35). Glucagon-like peptide-1 has both lipogenic, and insulinogenic actions, but it decreases glucose levels, as it is a powerful glucogenic factor (36). Whether the recently discovered and first known endogenous ligand of the GHS receptor (37) shares the observed GHRP-6 mediated changes metabolic parameters (and the possible subsequent changes in body composition) will be answered when data on the effects of Ghrelin administration will become available. Finally, changes in paracrine somatostatin activity within the pancreatic islets would either increase– or decrease insulin secretion with opposite changes in glucose levels. Possibly, with this study, we have observed direct GHS-R mediated effects on

glycolysis and insulin secretion.

Finally, Figure 1 clearly shows that in the fasting groups, the presence of pegvisomant was not significantly important for the changes observed in glucose, insulin and FFA levels. Apparently in this situation, loss of GH action and/or a drop in IGF-I levels, seem not to be the major players in fasting related changes in these parameters. In the non-fasting state, however, and in the presence of pegvisomant, a drop in free, but not total IGF-I could be observed. However, we observed an increase in IGF-I levels on day 2 in all subjects, which most likely was induced by the two stimulatory tests on day 1. After day 2, total IGF-I levels began to decrease, but this decrease did not reach the level of significance on day 4. Other studies are necessary to rule out confounding effects of changes in IGF-I on all noted findings. The question of which item (loss of GH action or the decrease in Free IGF-I concentrations) is responsible for the observed changes in glucose, insulin and FFA levels after injection of GHRP-6 remains to be answered.

In conclusion, we found that under the condition of GHR blockade, GHRP-6 has profound stimulatory effects on serum insulin, and glucose levels, which reflect a state of insulin resistance. These GHRP-6 induced changes only occur in the non-fasting state in the presence of GHR blockade. In the same condition there is an increase in lipogenesis, which indicates tissue specific differential changes in insulin sensitivity that actually could lead to undesired changes in body composition. We also conclude that these possible effects of GHRP-6 are GH independent, and that in man, some degree of GH action is necessary to prevent these GHRP-6-induced metabolic changes. Moreover, we postulate that direct GHS-R-mediated effects are involved in the induction of the metabolic alterations, as well as subsequent changes in body composition, which are characteristic for the insulin resistance syndrome.

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Chapter 4.1 - Acute Effect of Pegvisomant on Cardiovascular Risk Markers in Healthy Men. Implications for the Pathogenesis of Atherosclerosis in Growth Hormone Deficiency.

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Abstract

Cardiovascular risk is increased in Growth hormone deficiency (GHD).
GHD adults are frequently abdominally obese and display features of the
'metabolic syndrome'. Otherwise healthy abdominally obese subjects have
low growth hormone (GH) levels and show features of the 'metabolic
syndrome' as well. We investigated in healthy non-obese males the effect of
the GH receptor antagonist pegvisomant in different metabolic conditions.
This as a model for acute 'GHD' without yet the alterations in body
composition associated with GHD. We compared the effect of pegvisomant
with placebo before and after 3 days of fasting. In addition, we investigated
the effect of pegvisomant under normal – i.e. fed – conditions.
Three days of fasting as well as pegvisomant alone decreased serum free
IGF-I levels: 1.0±0.15 ng/mL vs 0.31±0.05 ng/mL and 0.86±0.23 ng/mL vs
0.46±0.23 ng/mL respectively. Fasting in combination with pegvisomant also
decreased serum free IGF-I levels: 1.0±0.15 ng/mL vs 0.31±0.07 ng/mL.
Treatment with pegvisomant had no additional influence on the decline of
free IGF-I induced by fasting. Pegvisomant alone had no influence on insulin
sensitivity. The increase in insulin sensitivity induced by fasting was
comparable to the increase in insulin sensitivity induced by fasting
combined with pegvisomant. Among serum lipid concentrations only serum
triglycerides increased significantly as a result of pegvisomant alone: 1.0±0.2
mmol/L vs 1.6±0.4 mmol/L. The changes in lipid concentrations induced by
fasting alone or with pegvisomant were not different from that induced by

pegvisomant alone. Von Willebrand Factor antigen levels declined significantly under the influence of pegvisomant alone: 1.1±0.07 U/mL vs 0.8±0.06 U/mL.

In conclusion, in different metabolic conditions the GH receptor antagonist pegvisomant induces no significant acute changes in the major risk markers for CVD. These data suggest that the secondary metabolic changes – e.g. abdominal obesity or inflammatory factors – that develop as a result of long standing GHD are of primary importance in the pathogenesis of atherosclerosis in patients with GHD.

Introduction

Adults with growth hormone deficiency (GHD) have an increased risk for cardiovascular disease (CVD) (1). Four relevant cohort studies investigating 1197 patients on routine replacement therapy without growth hormone (GH) have reported a decrease in life expectancy of patients with hypopituitarism (2-5). The overall Relative Risk for CVD has been estimated at 1.47 (95% C.I.: 1.27-1.70) (6). Compared with age- and sex-matched normal subjects, patients with GHD have reduced insulin sensitivity, pro-atherogenic hemostasis parameters and higher serum lipid concentrations. They thus display characteristic features of the 'metabolic syndrome' (7;8). Since central adiposity is present in GHD and because adiposity itself is associated with low growth hormone (GH) levels, it has been postulated that low GH levels play a role in the metabolic alterations associated with the 'metabolic syndrome' (7;9-11).

GH acts by binding to receptors on liver and other cells. One GH molecule binds to two receptor molecules on the target cell initiating dimerization of these receptor molecules finally resulting in the secretion of Insulin-like Growth Factor-I (IGF-I) (12;13). Pegvisomant is a genetically manipulated GH molecule, which disables functional dimerization of the two GH receptor molecules (14;15). In normal subjects and in patients with acromegaly, pegvisomant is an effective blocker of GH action and significantly decreases IGF-I concentrations (16;17).

The aim of the present study was to investigate whether GH receptor blockade – as a model of GHD but without the typical alterations in body composition – influences insulin sensitivity, hemostasis parameters and serum lipid concentrations in healthy non-obese males. Because fasting has marked effects on the GH-IGF-I axis we performed two studies: 1) A double blind placebo controlled cross-over study comparing the effects of fasting on

insulin sensitivity, hemostasis parameters and serum lipid concentrations with and without pegvisomant; 2) A single arm study investigating the effect of pegvisomant during normal – fed – conditions.

Subjects and methods

Study subjects

Ten healthy male subjects (mean ± SD age, 23.4 ± 2.7 year; range, 20 – 28) with a normal body weight (mean ± SD BMI, 21.8 ± 1.8 kg/m²; range, 19.7–25.8) were asked to participate. None of the subjects had a relevant medical history or used medication. All ten subjects participated in the cross-over study and five participated in the single-arm study. The local ethical committee approved the study and all subjects gave written informed consent.
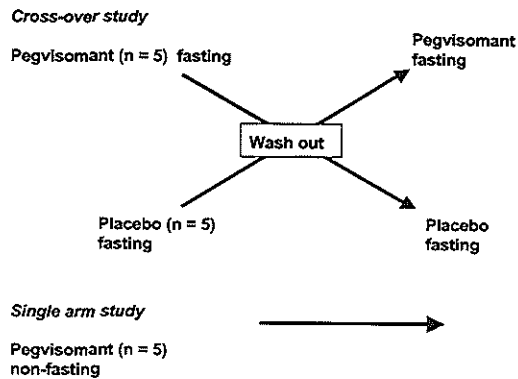
Experimental design

Figure 1: General study design.

The study consisted of two parts (figure 1):
A double blind placebo controlled cross-over study comparing GHR blockade with placebo before and after 3 days of fasting. After an overnight fast subjects were admitted to the Clinical Research Unit on day 1 at 7.30 AM. Blood was drawn at 8 AM, and at 6 PM a single dose of 80 mg pegvisomant (Sensus drug development corporation, Austin, Texas, USA) or placebo was administered subcutaneously (figure 2). From midnight until the end of the study - on day 4 at 8 PM - subjects fasted (while having free access to non-caloric fluids). Each morning at 8 AM blood was drawn. Between the study periods there was a wash out period of 3 to 7 weeks.
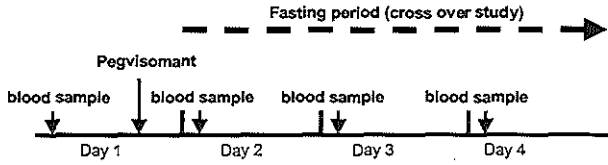
Figure 2: Overview of the study periods.

A single arm open label study in which the effect of GHR blockade under non-fasted conditions (subjects received a standardized diet) was investigated. After an overnight fast subjects were admitted to the Clinical Research Unit on day 1 at 7.30 AM, at 8 AM blood was drawn and at 6 PM a single dose of 80 mg pegvisomant was administered subcutaneously. After an overnight fast blood was drawn each morning at 8 AM. In this study a detailed analysis of hemostasis was performed. Hemostasis was assessed on day 1 and day 4 at 8 AM.

GH, total IGF-I, free IGF-I, glucose, insulin, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides and lipoprotein (Lp(a)) were determined from all blood samples. In the single arm study fibrinogen, plasminogen, antiplasmin, factor VIII activity (FVIII:c), von Willebrand factor antigen(vWF:ag), vWF Ristocetin cofactor (vWF:Rco) activity, vWF Collagen binding activity (vWF:Cba), plasminogen activator inhibitor-I (PAI-I) antigen, PAI-I activity, tissue plasminogen activator (t-PA) activity and t-PA antigen were determined at baseline and on day 4.

Assays
All assays were done in duplicate. Samples were measured for endogenous GH in a two-site immunoassay that does not cross-react with pegvisomant. The assay exhibits a lower detection limit of 0.02 µg/L GH, an upper end of the working range of 50 µg/L for 25 µL serum samples, and no cross-reaction with pegvisomant up to a concentration of 50,000 µg/L (16). The inter-assay coefficients of variation (CV) are 4.1% at 4.0 µg/L and 3.8% at 20 µg/L. The intra-assay CVs are 3.4% at 0.25 µg/L, 1.9% at 2.5 µg/L, and 4.5% at 25 µg/L (Med Klinik Innenstadt; LMU; Munich; Germany). Serum IGF-I was determined with a commercially available radioimmunoassay (Biosource Europe S.A., Nivelles, Belgium; intra- and interassay CV 5.0 and 9.6% respectively) and free IGF-I was determined with a commercially available immunoradiometric assay (Diagnostic System Laboratories, Webster, TX;

intra- and interassay CV 10.3 and 10.7% respectively). Glucose was assessed with a automatic hexokinase method (Roche, Almere, The Netherlands). Insulin was assessed with a radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium; intra- and interassay CV 13.7 and 8.0% respectively). Total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides were assessed with an enzymatic colorimetric test (Roche Diagnostics, Mannheim, Germany). Lipoprotein (a) was assessed with a radioimmunoassay (Mercodia AB, Uppsala, Sweden).

Blood for determination of hemostasis parameters was obtained by venipuncture under standardized conditions (overnight fasting, after 15 minutes rest, no tourniquet use) and collected in citrate (final concentration of 0.105M), in Stabilyte® (Biopool, Umeå, Sweden) for determination of t-PA and PAI-I activity, and in CTAD (Beckton Dickinson, Plymouth, UK) for determination of PAI-1 and t-PA antigen. Plasma was obtained by centrifugation at 2000xg for 20 min at 4° C. Plasma was stored at -80° C until use. FVIII:c was measured by a one stage clotting assay. vWF:ag was measured by an ELISA using rabbit anti-human vWF (Dako A/S, Glostrop, Denmark) as primary antibody and as secondary a horseradish peroxidase-conjugated antibody. vWF:Rco was assayed with formalin-fixed platelets using the PAP-4 aggregometer (BioData). vWF:Cba was assessed by measuring the amount of plasma vWF which binds to collagen by EIA. Fibrinogen was measured using the Clauss method. PAI-1 activity was measured using Chromolize™ PAI-1 bio-immunoassay (Biopool). (t-PA activity was determined using a bio-functional immunosorbent assay (Chromolize™, Biopool). PAI-1 antigen and t-PA antigen were measured using a TintElize® PAI-1 and t-PA ELISA also obtained from Biopool. Plasminogen and antiplasmin were determined using chromogenic substrates, S-2251 and S-2403 respectively, on an automated analyzer (Sysmex, Dade Behring, Marburg, Germany).

Homeostatic Model Assessment (HOMA)
Beta-cell function (%B) and insulin sensitivity (%S) were analyzed with the HOMA model (kindly provided by Dr Jonathan Levy, Diabetes Research Laboratories, Oxford, United Kingdom). This is a structural model of glucose-insulin interaction, which describes the functioning of the major effector organs. Simultaneous assessment of the glucose and insulin concentrations after an overnight fast in each person allows evaluation of the combination of beta-cell function and insulin sensitivity. Beta-cell

function and insulin sensitivity are expressed in relation to values in a 'standard individual', in which they are each accorded the value 100%. The HOMA model has been validated (18-21).

Assessment of body composition

Body composition was assessed with bioelectrical impedance assessment (Holtain Limited, Croswell, U.K.). Total body resistance was measured with a four-terminal portable impedance analyser. Measurements were made while the subjects lay comfortable on bed with the limbs abducted from the body. Current injector electrodes were placed just below the metacarpo-phalangeal/metacarpo-tarsal joint on the dorsal side of the right hand/foot. Detector electrodes were placed on the posterior side of the wrist. Impedance was measured after a 800µA at 50kHz current was injected. A computer program employing empirically derived formulas was used to calculate total body water (TBW), fat free mass (FFM) and fat mass (FM). This method has been shown to be a reliable and valid approach for the estimation of human body composition in healthy human beings (22).

Statistical analysis

All results are reported as mean ± SEM. As the maximal effect for all parameters studied was reached on day four all comparisons are reported as baseline vs day four. Because all study periods were exactly the same until 6 PM on day 1 and in order to minimize intra-individual differences we took the means of the values of the fasting study periods as baseline values. Means from baseline and day 4 were compared with the Wilcoxon signed ranks test. Correlations were calculated with Pearson correlation coefficient. All P-values are two-sided, P-values <0.05 were considered significant. Analyses were performed using SPSS version 9.0 for windows (SPSS Inc., Chicago, Illinois).

Results

GH, total- and free IGF-I

Cross over study: fasting non significantly increased GH levels: 0.3 ± 0.09 µg/L vs 0.6 ± 0.2 µg/L (figure 3a). Three days of fasting combined with pegvisomant resulted in a significant increase in GH levels: 0.3 ± 0.09 µg/L vs 1.8 ± 0.5 µg/L ($p = 0.005$). Serum total IGF-I levels did not change; fasting: 204.6 ± 19.2 ng/mL vs 214.6 ± 30.8 ng/mL; fasting and pegvisomant: 204.6 ± 19.2 ng/mL vs 181.5 ± 32.3 ng/mL (figure 3b). Three days of fasting as well as fasting in
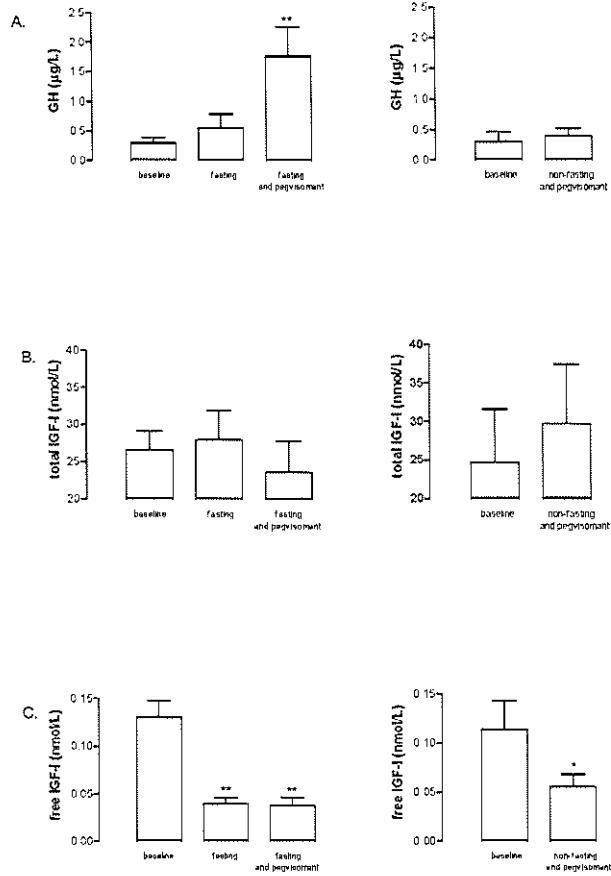
Figure 3: Mean values (±SEM) of GH (A), total IGF-I (B) and free IGF-I (C): effects of fasting, fasting with pegvisomant and pegvisomant alone.
*p<0.05, **p<0.01 compared to baseline (Wilcoxon signed rank test).

combination with pegvisomant decreased serum free IGF-I levels: 1.0±0.15 ng/mL vs 0.31±0.05 ng/mL (p = 0.005) and 1.0±0.15 ng/mL vs 0.31±0.07 ng/mL (p = 0.005) respectively (figure 3c). Treatment with pegvisomant had no influence on the decline of free IGF-I induced by fasting.

Single arm study: pegvisomant alone did non significantly increase GH levels: 0.3±0.2 μg/L vs 0.4±0.1 μg/L (figure 3a). Serum total IGF-I levels did not change: 190.7±52.3 ng/mL vs 229.2±59.2 ng/mL (figure3b), whereas serum free IGF-I levels decreased significantly: 0.86±0.23 ng/mL vs 0.46±0.08 ng/mL (p = 0.04) (figure 3c).

Cross over study vs single arm study: Compared to pegvisomant alone fasting alone and fasting in combination with pegvisomant had similar effects on

GH, total IGF-I and free IGF-I concentrations.

Body composition

Cross over study: Fat mass did not change (table 1). Fasting either with or without pegvisomant resulted in a significant decrease in fat free mass and total body water (table 1). As the change in fat free mass was significantly correlated with the change in total body water (p<0.001, for all study periods) the change in fat free mass during fasting was most likely a result of the expected decline in total body water.

Single arm study: Fat mass, fat free mass and total body water did not change (Table 1).

Table 1: Mean values (±SEM) in body composition: effects of fasting, fasting with pegvisomant and pegvisomant alone.

	Baseline	Day 4		
		Cross over study		Single arm study
		Fasting	Fasting & PegV	PegV
Fat mass (kg)	19.0±2.6	19.3±2.4	18.7±2.1	20.0±3.8
Fat free mass (kg)	57.0±2.1	53.1±1.9**	53.8±2.0*	57.6±4.0
Total body water (L)	41.6±1.5	38.7±1.4**	39.2±1.5*	42.1±2.9

*Note: all comparisons are pairwise. *p<0.05 baseline vs day 4, **p<0.01 baseline vs day 4 PegV denotes pegvisomant.*

Beta-cell function and insulin sensitivity

Cross over study: Beta-cell function as measured with the HOMA model remained unchanged; baseline vs fasting: 139.6±14.0 % vs 125.7±20.6 %; baseline vs fasting and pegvisomant: 139.6±14.0 % vs 156.0±32.5 % (figure 4a). Fasting alone or with pegvisomant resulted in a significant increase in insulin sensitivity: 166.4±47.7 % vs 637.3±139.1 % (p = 0.02) and 166.4±47.7 % vs 450.1±96.4 % (p = 0.008) respectively (figure 4b). The change in insulin sensitivity induced by fasting was not different from that induced by fasting combined with pegvisomant.

Single arm study: Beta-cell function as measured with the HOMA model remained unchanged; baseline vs non-fasting and pegvisomant: 145.8±25 % vs 166.5±15.2 % (figure 4a). Pegvisomant without fasting had no significant effect on insulin sensitivity: 163.3±78.6 % vs 82.2±8.0 % (figure 4b).

Cross over study vs single arm study: The changes in beta-cell function and insulin sensitivity induced by fasting alone or with pegvisomant were not different from that induced by pegvisomant without fasting.
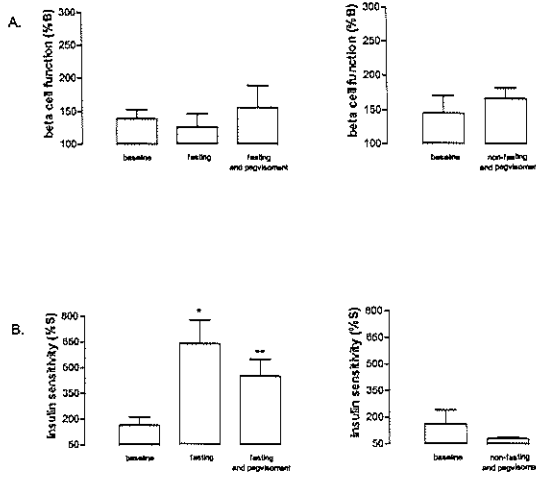
Figure 4: Mean values (±SEM) of beta cell function (%B) (A) and insulin sensitivity (%S) (B): effects of fasting, fasting with pegvisomant and pegvisomant alone. *p<0.05, **p<0.01 compared to baseline (Wilcoxon signed rank test).

Hemostasis (single arm study only)

VWF:ag changed significantly under the influence of pegvisomant alone (table 2). A similar trend was observed for FVIII:c (baseline vs three days of fasting: 1.7±0.1 IU/mL vs 0.9±0.2 IU/mL; p = 0.3) and vWF:activity (baseline vs

Table 2: Mean values (±SEM) of coagulation and fibrinolysis parameters: effect of pegvisomant alone.

	Baseline	Pegvisomant
Fibrinogen (g/L)	2.3±0.2	2.4±0.3
Plasminogen (IU/mL)	1.0±0.03	1.0±0.03
Antiplasmin (IU/mL)	0.9±0.04	1.0±0.08
FVIII:c (IU/mL)	1.1±0.1	0.9±0.2
vWF:ag (U/mL)	1.1±0.07	0.8±0.06*
VWF:Cba. (U/mL)	1.1±0.2	0.9±1.0
VWF:Rco (U/mL)	1.0±0.09	0.8±0.1
PAI-I activity (IU/mL)	10.9±3.8	19.9±6.8
PAI-I antigen (ng/mL)	19.3±6.0	22.2±5.5
t-PA activity (IU/mL)	0.4±0.2	0.4±0.2
t-PA antigen (ng/mL)	5.6±0.5	5.9±1.2

*p<0.05 baseline vs day 4

FVIII:c denotes Factor VIII activity, vWF:ag denotes von Willebrand Factor antigen, Cba denotes Collagen binding activity, Rco denotes Ristocetin cofactor activity, PAI-I denotes Plasminogen Activator Inhibitor-I, t-PA denotes Tissue Plasminogen Activator

three days of fasting: vWF:Rco 1.0±0.09 U/mL vs 0.8±0.1 U/mL; p = 0.08; vWF:Cba 1.1±0.2 U/mL vs 0.9 ±1.0 U/mL; p = 0.2). Fibrinolysis parameters, including PAI-I and t-PA did not change during treatment (table 2).

Lipids

Cross over study: Fasting alone or in combination with pegvisomant resulted in a significant increase in LDL: 2.2±0.2 mmol/L vs 2.9±0.3 mmol/L (p=0.01) and 2.2±0.2 mmol/L vs 3.1±0.2 mmol/L (p = 0.005); however total cholesterol levels did not change: 3.9±0.2 mmol/L vs 4.4±0.3 mmol/L and 3.9±0.2 mmol/L vs 4.7±0.2 mmol/L (figure 5a&b). HDL cholesterol levels decreased as a result of fasting alone: 1.2±0.1 mmol/L vs 1.0±0.1 mmol/L. This decrease was statistically significant if fasting was combined with pegvisomant: 1.2±0.1 mmol/L vs 1.1±0.07 mmol/L (p = 0.04) (figure 5c). As a result of the changes in total and HDL cholesterol the cholesterol-HDL ratio rose significantly as a result of fasting alone and fasting in combination with pegvisomant: 3.5±0.3 vs 4.7±0.4 (p = 0.005) and 3.5±0.3 vs 4.5±0.4 (p = 0.005) (figure 5d). Triglycerides rose after fasting with pegvisomant: 0.8±0.1 mmol/L vs 1.1±0.2 mmol/L (p = 0.02); but not after fasting alone: 0.8±0.1 mmol/L vs 1.0±0.09 mmol/L (p = 0.1) (figure 5d). Lipoprotein (a) increased significantly after fasting either without or with pegvisomant; 308.2±153.5 U/L vs 391.8±170.7 U/L (p = 0.008) and 308.2±153.5 U/L vs 359.3±153.0 U/L (p = 0.02) respectively (figure 5f). The changes in lipid levels induced by fasting alone were not significantly different compared to the changes in lipid levels induced by fasting combined with pegvisomant.

Single arm study: Total, LDL and HDL cholesterol levels did not change: 3.9±0.3 mmol/L vs 4.2±0.3 mmol/L, 2.2±0.2 mmol/L vs 2.4±0.2 mmol/L and 1.1±0.2 mmol/L vs 1.0±0.07 mmol/L respectively (figure 5a-c). Pegvisomant alone had no influence on the total cholesterol / HDL cholesterol ratio: 4.0±0.5 vs 4.5±0.6 (figure 5d). Lipoprotein (a) did not change: 567.0±313.5 U/L vs 599.8±333.7 U/L (figure 5f). The only lipid particle that changed significantly was triglyceride; 1.0±0.2 mmol/L vs 1.6±0.4 mmol/L (p = 0.04) (figure 5e).

Cross over study vs single arm study:
The changes in lipid concentrations induced by fasting alone or with pegvisomant were not different from that induced by pegvisomant without fasting.
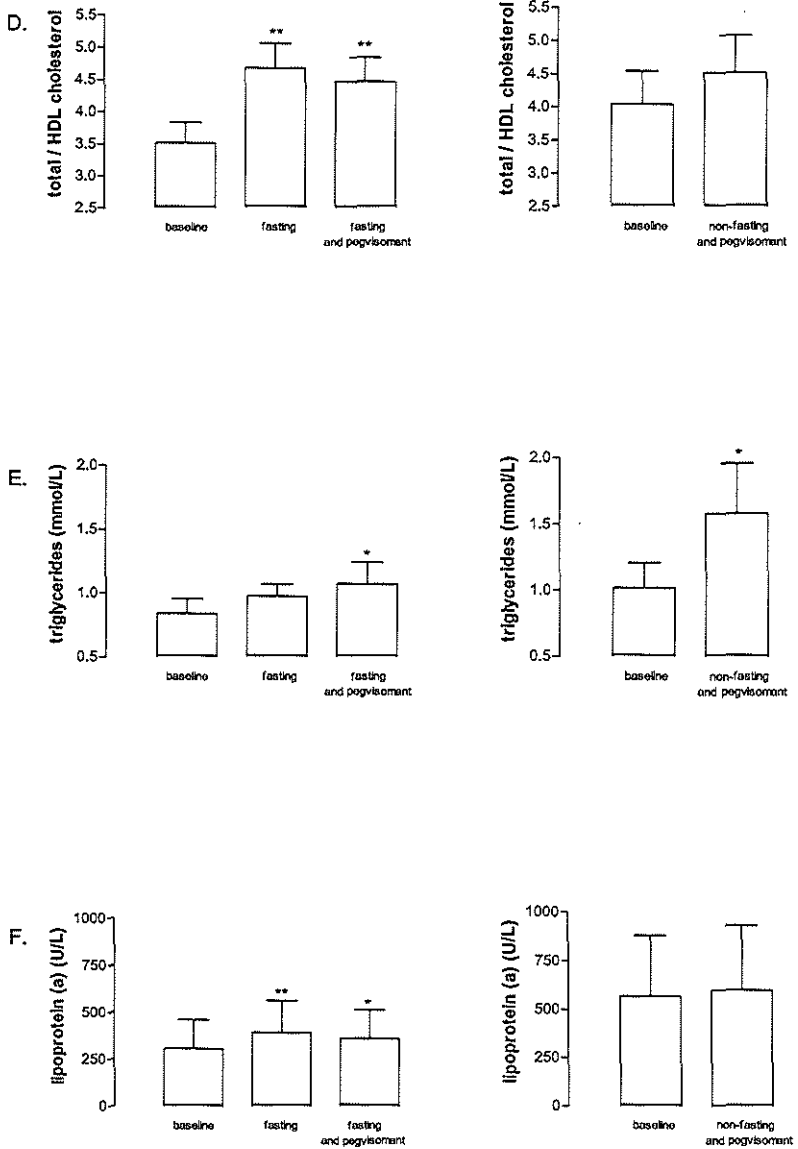
A.

B.

C.
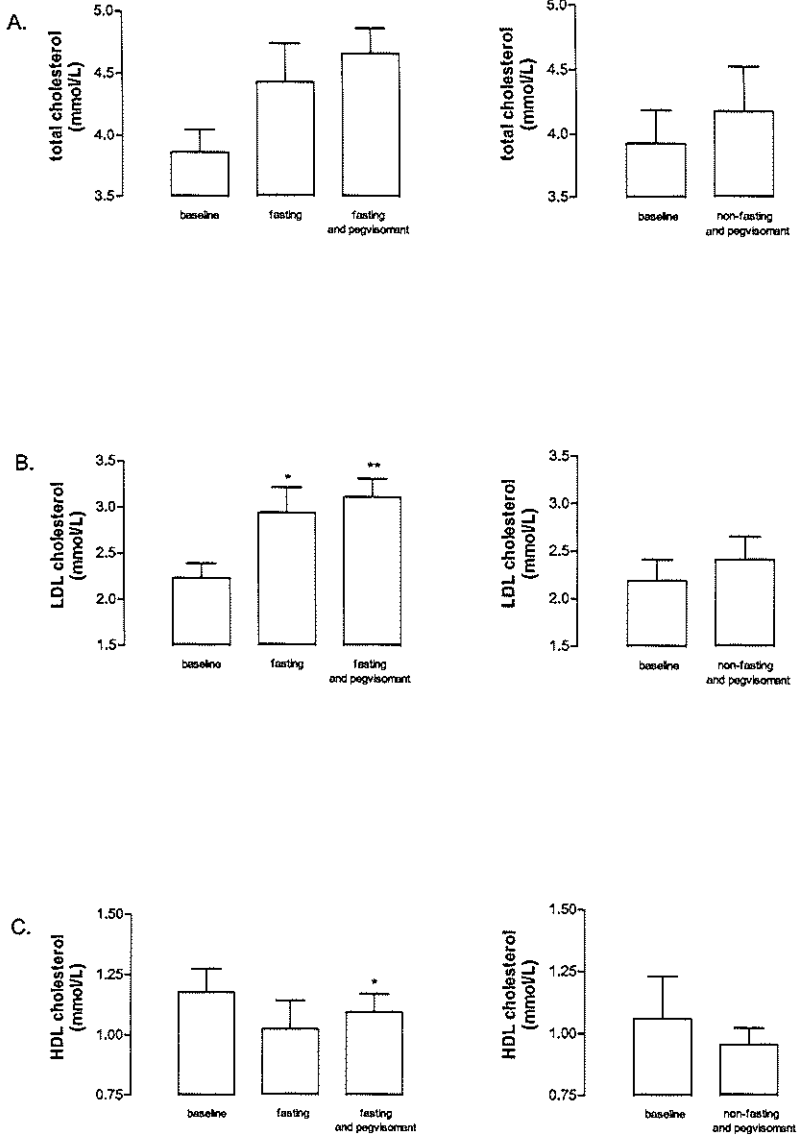
Figure 5: Mean values (±SEM) of total cholesterol (A), LDL cholesterol (B), HDL cholesterol (C), total / HDL cholesterol ratio (D), triglycerides (E) and lipoprotein (a) (F). *p<0.05, **p<0.01 compared to baseline (Wilcoxon signed rank test).

Discussion

GHD is associated with central obesity, insulin resistance, pro-atherogenic hemostasis parameters and elevated serum lipid concentrations (7;8;23). Treatment of GHD adults with GH attenuates central obesity and induces positive effects on lipid levels (24-27). Because adiposity in otherwise healthy subjects is associated with low levels of GH and treatment of middle aged obese males with GH resulted in a reduction of central adiposity with favorable effects on insulin sensitivity and lipid metabolism several authors have suggested an important role for GH in the development of central adiposity and the associated metabolic consequences (28;29).

By using the GH receptor antagonist pegvisomant we set out to study the effects of 'functional' GHD independently from the eventual alterations in body composition that are associated with long standing GHD (7). In our study maximal pegvisomant drug levels were achieved on day four (fasting in combination with pegvisomant vs pegvisomant only; 5228±745.6 ng/mL vs 4164±788.3 ng/mL [p=0.45, Mann Whitney U-test]), these levels were more than 2000-fold higher than endogenous GH levels. We used free IGF-I as a measure for assessing the efficacy of the GH receptor blockade (30-32). Indeed, pegvisomant induced a significant reduction in free IGF-I resulting in free IGF-I levels on day four comparable to those observed in hypopituitarism with GHD (33). Although, free IGF-I may not be a marker for all local (paracrine and/or autocrine) GH actions the observed decline in free IGF-I levels can be take to indicate efficient GH receptor blockade; and, as we have to assume that GH exerts its actions through the GH receptor. These data, indeed, indicate that the administration of pegvisomant creates a state of acute – functional - 'GHD'.

Fat mass was not altered in our study; however, fat free mass decreased as a result of fasting. This implies that lean body mass also decreased. It should be noted that this decrease in fat free mass could be fully accounted for by the concomitant – metabolically inert - decline in total body water. Pegvisomant alone had no effect on insulin sensitivity, and although insulin sensitivity increased as a result of fasting this increase was not influenced by additional GH receptor blockade. From these data we conclude that acute impairment of the GH signaling cascade has no immediate effect on insulin sensitivity. In a recent study Christopher et al. described a negative correlation between insulin sensitivity and IGF-I levels (34). In our study no significant correlations were observed between changes in free IGF-I and insulin sensitivity.

The increased risk of cardiovascular disorders in GHD has been partially ascribed to changes in hemostasis in these patients, such as an increase in serum fibrinogen concentration and increased PAI-I activity (8;35). High fibrinogen levels are associated with an increased incidence of stroke and myocardial infarction and high PAI-I activity or antigen is an independent risk factor for primary and recurrent myocardial infarction (36-38). Johansson et al. have shown that GHD adults have higher fibrinogen and PAI-I activity compared to healthy controls matched for sex, age and body mass index (8). In a subsequent study they observed a decrease in PAI-I after two years of GH therapy (35). In addition to GHD increased PAI-I activity has been found in abdominally obese subjects and a reduction of PAI-I levels has been observed after weight loss (39). Probably this is due to a decrease in insulin resistance as a result of weight loss (40). So it is unclear whether the observed changes in PAI-I activity, PAI-I antigen and t-PA antigen are due to a direct effect of GH itself or to changes in body composition. However, the fact that in our study we observed no significant changes in PAI-I levels makes a causative role of GH itself in the elevation of PAI-I observed in GHD less likely.

Interestingly, pegvisomant resulted in a significant decrease in vWF:ag and a similar trend was seen for FVIII and vWF activity. It has long been recognized that there is a relationship between GH and vWF. A rise in GH is associated with increased vWF activity in healthy subjects (41). Our study thus supports these data. In GHD subjects, Jorgensen et al. found only a non-significant increase in vWF:ag levels after 4 months of GH replacement therapy in 22 adult GHD subjects (42). Johansson et al. investigated the long term effects of GH replacement therapy on hemostasis and fibrinolysis and found a non-significant decrease 24 months after initiation of GH replacement therapy (35). However, vWF levels before start of GH replacement were not decreased compared to normals in this study (35). Besides vWF all other hemostatic parameters including fibrinogen, plasminogen, antiplasmin and t-PA remained unchanged by pegvisomant administration. Therefore GH does not seem to play an important – direct - role in the regulation of hemostasis. The changes in coagulation factors, as observed in GHD are probably caused by the metabolic changes induced by longstanding GHD.

Pegvisomant alone increased serum triglyceride concentration indicating that GH is directly involved in the regulation of serum triglyceride. However, in a previous cross-sectional study we observed a strong

negative correlation between free IGF-I and triglycerides (43). Moreover, administration of recombinant IGF-I has been reported to cause a decrease in triglyceride levels (44). Taken together these data indicate that GH and IGF-I are both involved in triglyceride metabolism. Fasting alone, fasting in combination with pegvisomant and pegvisomant alone had similar effects on all the assessed lipid particles. In accordance with the previously described data concerning insulin sensitivity and coagulation and fibrinolysis factors these data also point to a primary role of changes in body composition and not of GH itself in the metabolic changes seen in GHD patients.

Several authors have reported that GH increases and IGF-I decreases circulating Lp(a) (45-47). Surprisingly, pegvisomant induced no change in Lp(a). It could be argued that the four day study period is too short to induce significant changes. However, four days of fasting independently of GH receptor blockade was able to induce significant changes in Lp(a). Apparently GH and IGF-I are - at least in the short term - only of minor importance in the regulation of Lp(a).

In conclusion, in different metabolic conditions the GH receptor antagonist pegvisomant induces no significant changes in the major risk markers for CVD. Based on these data we hypothesize that the secondary metabolic changes - e.g. abdominal obesity or inflammatory factors (48) - that develop as a result of long standing GHD are of primary importance in the pathogenesis of atherosclerosis in patients with GHD.

Acknowledgements

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Chapter 4.2 - Control of Tumor Size and Disease Activity During Co-treatment with Octreotide and the Growth Hormone Receptor Antagonist Pegvisomant in an Acromegalic Patient

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Abstract

We describe the case of an acromegalic subject, who was the first patient ever treated with this growth hormone receptor (GHR) antagonist pegvisomant. Furthermore, in this particular patiënt progression in tumor size was encountered during treatment with pegvisomant. The patient described did benefit from co-treatment with pegvisomant and octreotide, including decreased growth hormone levels, normalization of serum IGF-I concentrations and improvement of visual field defects.

Report of the case

This 34-year old male patient was treated by transsphenoidal selective adenomectomy for a convex GH-producing pituitary macroadenoma in February 1997. He had, at that time, active acromegaly with fatigue, headaches, excessive perspiration, and joint pains. Before surgery, a single injection of 50 mg octreotide sc decreased the serum GH concentration from 70 ng/mL to minimally 6 ng/mL after 6 hrs Serum prolactin (PRL) was normal, whereas secondary hypothyroidism and hypogonadism were present These were subsequently treated by replacement therapy. The neurosurgical procedure was unsuccessful because 6 months after operation the tumor still extended up to the optic chiasm, although without signs or symptoms of compression of the chiasm. Because of the extension close up to the optic chiasm, it was decided not to treat him with radiotherapy. Because of tumor size and persistent disease activity, he started treatment with octreotide in sc dosages up to 200 mg three times daily (t.i.d.). This did not result in normalization of his serum

total insulin-like growth factor I (IGF-I) concentrations (untreated IGF-I levels, 6230 nmol/L; age-adjusted upper normal level, 50 nmol/L; nadir in serum total IGF-I levels during treatment with octreotide 200 mg sc t.i.d., 170 nmol/ L). Therefore, it was concluded that this patient was only partially sensitive to octreotide.

In April 1997, he received for the first time pegvisomant, as he participated as the first patient in a dose-finding study in our center. The pegvisomant dose administered was 0.3 mg/kg body weight (27.6 mg). A single sc administration of the GH receptor (GHR) antagonist resulted in a decline in the total serum IGF-I level on day 3 after the injection without reaching normal levels (from 233 nmol/L to 211 nmol/L).

In July 1997, he was enrolled in a Phase 2b study on the efficacy and safety of pegvisomant in the treatment of acromegaly. In this 6-week placebo-controlled study, he was randomized to receive 80 mg pegvisomant sc once weekly. A significant decrease in serum total IGF-I levels was observed (from 305 mmol/L to 190 nmol/L), although the result was still three times the upper age-adjusted normal level (,50 nmol/L).

Because temporarily no study drug was available, he was treated again with octreotide sc (200 µg sc t.i.d) between October 1997 and February 1998. In this period, the nadir in total serum IGF-I concentrations in the pegvisomant-free period remained the same as before the first period of treatment with the GHR antagonist (around 150 nmol/L; see Table 1), indicating that he still was only partially sensitive to octreotide treatment. From March 1998 onward, however, he was treated again with pegvisomant,

Table 1: Serum total IGF-I and tumor volume in cubic centimeters during medical treatment of a 34-yr-old acromegalic patien with octreotide alone, pegvisomant alone, and during coadministration with both compounds.

Date	Pegvisomant dose	Octreotide dose	Volume (cc's)	IGF-I (nmol/L)	comments
July-97	80 mg weekly	–	2,5	216	
October-97	–	Octreotide 200 µg t.i.d.	2,9	162	
March-98	10 mg daily	–		152	
May-98	10 mg daily	–	4,3	145	
June-98	15 mg daily	–		127	
August-98	20 mg daily	–		101	
October-98	25 mg daily	–		140	
November-98	30 mg daily	–		125	
February-99	30 mg daily	–	4,9	80	
June-99	35 mg daily	–		85	
July-99	40 mg daily	–		102	
September-99	40 mg daily	–	5,4	122	
October-99	40 mg daily	–		103	visual field defects
November-99	40 mg daily	30 mg monthly (LAR)		82	visual field defects
December-99	40 mg daily	30 mg monthly (LAR)		35	visual field defects
January-00	40 mg daily	30 mg monthly (LAR)		37	normalization of visual disturbances
March-00	40 mg daily	30 mg monthly (LAR)	5,4	37	

without interruptions. In this period, pegvisomant was administered daily by sc injections, instead of weekly injections. A dose-responsive reduction in IGF-I was observed as daily doses were gradually increased from 10 mg sc to the maximal allowed daily dose in this study of 40 mg (see Fig. 1), although the final serum IGF-I concentration was still slightly above the upper limit of normal. This resulted in high pegvisomant concentrations

Figure 1: *Serum GH (ng/mL), serum total IGF-I (nmol/L), and serum pegvisomant (ng/mL) concentrations in an acromegalic patient during treatment with the GHR antagonist pegvisomant in increasing daily sc doses of 10 - 40 mg. The initiation cotreatment with octreotide long-acting repeatable (30 mg monthly) is indicated by a dashed line.*
Middle, the inital decline in serum IGF-I concentrations during sc octreotide treatment (200 μg t.i.d.) before the start of pegvisomant therapy is depicted as an arrow.
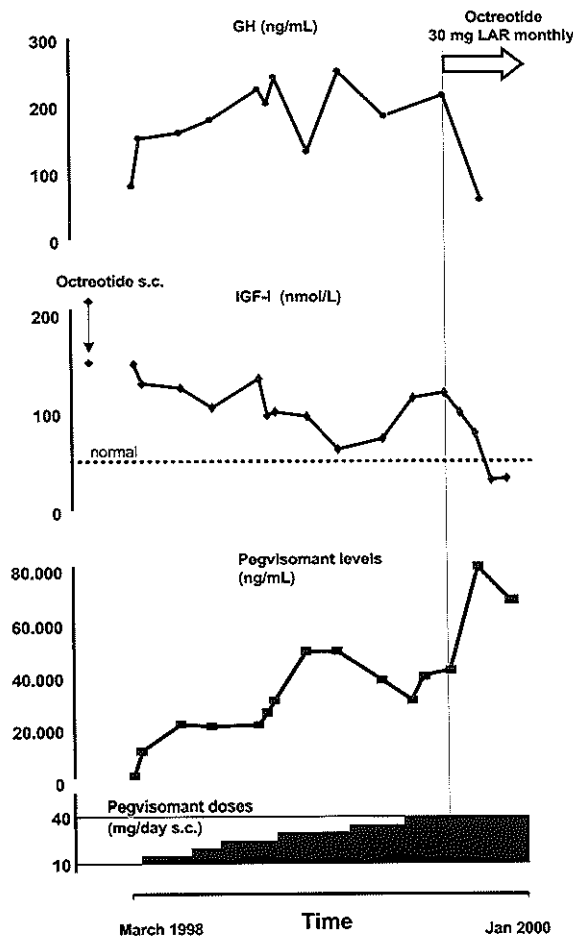
(concentrations around 50,000 ng/mL). Also, a very significant increase in serum GH levels was observed (maximal serum GH concentration during pegvisomant therapy;260 ng/mL).

During September and October 1999, serum total IGF-I concentrations started to increase again, while for the first time bitemporal visual fields defects were found because of a small, but significant, increase in tumor size, whereby the suprasellar extension was increased to the extent that compression of the chiasm was radiologically very likely (see Table 1). These observations were accompanied by an unexplained decrease in serum pegvisomant concentrations, without a change in dose. The patient's compliance was considered to be optimal throughout the whole period of observation, however (e.g. by the interpretation of drug accountability forms).

In November 1999, it was decided to start treatment with octreotide, together with 40 mg sc pegvisomant once daily. Therefore, 30 mg Sandostatin LAR therapy was initiated. This resulted in a rapid normalization of serum total IGF-I concentrations within 2 months (see Fig. 1), whereas the abnormalities in the visual field completely resolved. Also, no further increase in tumor size on magnetic resonance imaging (MRI) was observed between July 1999 and March 2000. At the same time, a striking decrease in serum GH concentrations was observed as well, down to levels comparable with concentrations before the start of pegvisomant treatment. At least up to April 2000, serum IGF-I levels remained well controlled with levels around 35 nmol/L. Signs and symptoms of acromegaly were considerably improved as well.

Discussion

The primary goal in the management of acromegaly is to reverse the effects of GH hypersecretion and to decrease tumor size as much as possible (1-3). The importance of "normalizing" the GH/IGF-I axis has been demonstrated in two long-term studies of surgery (4) (5) in which the mortality rates of patients with acromegaly in whom disease control was inadequate were not different than those of matched controls, in contrast to the 2.4- to 4.8-fold greater mortality in patients who had persistent disease. Therefore, tight control of the GH/IGFI axis is now considered to be an achievable and desired goal of therapy (6).

The reported efficacy data of the available long-acting somatostatin analogs in reducing serum IGF-I levels in acromegalic patients indicate that effective

control of disease activity can be achieved in two thirds of patients who are sensitive to somatostatin analogues (7-12). This leaves at least one third of the patients without an effective control of disease activity by medical intervention. To our knowledge, there are no reports available that describe an increase in size of a somatotropinoma during long-term treatment with somatostatin analogs.

The present case illustrates several important issues, regarding the future use of GHR antagonists on a large scale in the (near) future.

Pegvisomant concentrations
Pegvisomant is a genetically manipulated GH molecule that disables functional dimerization of the two GHR molecules involved in signal transduction, due to a single mutation at the site II of the GH molecule. Pegvisomant is pegylated to increase serum half-life time and to reduce the likelihood of antibody formation. Currently, the compound is under investigation in the treatment of acromegaly (13-15). Pegvisomant blocks GH action, instead of inhibiting GH secretion as somatostatin analogs. This implies that during pegvisomant therapy GH levels are not indicative for GH mediated action on IGF-I production. Although the unpegylated GHR antagonist does have a higher affinity at the site I of the GH, compared to endogenous GH, one must conclude that the pegylation process has major influences on this affinity, because in this particular patient pegvisomant concentrations of as high as 50,000 ng/mL were not able to block GH action of GH concentrations more than two hundred fold lower (250 ng/mL), although serum IGF-I concentrations in our patient nearly normalized when pegvisomant levels were around 50,000 ng/mL. Strikingly, in the phase II and III studies, no clear-cut correlation between pegvisomant concentrations, GH levels, and efficacy data was observed (13-15). In this particular patient, pegvisomant concentrations appear to be roughly correlated to the serum IGF-I response throughout the course of therapy. An unexpected finding was the twofold increase in serum pegvisomant concentrations, which was observed within one month after the initiation of octreotide treatment, while pegvisomant dosages were unchanged (40 mg daily). The reasons for this are unclear.

Growth hormone concentrations
As has been reported before, pegvisomant induces an increase in endogenous serum GH concentrations in acromegalic patients. Whether this dose-

dependant increase in serum GH concentrations reflects a reduction in receptor-mediated GH clearance, an increase in production, or modification of some other pathway remains unclear yet. Studies on the effect of GHR blockade on the pharmacodynamics of serum GH levels are currently being performed. The important observation in the present case, however, is that lowering serum GH concentrations by octreotide co-administration results in a synergistic decrease in serum IGF-I concentrations not achieved with octreotide or pegvisomant administrated alone. This synergistic effect might be predicted, based on the mechanism of action of pegvisomant as a competitive GH receptor antagonist. Lowering GH levels would therefore make a given concentration of pegvisomant more effective.

Tumor size

The present case describes the first patient in whom progression in tumor size was encountered during treatment with pegvisomant. No data are yet available that indicate whether or not the size of a GH producing tumor is modified by pegvisomant. It is therefore possible that some patients with aggressive tumors will show an increase in tumor size during long-term treatment with pegvisomant alone. Therefore, patients treated with pegvisomant should receive routine MRI monitoring of tumor size at least until more data become available. The aggressiveness of the tumor in this particular patient might reflect a subgroup of patients in whom a close follow up of tumor size is mandatory with any treatment. In this patient, co-treatment with octreotide and pegvisomant did result in an improvement in the visual field defects, while no further increase in tumor size was observed in between the last two MRI examinations eight months apart. In this period co-treatment was applied in the last four months.

Conclusion

We describe a 34-year-old male acromegalic patient in whom a high dose of 40mg pegvisomant (by sc daily injections) did not completely normalized serum IGF-I concentrations. Furthermore, he is the first patient in whom progression in tumor size was encountered during treatment with pegvisomant, and finally he was the first patient who was successfully treated with long-acting octreotide together with pegvisomant. This co-treatment resulted in an adequate control of biochemical disease activity, as well as an improvement of visual field defects and a further improvement in signs and symptoms.

▓▓▓▓ Reference List

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Chapter 5 - General Discussion and Conclusions

The first aim of this thesis was to investigate the effects of GHR blockade - by using the GHR antagonist pegvisomant – and fasting either alone or in combination on determinants of GH release. The second aim was to investigate the effect of GHR blockade on cardiovascular risk markers in healthy non-obese males. By using pegvisomant we were able to investigate the role of the GHR in fasting. Ten healthy non-obese subjects were investigated both before and after a 3 day fast and again before and after a 3 day fast in combination with pegvisomant. Secondly, five of the ten subjects that had already participated in the first part of our study were investigated again but now under non-fasting conditions. Apart from the fact that food intake was allowed and that in this second part of the study we also made a detailed assessment of hemostasis and fibrinolysis the study protocol was exactly the same as in the first – fasting – part of our study. In the next sections I will draw some inferences based on the findings presented to you in the preceding chapters.

Regulation of GH secretion

Three days of fasting with or without pegvisomant resulted in the same decrease in serum free IGF-I levels but not in total IGF-I levels. Concomitant with this decline in free IGF-I levels there was an increase in GH levels. Interestingly, the drop in free IGF-I was the same in both study conditions, but GH levels increased significantly more in the fasting + pegvisomant conditions, compared to fasting alone. These data are in accordance with Frystyk and co-workers, they too observed that three days of fasting led to a reduction in serum free – but not total – IGF-I levels with a concomitant rise in serum GH levels [1]. By using a multiple-parameter deconvolution method Hartman et al. have made a detailed analysis of the mechanisms whereby fasting leads to an enhancement of GH secretion [2]. They also found that fasting, albeit only 2 days in this particular study, does not result in lowered serum total IGF-I concentrations. From their model these authors suggested that starvation-induced enhancement of GH secretion is mediated by the dual effect of an increased GHRH- and a decreased somatostatin-tone. The data of our study suggest that free IGF-I, but not total IGF-I, is the major component in the negative feedback on GH secretion. As already stated above, there was no linear relationship between free IGF-I and GH.

This is clearly shown in chapter 3.1, figure 3 and chapter 3.2, figure 3: although the decline in free-IGF-I a result of fasting was not influenced by additional GHR blockade there was a clear difference in GH levels. Therefore, we have to conclude that factors other than free IGF-I are responsible for the additional increase in serum GH levels during fasting in the presence of pegvisomant.

In order to understand why GHR blockade in addition to fasting leads to higher GH levels we – and others (3) – postulate that pegvisomant does not cross the blood-brain-barrier. In this respect it is also important to realize that the pituitary gland lies outside the blood-brain-barrier and pegvisomant will thus block the pituitary GHR's leading to a reduction in ultra shortloop GH auto feedback (figure 1) (4). The GHR's at the hypothalamic level will not be blocked and therefore the hypothalamus will sense high GH levels that will lead to a reduction in GHRH output followed
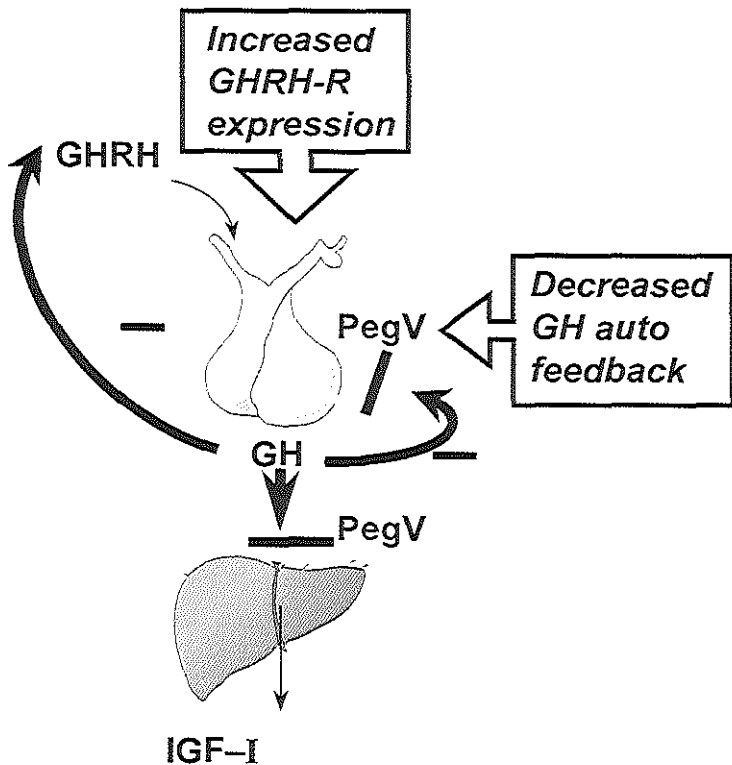

Figure 1:
Postulated effect of Pegvisomant on GHRH-Receptor (GHRH-R) expression and ultra short loop GH autofeedback.

by an upregulation in pituitary GHRH receptor expression. Still, at present it cannot be excluded that pegvisomant crosses the blood barrier. In such a scenario the hypothalamic GHRH neurones will sense no GH and the GHRH output will be increased leading to a reduction in pituitary GHRH receptors. But during GHR blockade alone we observed an increase instead of a decrease in GH release after GHRH indicating that pegvisomant does not enter the brain. Moreover, taking into account the fact that pituitary sensitivity to GHRH during fasting is not influenced by additional pegvisomant we assume that the blockade of ultra shortloop GH auto feedback is the major factor responsible for the significantly higher GH levels during fasting + pegvisomant, compared to fasting alone.

In conclusion, we have reconfirmed that in man free – and not total – IGF-I can exert negative feedback on GH secretion. By using pegvisomant in addition to fasting we have shown that other factors such as ultra shortloop GH auto feedback and pituitary GHRH receptor expression have a role as well.

GH regulation during fasting

As previously mentioned, data indicate that in fasting GH secretion is amplified (2;5;6). Both GHRH and somatostatin output are thought to play a role in the regulation of GH output during fasting. The recent discovery of ghrelin as an endogenous ligand of a GHS-R and the observation that in man the administration of ghrelin strongly stimulates GH release has led to the hypothesis that ghrelin is another important determinant of GH secretion during fasting.

In chapter 3.1 we show that in man fasting rapidly induces an acute and distinct diurnal rhythm in systemic ghrelin levels that is not present in the fed state. Because these changes in systemic ghrelin levels are followed by similar changes in serum GH levels we postulate that ghrelin is the driving force of the increase in GH secretion during fasting. Relevant data indicate that – laboratory animals as well as in man – ghrelin is mainly produced in the stomach (7). Taken together these data indicate that during fasting the pituitary is under direct gastric control. In other words the stomach can be considered as a classical endocrine organ.

In the previous section the role of increased pituitary GHRH receptor expression has already been alluded to. By performing GH stimulation tests with GHRH and GHRP-6 the pituitary sensitivity to these compounds and the influence of pegvisomant and fasting hereon has been formally investigated. Interestingly, fasting alone as well as pegvisomant alone

resulted in a similarly increased pituitary responsiveness to GHRH (chapter 3.2, figure 2). A relevant animal model in this respect is the transgenic growth retarded rat in which GH is only expressed in the GHRH producing hypothalamic neurones (8). In these animals, high hypothalamic GH concentrations induce a decrease in hypothalamic GHRH output with a subsequent increase in pituitary GHRH receptor expression. These animals are therefore highly sensitive to GHRH administration (9). In chapter 3.2 we describe that pegvisomant alone increased the sensitivity to exogenous GHRH. Thus our in vivo experimental data support the notion that pegvisomant does not cross the blood-brain barrier (3). Apparently a situation comparable to the one in the transgenic growth retarded rat ensues, whereby the increased serum GH concentration induced by pegvisomant decreases hypothalamic GHRH output and subsequently increases pituitary GHRH receptor expression. Furthermore, considering the peculiar analogy that fasting alone and pegvisomant alone have on the GHRH stimulated GH release our data suggest an important role for increased pituitary GHRH receptor expression in the mechanism whereby fasting leads to an increased GHRH activity.

Also, fasting alone – but not pegvisomant alone – increased pituitary responsiveness to GHRP-6. This observation is in line with animal data showing that the GHRP-6 induced activation of cells in the hypothalamic arcuate nucleus is much greater in animals that have fasted for 48 hours (10). Before attempting to integrate the above mentioned data it should be mentioned that it is becoming increasingly apparent that the actions of GHRP's and ghrelin parallel each other (11). In trying to explain the data the actions of ghrelin and GHRP's will be considered similar.

In conclusion the somatotroph hyperactivity of fasting can be explained at several levels in the regulation of GH (figure 2). Firstly, there is the previously described decrease in somatostatinergic tonus as well as an increase in GHRH activity (2;5;6;12). To this we add an increase in the production of ghrelin. Secondly, there is a concomitant effect of increased pituitary sensitivity to GHRP-6 (and thus ghrelin) and GHRH during fasting. Finally, in order to fully appreciate the meaning of these changes it should be noted that there is a well established in-vivo synergism existing between GHS's (and thus ghrelin) and GHRH (13).
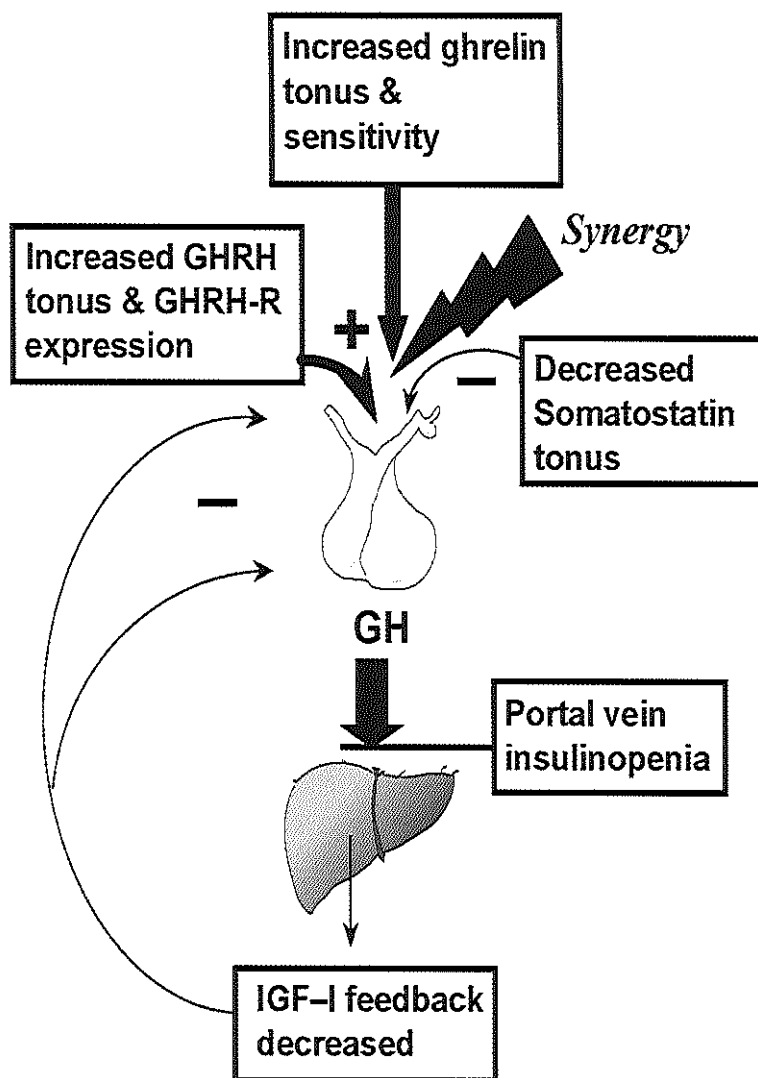
Figure 2:

Proposed mechanisms whereby fasting leads to an increase in GH levels.

GH independent effects of ghrelin

Chapter 3.3 describes GH independent effects of of a standard dose of 1 μg/kg of GHRP-6 on insulin, glucose and free fatty acid levels (14). Under the condition of GHR blockade and only under fed conditions GHRP-6 has profound stimulatory effects on serum insulin and glucose levels reflecting a state of insulin resistance at the liver and muscle level. In the same condition there is a decrease in FFA indicating either an increase in lipogenesis or a

decrease in lipolysis. Taken together these data indicate tissue-specific changes in insulin sensitivity. As these GHRP-6 induced changes in insulin, glucose and FFA levels were only found in the presence of pegvisomant one must conclude that a certain degree of GH action in peripheral target tissues, such as beta-cells, adipose tissue, liver and muscle is necessary to control these GHRP-6 mediated changes in insulin, glucose and FFA levels. The potential importance of these findings lies in the fact that: 1) GHRP-6 can be considered a synthetic analog of a naturally occurring hormone called ghrelin (11); 2) ghrelin is present in the circulation and is actively regulated (see chapter 3.1); 3) the use of pegvisomant might be a model for other states of low GH action/concentration such as obesity, ageing, syndrome-X (characterized by insulin resistance, obesity and hypertension) and protracted critical illness (15-20); 4) the combination of hyperinsulinemia with intact insulin sensitivity of the adipocytes will lead to an increase in fat mass; 5) in animal studies ghrelin also stimulates food intake (21-24). Indeed, the concept that ghrelin can lead to adiposity was subsequently proven by Tschop et al. (25). These authors have shown that once daily subcutaneous ghrelin administration to mice for 2 weeks leads to a significant increase in fat mass (25). Furthermore, in this and other studies it was shown that ghrelin leads to an increase in food intake (22-25). What might the physiological meaning of these tissue-specific changes in insulin sensitivity be? In chapter 3.1 data have been presented showing that during fasting ghrelin levels increase. So, ghrelin might be an important signal of the degree of fasting to the brain and adipocytes. In this way the body can achieve a metabolic control that is also dependent the previous ingestion of meals and possibly also on the relative content of fat, protein and carbohydrate. At least in animal studies ghrelin also stimulates food intake to increase the presence of sufficient nutrients. It should also be noted that the time course of such a GHS mediated metabolic control system is well equipped to fine tune the immediate active metabolic control system, involving glucose induced insulin release, and secondary the storage of nutrients as FFA in adipose tissue.

From a potential therapeutic point of view a ghrelin antagonist might be used to reduce the storage of ingested food in adipose tissue, and facilitate glucose handling in states of low GH action/concentration. However, in a recent study it was shown that obese subjects had lower plasma ghrelin concentrations than age matched lean control subjects (26). However, in this study ghrelin levels were measured in the early morning after overnight

fasting. Considering the data we presented in chapter 3.1 in which ghrelin levels were lowest in early morning these data should be interpreted with caution.

On the basis of the results described in this paragraph it is also tempting to speculate that the diurnal rhythm in insulin sensitivity is related to a ghrelin rhythm. Even more so after taking into account that GHS's have been reported to have an effect on sleep quality and sleep has been reported to have effects on both glucose and insulin levels as well as on GH levels (27-30).

Collectively, the data presented support a GH independent acute metabolic action of ghrelin. These effects can potentially lead to an increase in body fat. In order to further explore the (patho)physiological significance of these findings further research is neccessary.

Pegvisomant as a model for growth hormone deficiency

We postulated that we could use pegvisomant as a model of GH deficiency but without the typical alterations in body composition associated with long-standing GH deficiency to study the changes in several cardiovascular risk markers known to play a role in the excess cardiovascular mortality observed in GH deficiency (31). Chapter 4.1 describes the result of this study. In summary, in different metabolic conditions the GH receptor antagonist pegvisomant induced no significant changes in insulin sensitivity, beta-cell function or lipid levels including Lp(a).

Based on these data we hypothesize that secondary metabolic changes – e.g. abdominal obesity or inflammatory factors (32) – that develop as a result of long standing GHD and not the lack of GH per se are of primary importance in the pathogenesis of atherosclerosis in patients with GHD. Considering the postulated role of abdominal obesity – as opposed to GHD per se – on cardiovascular risk markers it would be interesting to speculate on the effect of GHD per se on ghrelin levels (33;34).

GHR blockade in the treatment of acromegaly

The biochemical and clinical efficacy of pegvisomant has been shown in a 12-week, double-blind placebo controlled study (35). In all patients on active treatment pegvisomant resulted in a significant dose-dependent decrease in serum IGF-I levels. Moreover, it was also shown that pegvisomant reduced clinical symptomatology (35).

The case we describe in chapter 4.2 is special on several accounts (36). He was

the first patient in whom progression in tumor size was encountered during treatment with pegvisomant. Paired sets of magnetic resonance imaging sets from before and during treatment with pegvisomant are available for more the 130 patients. In patients stratified by prior treatment with or without radiotherapy no change in pituitary volume was observed. However, on an individual basis two patients demonstrated a clinically significant increase in tumor size. None of these two patients had received radiotherapy prior to pegvisomant treatment (37). Considering the patient described in chapter 4.2 it is still unclear, whether the observed increase in size of the tumor in this patient has been part of the natural history of this particular tumor, in addition to growth secondary to the use of a pegvisomant. In this patient, cotreatment with octreotide and pegvisomant resulted in an improvement in the visual field defects, whereas no further increase in tumor size was observed in between the last two MRI examinations 8 months apart. In this period, cotreatment was applied in the last 4 months.

Another important observation in the case presented in chapter 4.2, is that lowering serum GH concentrations by octreotide coadministration results in a synergistic decrease in serum IGF-I concentrations that is not achieved with octreotide or pegvisomant administrated alone. This synergistic effect might be predicted, based on the mechanism of action of pegvisomant as a competitive GHR antagonist (38). Lowering GH levels would, therefore, make a given concentration of pegvisomant more effective.

In conclusion, lowering of GH levels probably makes pegvisomant more effective. In the future this might prove to be clinically important in those acromegalic patients that are difficult to manage when IGF-I normalization is concerned. In these patients pegvisomant should be seriously considered as an adjunct to treatments aimed at reducing GH secretion (dopamine agonists and somatostatin analogs). Moreover, long term monitoring of tumor size in acromegalic patients treated with pegvisomant is mandatory, especially in patients that have not received radiotherapy.

▨▨▨▨ Reference List

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Chapter 6.1 – Summary in English

In part I of the thesis we have investigated the effects of GHR blockade and fasting either alone or in combination on some known determinants of GH release.

In chapter 3.1 we show that fasting leads to a diurnal ghrelin rhythm that cannot be explained by changes in insulin, glucose, or free fatty acid levels. These changes in serum ghrelin levels during fasting are followed by similar changes in serum GH concentrations, indicating that ghrelin is the driving force of increased GH secretion during fasting. By using the GH receptor antagonist pegvisomant we also provide indirect evidence that these changes in serum ghrelin levels are not regulated by the GH receptor. Finally, we found that the administration of the synthetic GH secretagogue GHRP-6 was followed by a decrease of peak ghrelin levels, but this effect could only be observed after several hours, suggesting that ghrelin concentrations are – at least partially – regulated by a long-loop negative auto feedback control. In chapter 3.2 the effects of fasting and pegvisomant on basal and GHRH and GHRP-6 stimulated GH secretion are described. A period of three days of fasting as well as blockade of the GH receptor by pegvisomant results in a significant decrease in serum free IGF-I - but not in total IGF-I concentrations. This decrease in free IGF-I is accompanied by an increase in GH concentrations. Indicating that circulating free IGF-I, and not total IGF-I, exerts negative feedback on GH secretion. With respect to the GHRH and GHRP-6 stimulated GH release we made three observations. First, pegvisomant administration to non-fasting subjects mimics the effects of fasting on GHRH, but not GHRP-6 – mediated GH release. Second, fasting – in contrast to pegvisomant – is able to increase GHRP-6 mediated GH secretion. Finally, the combination of three days of fasting and pegvisomant has a synergistic effect on the GHRP-6, but not on the GHRH mediated GH secretion. Based on the animal model of the transgenic growth retarded rat these data suggest an important role for increased pituitary GHRH receptor expression in the mechanism whereby fasting leads to an increased GHRH activity. Lastly, these data also imply that in-vivo, GHRP-6 sensitivity seems to be primarily regulated by metabolic factors and not by changes in GH-IGF-I axis.

From the studies on both peptidyl and non-peptidyl GHRP's and ghrelin it is known that the maximum in-vivo GH releasing capacity of GHRP's and

ghrelin is dependent on the concerted – albeit ill understood – actions of GHRP with GHRH. Therefore, the observed increase in ghrelin output in combination with the postulated pituitary GHRH receptor upregulation could very well explain a significant part of the increase in GH secretion. The last section of part I, chapter 3.3, describes GH independent effects of GHRP-6 on insulin, glucose and free fatty acid levels. Under the condition of GHR blockade and only under fed conditions GHRP-6 has profound stimulatory effects on serum insulin and glucose levels reflecting a state of insulin resistance at the liver and muscle level. In the same condition there is a decrease in FFA indicating either an increase in lipogenesis or a decrease in lipolysis. Taken together these data indicate tissue-specific differential changes in insulin sensitivity. As these GHRP-6 induced changes in insulin, glucose and FFA levels were only found in the presence of pegvisomant one must conclude that a certain degree of GH action in peripheral target tissues, such as beta-cells, adipose tissue, liver and muscle is necessary to control these GHRP-6 mediated changes in insulin, glucose and FFA levels. Moreover, the observed hyperinsulinemia in combination with increased insulin sensitivity of the adipocytes can potentially lead to an increase in fat mass.

These data are in line with the data from Tschöp et al. who have shown that once daily sc ghrelin administration to wild-type mice for 2 weeks leads to an increase in fat mass. Therefore, it might be that ghrelin acts by signaling the degree of fasting to the brain and adipocytes. In this way the body can achieve a metabolic control that is also dependent on the previous ingestion of meals and – most likely – also on the relative content of fat, protein and carbohydrate. It is envisioned that in periods of low GH bioactivity such a metabolic control system involves the release of ghrelin from the gastro-intestinal tract that induces an instant insulin resistance for carbohydrates (leading to increased insulin concentrations), while leaving sensitivity of the adipose tissues for insulin intact. It also stimulates food intake to increase the presence of sufficient nutrients. Such states of low GH action/concentration include obesity, ageing, the so-called syndrome-X (characterized by insulin resistance, obesity and hypertension) and protracted critical illness with its marked glucose intolerance and protein wasting, and accumulation of fat stores.

In part II of the thesis we focus more on some pathophysiological insights considering GH deficiency and acromegaly that arose while using pegvisomant.

In chapter 4.1 we describe the use of GHR blockade as a model of GH deficiency but without the typical alterations in body composition to study the changes in several cardiovascular risk markers known to play a role in the excess cardiovascular mortality observed in GH deficiency. We observed that in different metabolic conditions the GH receptor antagonist pegvisomant induces no significant changes in the major risk markers for CVD. Based on these data we hypothesize that the secondary metabolic changes – e.g. abdominal obesity or inflammatory factors – that develop as a result of long standing GHD and not the lack of GH per se are of primary importance in the pathogenesis of atherosclerosis in patients with GHD. Considering the postulated role of abdominal obesity – as opposed to GHD per se – on cardiovascular risk markers it would be interesting to speculate on the effect of GHD per se on ghrelin levels.

In chapter 4.2 an acromegalic patient is described in whom progression of pituitary tumor size was encountered during pegvisomant treatment. Moreover, despite a high dose of pegvisomant serum IGF-I concentrations did not normalize. After the patient was treated with a combination of pegvisomant and octreotide the visual field defects completely resolved and adequate biochemical control was achieved. The important observation in the present case, is that lowering serum GH concentrations by octreotide co-administration results in a synergistic decrease in serum IGF-I concentrations not achieved with octreotide or pegvisomant administrated alone. This synergistic effect might be predicted, based on the mechanism of action of pegvisomant as a competitive GH receptor antagonist. Lowering GH levels would therefore make a given concentration of pegvisomant more effective.

Another item that this case teaches us that long term monitoring of tumor size in acromegalic patients treated with pegvisomant is mandatory, especially in patients that have not received radiotherapy.

In chapter 5 the general discussion and conclusions are presented. In this thesis we have investigated several aspects of GH regulation. We provide indirect evidence confirming that free IGF-I – and not total IGF-I – exerts negative feedback on GH secretion. Moreover, by using pegvisomant we infer an important role for ultra shortloop GH autofeedback in the regulation of GH secretion. Considering the increase in GH output during fasting we show that the release of the newly discovered gastric hormone ghrelin is increased during fasting. Indirect data also indicate a role for

increased pituitary GHRH-receptor expression in the somatotroph hyperactivity of fasting. In another study with pegvisomant we show that the GHS GHRP-6 has GH-independent metabolic actions that might have a physiological role in the storage of nutrients.

Pegvisomant induces no acute changes in some cardiovascular risk markers known to play a role in the excess cardiovascular mortality observed in GHD patients. Therefore, we hypothesize that not the lack of GH per se but secondary changes – eg. in body composition – as a result of long standing GHD are of primary importance in the pathogenesis of atherosclerosis in these patients.

In our last study we describe some salient aspects from an acromegalic patient treated with pegvisomant.

Chapter 6.2 - Samenvatting in het Nederlands

In deel I van dit proefschrift is het effect van groeihormoon receptor (GHR) blokkade en vasten – alleen of in combinatie – op enkele bekende determinanten van de groeihormoon (GH) afgifte bestudeerd.

In hoofdstuk 3.1 wordt aangetoond dat vasten aanleiding geeft tot een diurnaal ghrelin ritme dat niet door veranderingen in insuline, glucose of vrije vetzuren kan worden verklaard. Deze veranderingen in de ghrelin spiegels tijdens vasten worden gevolgd door soortgelijke veranderingen in de GH concentraties in het bloed. Dit impliceert dat ghrelin verantwoordelijk is voor de verhoogde GH afgifte bij vasten. Door de GHR antagonist pegvisomant te gebruiken is op indirecte wijze aangetoond dat deze veranderingen in de ghrelin spiegels niet gereguleerd worden via de GHR. Voorts bleek dat toediening van het synthetisch GH secretagoog (GHS) "GH-releasing-peptide-6" (GHRP-6) na een aantal uren gevolgd werd door een verlaagde ghrelin piek concentratie. Dit suggereert dat de ghrelin bloedspiegels – althans ten dele – door een langzaam terugkoppelings-mechanisme worden gereguleerd.

In hoofdstuk 3.2 worden de effecten van vasten en pegvisomant op de door "GH-releasing-hormone" (GHRH) en GHRP-6 gestimuleerde GH afgifte beschreven. Een periode van 3 dagen vasten of een periode van 3 dagen GHR blokkade leiden tot een vermindering in de concentratie van de vrije insuline-achtige groeifactor-I (IGF-I) maar niet tot een vermindering in de concentratie van totaal IGF-I. Deze daling in vrij IGF-I gaat gepaard met een gelijktijdige stijging in de GH concentratie. Dit geeft aan dat vrij IGF-I – en niet totaal IGF-I – negatieve terugkoppeling uitoefent op de GH afgifte. Met betrekking tot de GHRH en GHRP-6 gestimuleerde GH afgifte zijn er drie belangrijke bevindingen. Ten eerste, pegvisomant toediening aan gezonde, niet vastende proefpersonen imiteert het effect van vasten op de GHRH, maar niet op de GHRP-6 gestimuleerde GH afgifte. Ten tweede, in tegenstelling tot pegvisomant is vasten in staat om de GHRP-6 gestimuleerde GH afgifte verder te stimuleren. Ten derde, de combinatie van 3 dagen vasten en pegvisomant heeft een synergistisch effect op de GHRP-6, maar niet op de GHRH gestimuleerde GH afgifte.

Naar aanleiding van het diermodel van de transgene groei geretardeerde rat suggereren deze gegevens dat bij de mens een belangrijke rol voor een toegenomen expressie van de GHRH receptor is weggelegd in het

mechanisme waardoor vasten tot een toegenomen GH afgifte leidt.

Ook impliceren deze bevindingen dat de in-vivo gevoeligheid voor GHRP-6 vooral bepaald wordt door metabole factoren en niet door veranderingen in de GH-IGF-I as.

Uit eerdere studies met GHRP's en met ghrelin is bekend dat voor een maximaal in-vivo GH stimulerend effect van GHRP's de aanwezigheid van GHRH noodzakelijk is. De door ons gevonden toegenomen ghrelin afgifte tijdens vasten, in combinatie met de door ons gepostuleerde toegenomen GHRH receptor expressie tijdens vasten, kan zo de toegenomen GH afgifte tijdens vasten- ten dele - verklaren.

In het laatste hoofdstuk van deel I, hoofdstuk 3.3, worden een aantal GH onafhankelijke effecten van GHRP-6 op de concentraties van insuline, glucose en vrije vetzuren beschreven. Tijdens GHR blokkade en alleen bij niet vasten omstandigheden geeft GHRP-6 een stijging van de insuline en glucose in het bloed, hetgeen wijst op insuline resistentie op het niveau van de lever en spieren. Onder dezelfde condities is er een gelijktijdige afname van de concentratie van vrije vetzuren in het bloed wat wijst op een toename in lipogenese of een afname in lipolyse. Er ontstaan dus weefsel-specifieke veranderingen in insulinegevoeligheid. Omdat de zojuist beschreven veranderingen in concentraties van insuline, glucose en vrije vetzuren alleen gezien werden na toediening van pegvisomant, moeten we concluderen dat een bepaalde mate van GH activiteit in de perfere weefsels - zoals beta-cel, vetweefsel, lever en spier – noodzakelijk zijn om deze door GHRP-6 veroorzaakte veranderingen in insuline, glucose en vrije vetzuur-spiegels te controleren. Bovendien, kan de toegenomen insuline-gevoeligheid van de vetcellen in combinatie met de hyperinsulinaemie tot een toename van de vetmassa leiden.

Recent hebben Tschöp en medewerkers laten zien dat in knaagdieren ghrelin, gedurende 2 weken 1 maal daags subcutaan toegediend, inderdaad tot een toename van de vetmassa kan leiden. Mogelijk is ghrelin voor de hersenen en vetcellen een signaal dat de mate van vasten aangeeft. Op deze wijze kan het lichaam de metabole controle aanpassen aan het tijdstip – en mogelijk zelfs de samenstelling – van de laatste maaltijd. In een dergelijk systeem zal tijdens perioden met lage GH activiteit de afgifte van ghrelin uit de maag een insuline resistentie voor carbohydraten veroorzaken en tegelijkertijd de insulinegevoeligheid van vetweefsel intact laten. Bovendien stimuleert ghrelin de voedselinname. Verminderde GH activiteit/concentratie wordt bijvoorbeeld gezien bij overgewicht, veroudering, syndroom-X

(gekarakteriseerd door insuline resistentie, overgewicht en hypertensie) en langdurige "critical illness" gekenmerkt door glucose-intolerantie, eiwit-verbruik en toename van vetmassa.

In deel II van dit proefschrift besteden we aandacht aan pathofysiologisch inzichten die ontstonden bij gebruik van pegvisomant en GH deficientie en acromegalie betreffen.

In hoofdstuk 4.1 wordt het gebruik van GHR blokkade als model voor GH deficiëntie maar zonder de typische veranderingen in lichaamssamenstelling beschreven. In deze studie is het effect van GHR blokkade op een aantal cardiovasculaire risico kenmerken die een rol spelen bij de verhoogde cardiovasculaire mortaliteit van patienten met langdurige GH deficiëntie onderzocht. Onder verschillende metabole omstandigheden gaf GHR blokkade met pegvisomant geen veranderingen in cardiovasculaire risico kenmerken te zien. Op grond hiervan is het waarschijnlijk zo, dat niet het GH tekort zelf maar de secudaire veranderingen in lichaamssamenstelling - met name de abdominale obesitas - als gevolg van dit tekort een primaire rol spelen in de pathogenese van atherosclerose bij patiënten met GH deficiëntie.

In hoofdstuk 4.2 wordt een 42-jarige patiënt met acromegalie beschreven, waarbij groei van de hypofyse tumor met gezichtsveld uitval werd gezien tijdens gebruik van pegvisomant. Gebruik van hoge doseringen octreotide leidde niet tot een normalisering van de IGF-I spiegel. Echter de combinatie van pegvisomant en octreotide resulteerde in normalisering van de gezichtsvelden en adequate biochemische ziektecontrole. Dus gelijktijdig gebruik van pegvisomant en octreotide heeft een synergistisch effect ten aanzien van de IGF-I concentratie. Deze waarneming is op grond van het werkingsmechanisme van pegvisomant goed te begrijpen. Immers, pegvisomant is een competetieve GHR antagonist, een vermindering van GH - door octreotide - maakt dus een gegeven concentratie pegvisomant effectiever.

Een andere belangrijke les uit deze ziektegeschiedenis is dat bij gebruik van pegvisomant langdurig vervolgen van tumorvolume noodzakelijk is, met name bij patiënten die niet behandeld zijn geweest met radiotherapie.

In hoofdstuk 5 worden de algemene discussie en conclusie beschreven. In dit proefschrift zijn verschillende aspecten van de GH regulatie beschreven. We

leveren indirect bewijs dat vrij – en niet totaal – IGF-I verantwoordelijk is voor de negatieve terugkoppeling op de GH afgifte. Door het gebruik van pegvisomant kunnen we ook een belangrijke rol voor ultra korte negatieve terugkoppeling van GH op zijn eigen afgifte aannemelijk maken. Ten aanzien van de toegenomen GH afgifte bij vasten laten we zien dat de afgifte ghrelin – een recent geïsoleerd hormoon dat onder andere in de maag wordt geproduceerd – toeneemt bij vasten. Ook zijn er indirecte gegevens die erop wijzen dat een toegenomen expressie van de GHRH-receptoren een rol spelen in het mechanisme waardoor vasten tot een grotere GH afgifte leidt. Uit een andere studie met pegvisomant blijkt dat het GHS GHRP-6 GH-onafhankelijke metabole effecten heeft die een belangrijke rol kunnen spelen in de opslag van voedingsstoffen.

Pegvisomant induceert geen veranderingen in een aantal cardiovasculaire risico kenmerken waarvan bekend is dat ze een rol spelen in de verhoogde cardiovasculaire morbiditeit van patiënten met GHD. Waarschijnlijk is dus niet het tekort aan GH zelf, maar zijn secundaire veranderingen – in bijvoorbeeld lichaamssamenstelling – van primair belang in de pathogenese van atherosclerose in deze patiënten.

Als laatste bespreken we een aantal belangrijke aspecten van een patiënt met acromegalie die behandeld is met pegvisomant.

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