PITUITARY AND MAMMARY GROWTH HORMONE IN DOGS

Sofie Bhatti

Utrecht, 2006

Wat was dus het leven?
Het was warmte, het warmteproduct van vormaannemende ongedurigheid,
een koorts van de materie, waarmee het proces van onophoudelijke ontbinding en herstel
der onhoudbaar ingewikkeld, onhoudbaar kunstig opgebouwde eiwitmoleculen gepaard ging.
Thomas Mann, "De Toverberg"
(1875-1955)

Voor mijn ouders

Voor Sarne

PITUITARY AND MAMMARY GROWTH HORMONE IN DOGS

Hypofysair en mammair groeihormoon bij de hond

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Chapter 1

Aims and outline of the thesis

The pulsatile secretion of growth hormone (GH) by pituitary somatotrophs is regulated by two antagonistic hypothalamic peptides: GH-releasing hormone (GHRH) and somatostatin. In addition, GH release can be stimulated by synthetic GH secretagogues (GHSs), such as growth hormone-releasing peptide-6 (GHRP-6), by acting through receptors different from those for GHRH. In 1999, the endogenous ligand for this GHS-receptor was purified and characterized from rat and human stomach and was called 'ghrelin'. Ghrelin has also been identified in the fundus of the canine stomach. The general aim of **Part I** of this thesis was to document spontaneous, GHS-, and ghrelin-induced GH release in healthy dogs and dogs with a pituitary disorder. In addition, ghrelin secretion was studied in healthy dogs.

The pituitary gland is not the only site of GH production. Under the influence of endogenous progesterone or the administration of progestins, the canine mammary gland is also able to secrete considerable amounts of GH into the systemic circulation. This mammary-derived GH is identical to pituitary GH. **Part II** of this thesis concentrates on several aspects of this progestin-induced mammary-derived GH in dogs.

The first part of the general introduction is an overview of pituitary GH secretion and its regulation, and of the diverse endocrine and nonendocrine effects of synthetic GHSs and ghrelin **(Chapter 2, part I)**. The second part of the general introduction **(Chapter 2, part II)** concentrates on the effects of progesterone and synthetic progestins in the bitch.

Besides the physiological effects of several hormones on pituitary GH secretion, the secretion pattern of GH may also change as a result of pathological hypersecretion of hormones such as, for example, cortisol. In **Chapter 3** and **Chapter 4** the effects of pituitary-dependent hyperadrenocorticism on the plasma GH profile and the GH response to various GHSs (ghrelin, GHRP-6, and GHRH) are reported.

In humans, not only diseases such as hypercortisolism, but also ageing and obesity affect pituitary GH secretion and cause a reduced response to GH stimulating factors. In dogs, little is known about the effect of age on the plasma GH response to GH-releasing stimuli. **Chapter 5** reports on the effects of several GHSs (ghrelin, GHRP-6, and GHRH) on the release of GH in young and old healthy Beagle dogs. In a search for the specificity of these stimulations, the effects of GHRP-6 and ghrelin administration on plasma adrenocorticotrophic hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and prolactin (PRL) release were also studied.

Ghrelin is a potent stimulator of GH release. The option of using ghrelin in the diagnosis of congenital GH deficiency was studied by measuring the effect of ghrelin administration on the plasma GH concentration in German shepherd dogs with pituitary

dwarfism. The dwarfism in German shepherd dogs is a combined pituitary hormone deficiency. Therefore, also the plasma concentrations of ACTH, cortisol, TSH, LH, and PRL were determined before and after ghrelin administration **(Chapter 6)**.

Through activation of pathways distinct from those involved in the stimulation of GH secretion, ghrelin also functions as a potent orexigenic peptide. Ghrelin induces weight gain by increasing food intake and reducing fat utilization. In several mammalian species it also plays a role in meal initiation. **Chapter 7** reports on the physiological effects of food intake and fasting on the circulating concentrations of ghrelin, GH, glucose, insulin, and insulin-like growth factor-I (IGF-I) in healthy Beagle dogs.

In **Part II** of this thesis several aspects of progestin-induced mammary-derived GH in dogs are presented. Cystic endometrial hyperplasia (CEH) is frequently seen in bitches treated repeatedly with progestins for prevention of oestrus. The condition may also develop spontaneously in the luteal phase of the oestrous cycle of middle-aged or elderly bitches, i.e. bitches that have gone through several luteal phases. Because of the similarity of the progestin-induced epithelial changes in both the mammary gland and the uterus, it was hypothesized that mammary GH is involved in the pathogenesis of progestin-induced CEH. Therefore, the effect of chronic administration of a synthetic progestin on the development of CEH was investigated in bitches with surgically excised mammary glands and in healthy control bitches **(Chapter 8)**.

It is not clear whether the oestrus-preventing properties of progestins in the bitch are due to effects at the level of the hypothalamus, the pituitary gland, or the ovary. In **Chapter 9** the effects of chronic administration of a synthetic progestin on adenohypophyseal function are reported, including the effects on the GH-IGF-I axis.

The presence of progesterone receptors in mammary gland tissue of dogs allows for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH overproduction. The effects of treatment with the progesterone receptor blocker aglépristone in Beagle dogs with progestin-induced mammary-derived GH excess are reported in **Chapter 10**.

In Chapter 11 the results of the studies are summarized and discussed.

Chapter 2

General introduction

Part of this review has been published:

Ghrelin, an endogenous growth hormone secretagogue with diverse endocrine and nonendocrine effects

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General introduction - Part I

Pituitary growth hormone secretion and its regulation, and the diverse endocrine and nonendocrine effects of synthetic growth hormone secretagogues and ghrelin

1. Nomenclature of the canine pituitary gland

According to the Nomina Anatomica Veterinaria, the canine pituitary gland is composed of two main parts 1) the adenohypophysis and 2) the neurohypophysis (Hullinger, 1993). The pituitary gland is suspended from the midline of the hypothalamus by a cylindrical stalk. This stalk is an extension of the median eminence of the hypothalamus, and is called the pars proximalis neurohypophysis (also called the infundibulum). The third ventricle continues as an invagination into the infundibulum. The pars proximalis neurohypophysis is continuous with the distal enlargement, the pars distalis neuohypophysis, which is the major portion of the neurohypophysis (Figure 1).

The adenohypophysis can be divided into two functional units 1) the anterior lobe (pars infundibularis adenohypophysis and pars distalis adenohypophysis) and 2) the pars intermedia (pars intermedia adenohypophysis). In the dog, the largest portion of the anterior lobe (AL) lays ventrorostral to the pars distalis neurohypophysis, which is almost entirely surrounded by the AL. The canine AL also extends as a cuff or collar around the pars proximalis neurohypophysis and even envelops part of the median eminence (Figure 1).

The pars intermedia (PI) is in direct contact with the pars distalis neurohypophysis and is separated from the AL by the hypophyseal cleft or cavity, which is a remnant of the embryonic Rathke's pouch.

The AL is populated by at least five highly differentiated types of endocrine cells, which are classified according to the trophic hormones they produce: somatotrophic cells secreting growth hormone (GH), lactotrophic cells secreting prolactin (PRL), thyrotrophic cells secreting thyroid-stimulating hormone (TSH), gonadotrophic cells secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and corticotrophic cells synthesizing the precursor molecule pro-opiomelanocortin (POMC), which gives rise to adrenocorticotrophic hormone (ACTH) and related peptides. Somatotrophic cells account for

50 % or more of the endocrine AL cells, with the other cell types each representing about 5-15 % of the AL cell population (Rijnberk, 1996; Meij, 1997).

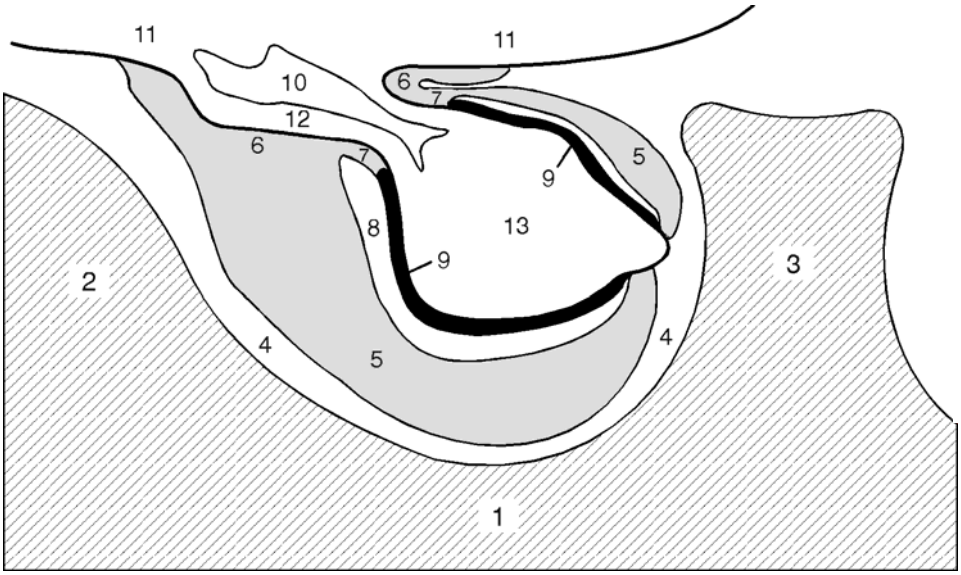

Figure 1. Schematic illustration of the median sagittal section through the canine pituitary gland (adapted from Meij, 1997). Left is rostral, right is caudal. 1 = sphenoid bone, 2 = tuberculum sellae, 3 = dorsum sellae, 4 = pituitary fossa, 5 = pars distalis adenohypophysis, 6 = pars infundibularis adenohypophysis, 7 = transitional zone, 8 = hypophyseal cleft or cavity, 9 = pars intermedia adenohypophysis, 10 = third ventricle, 11 = hypothalamus (median eminence), 12 = pars proximalis neurohypophysis, 13 = pars distalis neurohypophysis.

2. The hypothalamic-pituitary axis

The hypothalamic-pituitary axis constitutes the main axis of the neuroendocrine system of the body. In this axis, the pituitary is an essential regulatory interface integrating signals from the periphery and brain to control vital functions such as growth, reproduction, lactation, basal metabolism and the stress response (Treier and Rosenfeld, 1996). Hormonal control of pituitary gene expression and cellular proliferation is initiated during embryogenesis and continues through adulthood. At all stages, the cells of the pituitary have a remarkable ability to proliferate in response to demand for a specific hormone.

The hypothalamic-pituitary axis consists of three major systems: 1) a neuroendocrine system connected to an endocrine system by a portal circulation, 2) a neurosecretory pathway, and 3) a direct neural pathway that regulates endocrine secretion. The neuroendocrine system connects clusters of peptide- and monoamine-secreting cells in the anterior and middle

portion of the ventral hypothalamus to the AL (Swanson, 1987). Releasing and inhibiting factors such as GH-releasing hormone (GHRH), somatostatin (SS), thyrotrophin-releasing hormone (TRH), corticotrophin-releasing hormone (CRH), and gonadotrophin-releasing hormone (GnRH), are transported along nerve fibres from the hypothalamus to the median eminence. From the median eminence these factors are released into the capillary vessels of the hypothalamic-pituitary portal system and are transported to the pituitary to regulate the secretion of hormones from the AL (Figure 2). Specificity is achieved by the presence of specific receptors on individual types of AL cells. In addition to the hypothalamic hypophysiotrophic hormones, the secretion of AL hormones is regulated by feedback from target organs such as the thyroid, adrenals, and gonads (Figure 2).

The neurosecretory pathway is involved in osmoregulation through the production of vasopressin, and in parturition and nursing through the secretion of oxytocin. The two neurohypophyseal hormones are synthesised by populations of magnocellular neurons grouped in the paraventricular and supraoptic nuclei of the hypothalamus (Swanson, 1987), from which axons extend through the pituitary stalk and terminate in the neurohypophysis on fenestrated blood vessels. Vasopressin and oxytocin are stored in secretory granules within these nerve terminals and are released by exocytosis into the bloodstream in response to appropriate stimuli.

The pituitary PI is poorly vascularized and is directly innervated by predominantly dopaminergic nerve fibres from the hypothalamus. This direct neural control is mainly inhibitory in nature. Despite high levels of bioactive ACTH in the canine PI (Halmi et al., 1981), the main hormone secreted by the PI is α-melanocyte-stimulating hormone (α- MSH) (Figure 2).

3. Regulation of growth hormone secretion

3.1 Secretion and effects of growth hormone

In mammals, secretion of GH from the adenohypophysis is regulated by two hypothalamic hormones with antagonistic actions: a stimulatory GHRH that is produced in the arcuate nucleus, and an inhibitory hormone, SS, synthesized in the paraventricular nucleus (Figure 3) (Plotsky and Vale, 1985).

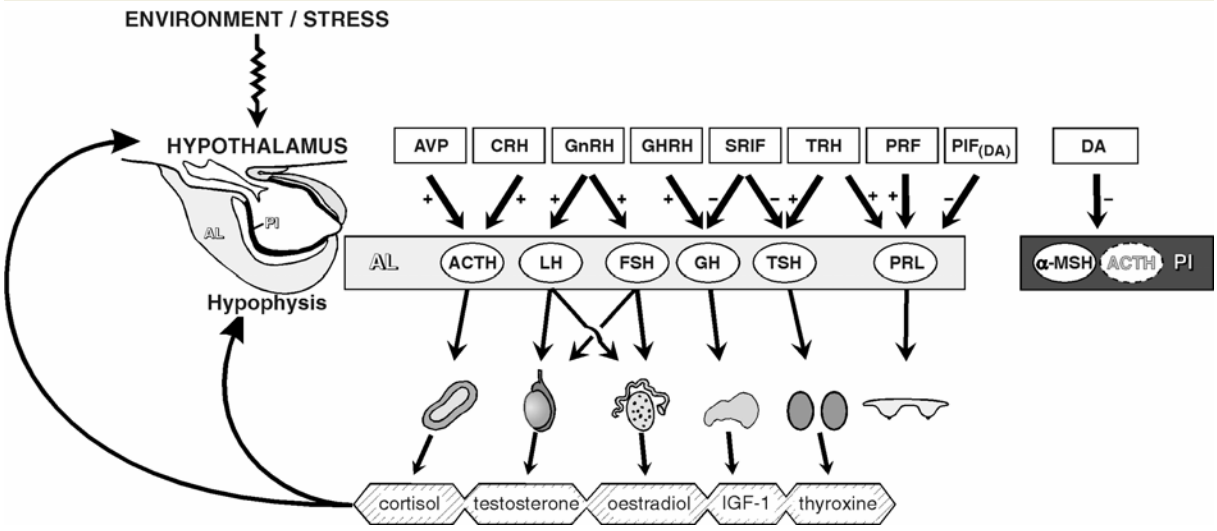

Figure 2. Simplified diagram of the hypophysiotrophic regulation of the secretion of hormones in the adenohypophysis, and with some target glands. Modified from Meij (1997).

Both hormones are transported from the hypothalamus to target cells in the pituitary gland via the hypothalamo-hypophyseal portal system in the median eminence. Alternation in secretion of GHRH and SS is responsible for the pulsatile pattern of GH release (Tannenbaum and Ling, 1984; Kooistra et al., 2000a). Measurement of GHRH and SS in hypophyseal-portal blood in humans and animals reveals that the episodic pattern of secretion of GHRH and SS does not fully account for all pulses of GH secretion (Frohman et al., 1992). The amplitude and frequency of GH secretory pulses are regulated by a complex array of external and internal stimuli, including body compostion, age, sleep, gender, disease status, menstrual cycle phase, genetic background, and nutritional status (Vigneri et al., 1976; Ho et al., 1988; Van Cauter et al., 1998). Furthermore, the secretion of GH is influenced by several hormones, such as progesterone, thyroid hormones, and glucocorticoids.

Growth hormone, a 191 amino acid single-chain polypeptide, mediates growth and metabolic functions through binding with the GH receptor. Growth hormone forms complexes with two peripheral GH-receptor components, leading to dimerization of the receptor, an event that is necessary for subsequent GH signalling (Hoech and Mukku, 1994). Growth hormone receptor dimerization elicits an intracellular phosphorylation cascade involving the JAK (Janus kinase)/STAT (signal transducers and activators of transcription) pathway (Xu et al., 1996).

The liver contains abundant GH receptors. Several other peripheral tissues, including muscle and fat, also express modest amounts of GH receptors (Barnard and Waters, 1997). In

contrast to most other pituitary hormones, the action of GH is not confined to a single target tissue and the hormone has both slow anabolic and rapid catabolic activities (Eigenmann et al., 1984). The catabolic effects are exerted via direct interaction with target cells, resulting in enhanced lipolysis in fat cells, and restriction of glucose transport across the cell membrane, caused by anti-insulin activity (Eigenmann et al., 1984; Casanueva, 1992; Carrel and Allen, 2002). The anabolic effects (i.e., growth and cell proliferation) of GH are exerted indirectly, mainly mediated by growth factors known as insulin-like growth factors (IGFs) or somatomedins (Figure 3) (Daughaday et al., 1972). The liver is the primary source of circulating IGFs. Growth hormone also promotes the production of IGFs in peripheral tissues (e.g., muscle, bone, cartilage, kidney, and skin), where they appear to have autocrine and paracrine effects (Daughaday et al., 1972).

Insulin-like growth factors have approximately 50 % sequence similarity with insulin (Tamura et al., 1989). In contrast to free circulating insulin, IGFs are bound to plasma proteins, which prolongs their half-life and contributes to their long-term growth-promoting effects. Circulating IGFs are important determinants of body size, because they stimulate protein synthesis, chondrogenesis, and body growth. Insulin-like growth factor-I has an inhibitory effect on GH secretion, most likely by stimulating the release of SS and by a directly inhibitory influence at the level of the pituitary gland (Figure 3) (Ceda et al., 1987). Additionally, GH has a negative feedback effect on its own production at the level of the hypothalamus (Figure 3) (Pelligrini et al., 1996).

3.2 Effects of gender and age on growth hormone secretion

Gender- and age-related differences in GH secretion have been established in various mammalian species. It appears that the sexually dimorphic pattern of GH secretion differs considerably between species. Before puberty, there is no apparent difference in GH secretion patterns between males and females in both humans (Zadik et al., 1985) and rats (Eden, 1979; Gabriel et al., 1992). In sheep, however, the plasma profiles of GH are already sexually dimorphic before puberty (Gatford et al., 1996). In humans (Zadik et al., 1985) and rats (Eden, 1979; Gabriel et al., 1992), GH secretion increases gradually till puberty. After puberty, GH release differs between males and females in most mammalian species. Mean concentrations of GH and IGF-I in blood are higher in adult males than in adult females in rats (Eden, 1979; Gabriel et al., 1992), mice (MacLeod et al., 1991), cattle (Plouzek and Trenkle, 1994), and horses (Thompson et al., 1994). In contrast, in primates mean circulating concentrations of IGF-I are higher in females than in males (Zadik et al., 1985; Ho et al.,

1987). Also the characteristics of GH pulses differ after puberty between males and females, albeit with some variation between species (Plouzek and Trenkle, 1994; Thompson et al., 1994).

In adulthood, the plasma GH concentration declines with increasing age in both primates (Finkelstein et al., 1972; Zadik et al., 1985; Ho et al., 1987; Corpas et al., 1993; Arvat et al., 1997a) and rodents (Sonntag et al., 1980; Crew et al., 1987). In humans it has been estimated that plasma GH decreases by 14 % with each decade (Iranmanesh et al., 1991). In both humans (Ho et al., 1987; Veldhuis et al., 1995) and rats (Sonntag et al., 1980) the amplitude of GH pulses is significantly lower in old individuals than in young individuals, whereas the frequency of GH pulses does not change with age. Several factors affect GH secretion in the elderly, such as age-related changes in body composition, reduced physical fitness, and medication use. Obesity is probably the strongest inhibitor of GH secretion (Veldhuis et al., 1995). Information on the effects of gender and age on the plasma profile in dogs is scarce. A recent study demonstrates that, in agreement with many other mammalian species, ageing in dogs is associated with a reduction in GH secretion. However, in contrast with findings in other mammalian species, no sex-related differences were detected in the pulsatile plasma profile in dogs (Lee, 2004).

3.3 Growth hormone secretion in endocrine disease

The pattern of GH secretion may also change in hyper- or hyposecretion syndromes, such as pituitary-dependent hyperadrenocorticism and pituitary dwarfism.

3.3.1 Pituitary-dependent hyperadrenocorticism

Cushing's disease or pituitary-dependent hyperadrenocorticism (PDH) is the most common endocrine disorder in the dog. Pituitary-dependent hyperadrenocorticism is most often caused by a corticotroph adenoma that may originate in the AL or the PI (Peterson et al., 1986). These corticotroph tumours produce an excessive amount of ACTH, resulting primarily in hypersecretion of glucocorticoids and in hyperplasia of the two inner zones of the adrenal cortices.

Glucocorticoids are important physiological regulators of GH synthesis and secretion. In humans and rats, glucocorticoids enhance GH gene transcription (Evans et al., 1982; Karin et al., 1990) and increase the number of pituitary GHRH receptors (Seifert et al., 1985; Ohyama et al., 1997). Consequently, both spontaneous and GHRH-induced GH secretion are stimulated by acute administration of dexamethasone (Casanueva et al., 1990). A minimum

level of cortisol is essential for normal GH production. Individuals with hypoadrenocorticism (Addison's disease) may become GH-deficient because of diminished GH synthesis (Allen, 1996). In humans with persistently elevated secretion of glucocorticoids, as in Cushing's disease, both the spontaneous and the stimulated GH secretion are blunted (Takahashi et al., 1992). Pituitary-dependent hyperadrenocorticism in dogs has many similarities to Cushing's disease in humans (Kemppainen and Peterson, 1993). Consequently, changes in spontaneous and stimulated pituitary GH secretion may also be expected in dogs with PDH.

3.3.2 Pituitary dwarfism

In dogs, congenital GH deficiency or pituitary dwarfism is the most striking example of adenohypophyseal hormone deficiency. Congenital GH deficiency has been mentioned to occur in different dog breeds, including Saarloos Wolfshounds and Carelian bear dogs. However, the condition is encountered most often as a simple, autosomal, recessive inherited abnormality in the German shepherd dog (Andresen and Willeberg, 1976).

Functionally, German shepherd dwarf dogs have a combined pituitary hormone deficiency. An absolute deficiency of GH, PRL, and TSH is associated with an impaired release of gonadotrophins, whereas ACTH secretion is preserved (Kooistra et al., 1998; Kooistra et al., 2000b). The abnormality in these dwarfs is most likely caused by a mutation in a developmental transcription factor that precludes effective expansion of a pituitary stem cell after the differentiation of the corticotroph cells (Kooistra et al., 2000b). To date, sequence analysis of genomic DNA from German shepherd dwarfs has not revealed causative mutations in candidate genes (Lantinga-van Leeuwen et al., 2000a; Lantinga-van Leeuwen et al., 2000b; Van Oost et al., 2002).

4. Ultradian pulsatile hormone secretion

4.1 Biological relevance of ultradian pulsatile hormone secretion

It is now generally accepted that all adenohypophyseal hormones are secreted in an ultradian pulsatile fashion and that this pattern of secretion represents an important component of neuroendocrine signalling (Negro-Vilar et al., 1987; Brabant et al., 1992). The ultradian patterns of adenohypophyseal hormone release are primarily driven by hypothalamic signals acting on responsive pituitary cells, which are modulated in turn by intrapituitary paracrine and autocrine factors, and also by systemic feedback of hormones derived of target organs.

Pulsatile hormone secretion allows for encoding information not only in the absolute concentration of the ligand but also in a time-dependent fashion. The pulsatile secretion of adenohypophyseal hormones is essential for target-cell regulation and is reproducible under comparable environmental conditions (Brabant et al., 1990). Physiological and pathophysiological situations may modulate pulsatile secretion patterns by altering the pulse amplitude, pulse frequency, or both (Brabant et al., 1992).

4.2 Analysis of pulsatile growth hormone secretion

The pulsatile pattern of GH secretion can be determined by collecting blood samples serially over a period of hours or days. The samples are assayed and the results are plotted graphically to enable recognition of the pulsatile secretion pattern. Different methods can be used to analyse the data. Traditionally, the graphs have been given to a blinded scorer who identifies the peaks by visual inspection. With this technique it is difficult to state precisely the criteria used for pulse identification, which complicates communicating among different centres studying similar problems and points to the need for more objective methods (Merriam and Wachter, 1982). Nowadays, a number of computer programmes are available for computer-assisted analysis of pulsatile secretion patterns. Commonly used detection methods such as the Pulsar programme (Merriam and Wachter, 1982) employ mathematical assumptions to identify pulses in time series of hormone concentrations. The statistical parameters used have to be adjusted for each hormone and different sampling frequencies.

Several methodological aspects should be borne in mind when interpreting patterns of pulsatile hormone secretion. Analysis of the profile of pulsatile hormone secretion requires that detection methods are precise and sensitive. A high intra-assay coefficient of variation will make it difficult to distinguish significant hormone pulses from assay noise. Secondly, hormone-specific responses are determined by the bioactivity of the hormone. However, plasma GH concentrations are measured by an immunoassay rather than by a bioassay. Thirdly, pulsatile plasma profiles are measured as a relatively small number of points, because “on-line” measurement of GH is not yet available. The shorter the sampling interval the higher the chance that significant GH pulses are detected. Therefore, the Pulsar programme is not able to extract the “true” underlying pattern of information. Nevertheless, the method is an objective way to compare different data sets (Brabant et al., 1992).

5. Synthetic growth hormone secretagogues

In 1975, before the discovery of GHRH, the GH-releasing properties of enkephalins were reported (Bowers et al., 1977). Chemical modification of the structure of met-enkephalin led to development of a highly potent GH-releasing hexapeptide, GHRP-6 [(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)^3] in 1980 (Bowers et al., 1984). One of the most remarkable properties of GHRP-6 was the strong GH-releasing activity induced following oral administration (Bowers et al., 1984). The hexapeptide was the basic structure from which synthetic GH secretagogues (GHSs), of either peptidergic structure such as hexarelin or non-peptidergic structure such as MK-0677 (Ghigo et al., 1994; Chapman et al., 1996), were subsequently produced. Current synthetic GHSs are highly bioavailable and may be administered via intravenous, intramuscular, intranasal, subcutaneous, oral, and transdermal routes (Casanueva and Dieguez, 1999). Because GH is a large protein that must be administered via injection or inhalation, administration of synthetic GHSs is often preferred over administration of GH. In addition, GHSs induce a more physiologic pulsatile profile of GH release (Laron, 1995; Casanueva and Dieguez, 1999). For example, a single orally administered dose of MK-0677 increases mean 24-h plasma GH concentrations (Chapman et al., 1996; Jacks et al., 1996; Smith et al., 1997).

The synthetic GHSs have potent GH-releasing activity in several species, including humans, mice, rats, swine, goats, cows, and dogs (Hayashida et al., 2001; Bhatti et al., 2002; Bhatti et al., 2006 in press). In humans, nearly all synthetic GHSs induce the release of more GH than GHRH (Casanueva and Dieguez, 1999). However, the hormone-releasing action of synthetic GHSs is not specific in all instances (Casanueva and Dieguez, 1999). In humans, synthetic GHSs such as GHRP-6 also have a stimulatory effect on the secretion of PRL, ACTH, and cortisol (Massoud et al., 1996; Casanueva and Dieguez, 1999; Arvat et al., 2001). Newer selective GHSs, such as ipamorelin, do not have ACTH- or PRL-releasing actions (Raun et al., 1998; Broglio et al., 2002).

Interest in GHSs faded after the isolation and characterization of GHRH in 1982 (Guillemin et al., 1982; Rivier et al., 1982), but was later revived when it was discovered that GHSs operated through receptors that are different from those for GHRH (Howard et al., 1996; Guan et al., 1997; Casanueva and Dieguez, 1999). Growth hormone secretagogues and GHRH have strongly synergistic actions, which indicates that synthetic GHSs are not physiologic surrogates of GHRH (Bowers et al., 1990). In 1996, the GHS-receptor (GHS-R), a G-protein-coupled seven-transmembrane receptor, was identified (Pong et al., 1996). This receptor has been cloned from cells of the pituitary gland in humans (Howard et al., 1996; McKee et al., 1997a) and rats (McKee et al., 1997b).

Two types of GHS-Rs, which are presumably the result of alternate processing of pre-mRNA, have been identified and designated as receptors 1a and 1b (Howard et al., 1996; Smith et al., 1997). The human GHS-R 1a shares 96 and 93 % sequence identity with rat and pig receptors, respectively. The existence of this receptor can be traced to animals in the pre-Cambrian era because amino acid sequences highly similar to those in the human GHS-R 1a have been detected in teleost fish (Palyha et al., 2000). These observations indicate that the GHS-R 1a is highly conserved across species and likely has an essential biological function. This receptor is largely confined to somatotroph cells in the pituitary gland and to several hypothalamic nuclei (e.g., the supraoptic, arcuate, and paraventricular nuclei) in humans and rats (Howard et al., 1996; Guan et al., 1997; Smith et al., 1997; Shuto et al 2001). The presence in the pituitary gland of mRNA coding for GHS-R 1a indicates that GHSs can act directly on somatotrophs to stimulate GH release. This is in accordance with an earlier observation (Cheng et al., 1993) that GHSs are able to directly stimulate GH release from rat pituitary cells *in vitro*. The hypothalamic localization of the GHS-R 1a, especially in the supraoptic and paraventricular nuclei, supports the notion that GHSs may also indirectly regulate GH release by interacting with GHRH-producing neurons, SS-producing neurons, or both, in the hypothalamus (Dickson et al., 1995). The GHS-R 1a is also expressed in other areas of the brain and certain peripheral tissues (Papotti et al., 2000), indicating that GHSs may also be involved in other physiologic functions (Guan et al., 1997; Kojima et al., 2001). The importance of the widespread expression of GHS-R 1b in endocrine and non-endocrine tissues has not been determined (Howard et al., 1996; Gnanapavan et al., 2002).

The GHS-Rs are distinct from the GHRH receptor (Howard et al., 1996; Guan et al., 1997; McKee et al., 1997a). Although binding of GHRH to the GHRH receptor increases cAMP in somatotroph cells and stimulates GH release via activation of the kinase A pathway, the binding of ghrelin and synthetic GHSs to the GHS-R 1a activates the phospholipase C signalling pathway, leading to an increase in inositol triphosphate and protein kinase C activation, followed in turn by release of calcium from intracellular stores (Pong et al., 1996). Unlike GHS-R 1a, GHS-R 1b does not bind ghrelin or synthetic GHSs, and its function awaits clarification (Howard et al., 1996; McKee et al., 1997a; Gnanapavan et al., 2002).

6. Ghrelin

The 1999 discovery of the endogenous or natural ligand of the GHS-R, termed ghrelin (*ghre* is the proto-Indo-European root of the word grow, and *relin* indicates release), provided

a new dimension to GH research (Figure 3) (Kojima et al., 1999). The usual sequence of discovery in endocrinology is isolation of a hormone, cloning of its receptor, and development of analogues of the hormone for clinical use. With ghrelin, this sequence was reversed: first, analogues were synthesized, then the receptor was cloned, and lastly, the natural ligand of the orphan receptor was isolated.

Ghrelin releases GH *in vitro* and *in vivo*. The 28-amino acid peptide was isolated from the stomach, where its expression is higher than in any other tissue (Kojima et al., 1999). Although this source may initially seem strange, it should be remembered that most circulating SS is synthesized primarily in the gut and pancreas and that GHRH was first isolated not from the hypothalamus but from a pancreatic tumour (Casanueva and Dieguez, 2002). Thus, the 3 neurohormones (i.e., SS, GHRH, and ghrelin) responsible for regulation of GH secretion are highly expressed in gastrointestinal tissues.

In humans, rats, and domestic animals, expression of ghrelin mRNA and the ghrelin peptide have been primarily detected in the entero-endocrine or *X/A-like* cells of the fundic gland in the stomach (Hayashida et al., 2001; Tomasetto et al., 2001) and have now been renamed Ghr-cells. The cells containing ghrelin do not communicate with the lumen of the fundic gland. Like all entero-endocrine cells, they are positioned adjacent to capillaries, indicating that their primary action is secretion of hormone into plasma and not into the intestinal lumen (Date et al., 2000).

The degree of structural heterogeneity of ghrelin among species appears to be minor, suggesting that there is little functional heterogeneity. Such preservation of structure throughout evolution reflects the physiologic relevance of the peptide (Tomasetto et al., 2001; Casanueva and Dieguez, 2002). For example, human and rat ghrelin differ in only two amino acids (Table 1) (van der Lely et al., 2004). Alternative splicing of mRNA segments encoding ghrelin yields two different peptides, ghrelin and des-Gln14-ghrelin (Hosoda et al., 2000). The latter is homologous with ghrelin except for the absence of a single glutamine residue. Des-Gln14-ghrelin is expressed in the stomach in low quantities (Kojima et al., 2001), but, like ghrelin, it increases the intracellular concentration of calcium in cells that express the GHS-R 1a and increases plasma GH concentrations (Kojima et al., 1999; Hosoda et al., 2000).

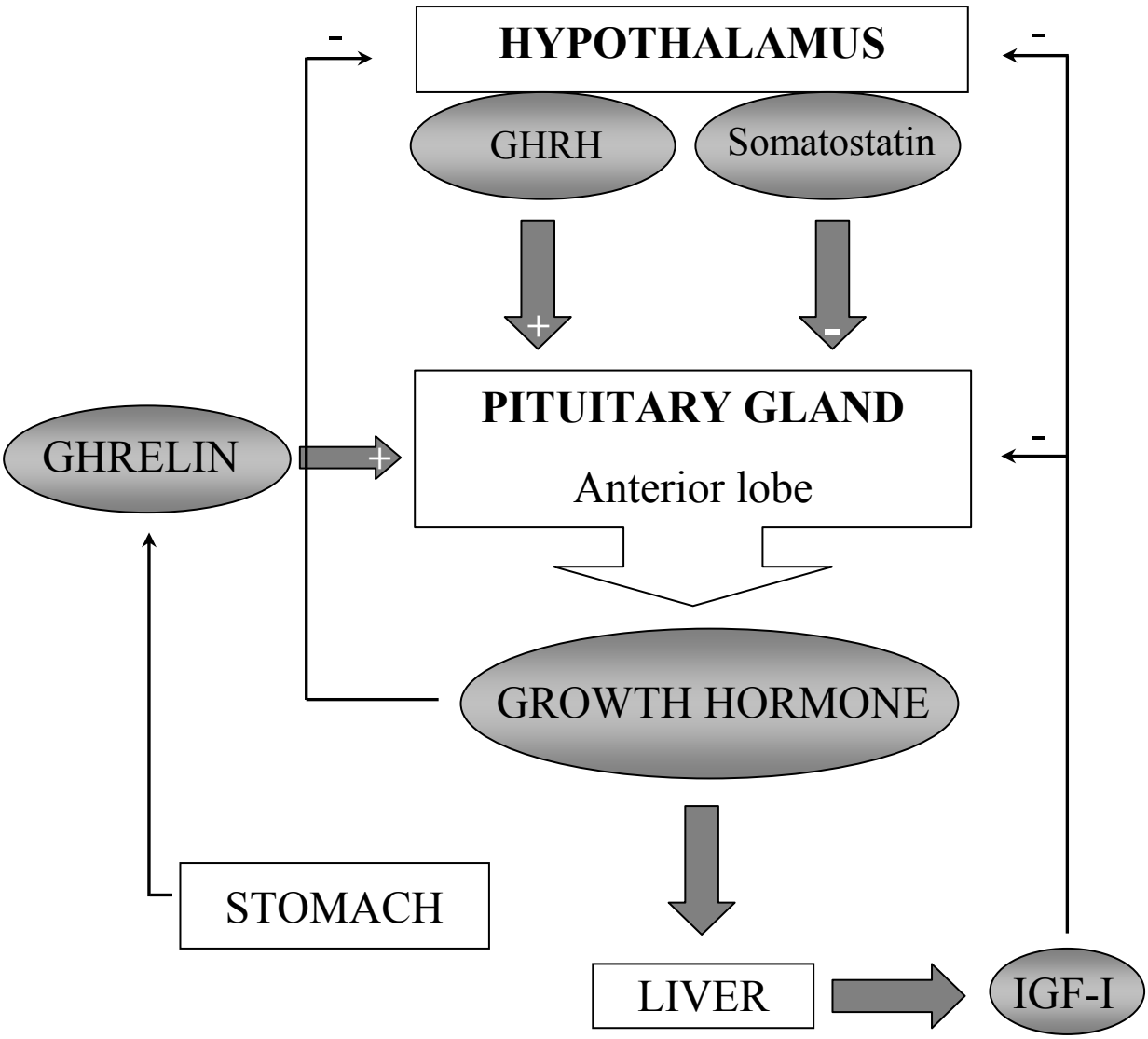

Figure 3. Regulation of the hypothalamic-pituitary-growth hormone (GH) axis. Growth hormone secretion by the pituitary gland is stimulated by growth hormone releasing hormone (GHRH) and is inhibited by somatostatin (SS). Negative feedback control of GH secretion is exerted at the pituitary and hypothalamic level by insulin-like growth factor-I (IGF-I). Growth hormone itself exerts a short-loop negative feedback by activation of SS neurons. The gastric peptide ghrelin is the natural ligand for the GH secretagogue receptor that stimulates GH secretion at the pituitary level.

Table 1. Primary structure of ghrelin from domestic mammalian species. Adapted from van der Lely et al. (2004) with permission *(Copyright 2004, The Endocrine Society).* Bold indicates sites at which a residue is different from that in the human peptide.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	Species
G	S	S	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Human
G	S	S	F	L	S	P	E	H	Q	**K**	**T**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Gerbil
G	S	S	F	L	S	P	E	H	Q	**K**	**A**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Mouse
G	S	S	F	L	S	P	E	H	Q	**K**	**A**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Rat
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	Dog
G	S	S	F	L	S	P	E	H	Q	**K**	V	Q	Q	R	K	E	S	K	K	P	A	A	K	L	**K**	P	R	Pig
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	**A**	K	K	P	**S**	**G**	**R**	L	**K**	P	R	Cattle
G	S	S	F	L	S	P	E	H	Q	**K**	**L**	Q	Q	R	K	E	**P**	K	K	P	S	**G**	**R**	L	**K**	P	R	Sheep
G	S	S	F	L	S	P	**T**	**Y**	**K**	**N**	**I**	Q	Q	**Q**	K	**D**	**T**	**R**	K	P	**T**	A	**R**	L	**H**	**R**	R	Chicken

Before being secreted, *n*-octanoic acid is added to the third serine residue of ghrelin and des-Gln14 ghrelin (Figure 4) (van der Lely et al., 2004). This acylation step, unique in mammalian species, is essential for binding to and activating the GHS-R 1a (Bednarek et al., 2000) and hence for the peptide's GH-releasing action. The acylation is most likely also necessary for the other endocrine actions of the ghrelin molecule (Kojima et al., 2001; Broglio et al., 2003). Addition of the *n*-octanoyl group confers a hydrophobic property to the N terminus of the peptide. It has been suggested that the octanoylation of ghrelin is critical to the peptide's ability to cross the blood-brain barrier. It may also facilitate distribution of the peptide in the brain although there are presently no data to support this speculation (Horvath et al., 2001). Non-acylated ghrelin is found in far greater quantities in human serum than acylated ghrelin, but it seems to be devoid of any endocrine activity. However, this peptide does have certain nonendocrine actions, such as cardiovascular and anti-proliferative effects and these are probably mediated through binding to a novel, as yet unidentified, GHS-R subtype (Cassoni et al., 2004). Non-acylated ghrelin is able to inhibit proliferation of human prostate cancer cell lines and neoplastic cell growth in thyroid, breast and lung tumours. Also, cardioprotective and negative inotropic effects have been described (Date et al., 2000; Cassoni et al., 2004).

Lower amounts of ghrelin have been detected in various other tissues, including the intestines (Date et al., 2000), the pituitary gland (Korbonits et al., 2001), the hypothalamus (Kojima et al., 1999; Date et al., 2000), the kidney (Mori et al., 2000), the placenta (Gualillo et al., 2001), the heart (Casanueva and Dieguez, 2002), the testes (Barreiro et al., 2002), the thyroid gland (Kanamoto et al., 2001), the pancreas (Volante et al., 2002a), the lung (Volante, 2002b), the ovary (Gyatan et al., 2003), the immune system (Hattori et al., 2001), and

neoplastic tissue (Papotti et al., 2000). The physiologic importance of ghrelin as a paracrine factor in these tissues is the subject of current research. An endocrine role for non-stomach-derived ghrelin is thought to be unlikely. Removal of the stomach in humans and rats decreases the plasma concentration of ghrelin by approximately 65 % and 80 %, respectively (Date et al., 2000; Ariyasu et al., 2001). However, plasma ghrelin concentrations gradually increase after gastrectomy (Hosoda et al., 2003). Taken together, these findings indicate that the stomach is the major source of circulating ghrelin but other tissues may contribute to the secretion of ghrelin in a compensatory manner (Moller et al., 2003).

6.1 Endocrine effects of ghrelin

Ghrelin has pronounced, dose-related GH-releasing actions that are more marked in humans than in animals (Smith et al., 1997; Kojima et al., 1999; Arvat et al., 2000; Date et al., 2000; Seoane et al., 2000; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001; Hayashida et al., 2001; Bhatti et al., 2002; Bhatti et al., 2006 in press). The GH-releasing activity of ghrelin is greater *in vivo* than *in vitro*, because ghrelin and GHRH act synergistically, consistent with the fact that their actions are at least partially mediated via different mechanisms (Smith et al., 1997; Tannenbaum and Bowers, 2001). Nevertheless, GHRH activity is required for full expression of ghrelin's GH-releasing activity (Smith et al., 1997; Tannenbaum and Bowers, 2001). The GH response to ghrelin is inhibited, although not completely, by GHRH receptor antagonists and by hypothalamo-pituitary disconnection (Hickey et al., 1996; Popovic et al., 2003). This supports the assumption that the effect of ghrelin on GH secretion is primarily mediated by GHRH-secreting neurons at the level of the hypothalamus (Bowers et al., 1991; Smith et al., 1997; Tannenbaum and Bowers, 2001; Popovic et al., 2003).

In anaesthetized rats, intravenously administered ghrelin stimulates GH release without affecting the secretion of other adenohypophyseal hormones (Kojima et al., 1999). Also, in cultured rat pituitary cells, ghrelin stimulates GH release in a dose-dependent manner without affecting the release of other pituitary hormones, even at high concentrations (Kojima et al., 1999). However, in healthy humans, ghrelin is not fully specific for GH release, because it also has stimulatory effects on lactotroph and corticotroph cells (Arvat et al., 1997b; Peino et al., 2000; Takaya et al., 2000; Arvat et al., 2001). The effect of ghrelin on PRL secretion is independent of gender and age, and likely results from direct stimulation of somatomammotrophs (Renner et al., 1994; Arvat et al., 1997b; Takaya et al., 2000; Muccioli et al., 2002).

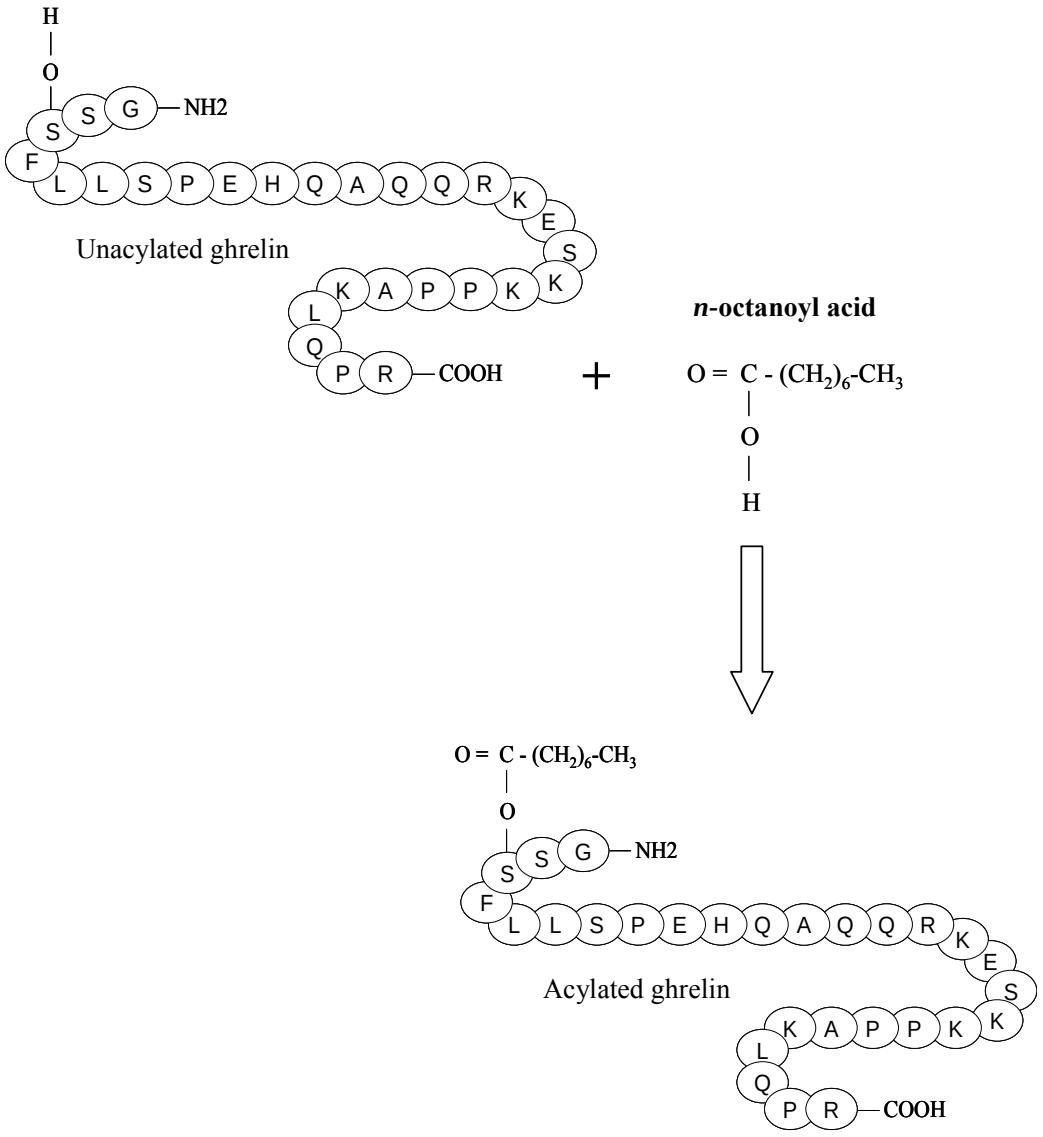

Figure 4. Acylation of the ghrelin molecule. A hydroxyl group on the serine residue at position 3 of the ghrelin molecule is octanoylated. This esterification is unique to mammals and is essential for ghrelin binding to and activating the growth hormone secretagogue receptor type 1a and, consequently, for the growth hormone-releasing action of ghrelin. The other endocrine actions of ghrelin are also likely dependent on the acylation of the peptide. Figure adapted from van der Lely et al. (2004) with permission *(Copyright 2004, The Endocrine Society).*

In rats (Kojima et al., 1999), synthetic GHSs do not stimulate PRL release. This species-related difference may be explained by differences in the number of somatomammotrophs in various species, with humans having a high proportion of those cells (Frawley and Boockfor, 1991; Raun et al., 1998). The mechanism by which ghrelin stimulates the pituitary-adrenocortical axis is still unknown, but may be mediated via the hypothalamus, because the stimulatory effect is lost after sectioning of the pituitary stalk (Loche et al., 1995). Ghrelin may also interact with hypothalamic peptides that control ACTH release, probably via arginine vasopressin (Thomas et al., 1997; Korbonits et al., 1999; Broglio et al., 2003).

6.2 Orexigenic actions and role in energy homeostasis

Evidence for the involvement of ghrelin in the regulation of appetite was first described in humans. Healthy human volunteers reported hunger after administration of ghrelin in a clinical study in which GH release was analyzed (Arvat et al., 2000). In rodents, ghrelin stimulates food intake and increases body weight while reducing mobilization of adipose stores (Tschop et al., 2000; Wren et al., 2000; Shintani et al., 2001). The effects of ghrelin on food intake are likely mediated through mechanisms other than those implicated in GH regulation, compatible with the concept of distinct GHS-R subtypes (Tschop et al., 2000; Toogood and Thorner, 2001).

Traditionally, adipocytes have been viewed as energy depots that store triglycerides during feeding and release fatty acids during fasting to provide fuel for other tissues. However, it has become clear that adipose tissue has major integrative physiologic functions, including the secretion of numerous proteins (Friedman an Halaas, 1998; Miner, 2004). The realization that adipose tissue functions as an endocrine organ has important implications for our understanding of the associations between excess body fat and pathologic states like insulin resistance and type-2 diabetes mellitus (Miner, 2004).

An important finding that linked central regulation of metabolism to peripheral energy stores was the discovery of the adipose hormone, leptin (from the Greek root *leptos*, meaning thin). Leptin, a peptide hormone discovered in 1994, is produced principally by white adipose tissue (Zhang et al., 1994). Leptin crosses the blood-brain barrier to act via receptors in the arcuate nucleus of the hypothalamus to inhibit the release of orexigenic neuropeptides and stimulate the release of anorexigenic neuropeptides (Friedman and Halaas, 1998; Neary et al., 2003). There is a direct relationship between plasma leptin concentrations and percentage body fat. Plasma leptin levels in humans are generally proportional to adipose mass. A reduction in leptin levels occurs because of loss of adipose mass such as anorexia nervosa,

diet- or exercise-induced weight loss, or starvation. Concentrations of circulating leptin decrease rapidly within 12h after initiation of starvation, whereas concentrations increase in response to overfeeding. Thus, plasma leptin concentrations reflect adipose tissue mass and provide a signal that informs the central nervous system about the body's energy reserves (Friedman and Halaas, 1998).

The similarities and complementary interplay of actions between leptin and ghrelin are intriguing. The effects of ghrelin on metabolism appear to be the opposite to those of leptin (Tschop et al., 2000; Bowers, 2001; Kojima et al., 2001; Spiegelman and Flier; 2001). Leptin reduces food intake and selectively reduces fat mass without altering lean body mass (Farooqi et al., 1999). Ghrelin, in contrast, increases food intake and selectively enhances fat mass (Tschop et al., 2000).

Ghrelin stimulates food intake in rodents when administered via central (intracerebroventricular) or peripheral (subcutaneous) routes, although the effect is more powerful after central administration (Tschop et al., 2000). There is evidence that the appetite-stimulating effects of ghrelin are mediated by secretion of 2 potent orexigenic hypothalamic hormones (neuropeptide Y and agouti-related peptide) and by inhibition of pro-opiomelanocortin and α-melanocyte-stimulating hormone (α-MSH) (Dickson et al., 1997; Hewson and Dickson, 2000; Kamegai et al., 2001; Cowley et al., 2003). Furthermore, the orexigenic action of ghrelin is eliminated when neuropeptide Y and agouti-related peptide are antagonized (Kamegai et al., 2001). By stimulating the release of orexigenic peptides and neurotransmittors, ghrelin mediates a novel regulatory circuit regulating energy homeostasis (Cowley et al., 2003; Kohno et al., 2003; Olszewski et al., 2003; Riediger et al., 2003; Seoane et al., 2003).

In humans and rats, concentrations of circulating ghrelin decrease in chronic (obesity) (Tschop et al., 2001) and acute (caloric intake) (Cummings et al., 2001) states of positive energy balance, whereas ghrelin levels increase in states of negative energy balance (e.g., fasting) (Tschop et al., 2000). In cattle, plasma ghrelin concentrations are low 1h after feeding and then return to the pre-feeding concentration (Hayashida et al., 2001). In sheep, the preprandial ghrelin surge is higher in animals fed twice daily than in animals fed four times daily, highlighting the influence of different feeding regimens on ghrelin concentrations (Sugino et al., 2002). The preprandial rise and postprandial fall in plasma ghrelin concentrations suggest a possible role for ghrelin as a hunger signal, triggering meal initiation (Cummings et al., 2001). Because ghrelin is a potent stimulator of GH release, these observations are in accordance with the low plasma GH concentrations associated with

obesity (Bowers, 1993) and the high concentrations observed in the malnutrition and fasting states (Dieguez and Casanueva, 1995).

It may be concluded that nutritional state is an important determinant of plasma ghrelin concentration (Tolle et al., 2002). Ghrelin peptide reaches ghrelin receptors in the hypothalamo-pituitary region via the general circulation, where it stimulates GH release and regulates energy homeostasis. It is unclear whether ghrelin must cross the blood-brain barrier to influence the activity of these central structures (van der Lely et al., 2004). In the general circulation, ghrelin is bound to high-density lipoproteins in the serum and presumably to other proteins, such as albumin, as well. Ghrelin may also signal the brain directly, by activating the afferent portion of the vagal nervous system as either an endocrine or a paracrine signal, at the level of the stomach. Ghrelin-responsive GHS-Rs are expressed on gastric vagal nerves, and vagotomy prevents some of the effects of ghrelin on energy balance. On the other hand, the extent and direction of ghrelin transport across the blood-brain barrier may be determined by its unique primary structure (Banks et al., 2002). There is still debate on the routes by which ghrelin in the peripheral circulation activates receptors in the central nervous system of different species.

6.3 Gastric prokinetic action

Ghrelin induces strong prokinetic activity in the stomach (Masuda et al., 2000; Asakawa et al., 2001). The peptide dramatically accelerates gastric and intestinal emptying in rats, and circulating ghrelin concentrations are correlated with gastric emptying time in humans (Masuda et al., 2000). In addition, ghrelin stimulates gastric acid secretion (Asakawa et al., 2001).

In this context, it is interesting to consider the structural and functional similarities between ghrelin and motilin (Folwaczny et al., 2001). In addition to their prokinetic effect on the gastrointestinal tract, both peptides have orexigenic properties (Garthwaite, 1985) and stimulatory effects on pituitary GH release (Samson et al., 1984). Also, the G-protein-coupled receptors of ghrelin and motilin have a high degree of structural homology (Feighner et al., 1999). In contrast to ghrelin, motilin is primarily expressed in the small intestine (Brown et al., 1971). Motilin stimulates motor activity in the gastric antrum and proximal portion of the duodenum, and plays a key role in the regulation of interdigestive motility (Itoh, 1997).

The gastrokinetic effects of ghrelin and motilin may prove beneficial in the treatment of postoperative gastric ileus. In humans and other mammalian species, abdominal surgery and attendant manipulation of the viscera inhibit gastric emptying and digestive motor

activity, which may result in postoperative ileus. Attempts to stimulate smooth muscle activity with various prokinetics (eg, cisapride and acetylcholine) are often unsuccessful (Asakawa et al., 2001). In rats, ghrelin reverses postoperative gastric ileus (Masuda et al., 2000). Further studies may elucidate the pharmacologic potential of ghrelin and motilin in gastroenterologic applications.

6.4 Effects on the endocrine pancreas

Ghrelin and GHS-R 1a mRNA are expressed in endocrine cells of the pancreas (Guan et al., 1997; Date et al., 2002; Gnanapavan et al., 2002; Rindi et al., 2002). Expression of ghrelin has been reported in the pancreatic α-cells (Date et al., 2002), although other investigators have reported that ghrelin is expressed in the pancreatic β-cells (Volante et al., 2002a). Ghrelin is not co-expressed with any known islet-derived hormone; thus, ghrelin-producing cells may be a newly recognized type of islet cell (Wierup et al., 2002).

Published information regarding the effect of ghrelin on insulin secretion in humans and rats is conflicting (Caixas et al., 2002; Date et al., 2002; Lee et al., 2002). However, most findings suggest a negative association between ghrelin concentrations and insulin secretion (Broglio et al., 2001; Cummings et al., 2001; Tschop et al., 2001; Adeghate and Ponery, 2002; Date et al., 2002). In humans, ghrelin induces a significant increase in plasma glucose concentrations and a decrease in insulin secretion (Broglio et al., 2001; Broglio et al., 2003). Coupled with the observation that treatment with GHSs, particularly the non-peptidyl derivatives, induces hyperglycaemia and insulin resistance in the elderly and in obese human patients, those findings suggest that ghrelin has an important role in the regulation of insulin secretion and glucose metabolism (Svensson et al., 1998; Muller et al., 2001).

In healthy humans, hyperglycaemia suppresses both baseline plasma concentrations of GH and GH release induced by GHRH (Masuda et al., 1985). The mechanism of the hyperglycaemia-induced decrease in circulating GH is unclear. Acute hyperglycaemia substantially decreases plasma ghrelin concentrations in healthy humans (Nakagawa et al., 2002). Because ghrelin markedly stimulates GH secretion, the hyperglycaemia-induced suppression of GH release may be caused, at least partly, by the decrease in plasma ghrelin concentrations (Casanueva, 1992).

6.5 Cardiovascular effects

Ghrelin receptors are widely distributed in cardiovascular tissues. In humans and rats, GHS-R 1a mRNA has been detected primarily in the heart, coronary arteries, and aorta

(Nagaya et al., 2001, Gnanapavan et al., 2002). Ghrelin is synthesized and secreted by isolated human cardiomyocytes, in which it likely has paracrine or autocrine effects and may protect the cells from apoptosis (Iglesias et al., 2004).

Growth hormone improves cardiac performance in experimentally induced heart failure (Yang et al., 1995; Fazio et al., 1996). In one study, prolonged treatment with GHSs protected aged rats against cardiovascular damage and improved cardiac performance after myocardial infarction, and enhanced left ventricular contractility in pigs with dilated cardiomyopathy (Muccioli et al., 2002). Long-term ghrelin administration improves cardiac contractility and cardiac output and reduces systemic vascular resistance in humans with chronic heart failure (Nagaya et al., 2001). Furthermore, it induces myocardial growth, improving the structure and function of the left ventricle (Nagaya et al., 2003; Nagaya et al., 2004). Interestingly, hexarelin, acylated ghrelin, and even unacylated ghrelin all prevent doxorubicin-induced death in cultured cardiomyocytes (Filigheddu et al., 2001). Because unacylated ghrelin does not activate the GHS-R 1a (Bednarek et al., 2000), these data indicate that another subtype of GHS-R exists in cardiac tissue and that unacylated ghrelin has some biological activity (Muccioli et al., 2002). Thus, long-term administration of ghrelin may become a treatment strategy for patients with heart failure (Nagaya et al., 2003).

6.6 Anti-proliferative effects

GHS-Rs are also found in human neoplastic tissues, such as mammary gland tumours and thyroid carcinoma cells (Cassoni et al., 2001; Kanamoto et al., 2001). Ghrelin and GHSs inhibit cell proliferation in thyroid tumour cells (Kanamoto et al., 2001; Cassoni et al., 2002) and breast cancer cells (Cassoni et al., 2001). Nonacylated ghrelin also exerts anti-proliferative actions (Cassoni et al., 2001). Because unacylated ghrelin is unable to bind to the GHS-R 1a, these data suggest that the anti-proliferative effects of acylated and unacylated ghrelin on cancer cells are mediated via a GHS-R subtype that is different from GHS-R 1a (Muccioli et al., 2001).

6.7 Conclusion

The isolation and characterization of ghrelin are landmarks in GH research and represent a major advancement in our understanding of GH regulation. Ghrelin is a gastric peptide that is active in the central nervous system, where it is involved in regulation of GH secretion and control of food intake. The widespread expression of GHS-Rs in central and

peripheral tissues suggests that ghrelin may have many endocrine, paracrine, and possibly autocrine effects.

Future challenges lie in improving our ability to diagnose and treat the different diseases associated with altered GH secretion. For example, the potential use of ghrelin in GH deficiency requires investigation. In addition, ghrelin or ghrelin analogues may be useful in pathologic catabolic states such as wound and fracture healing, osteoporosis, severe burns, sepsis, excessive inflammation, multiple organ failure, and weakness in critically ill patients, all conditions in which the administration of moderate doses of GH has been effective (Van den Berghe 2000; Petersenn 2002). The orexigenic actions of ghrelin and its analogues may be harnessed to treat the pathologic forms of anorexia that accompany cancer and ageing (Torsello et al., 1998). Whether ghrelin antagonists can be used to reduce food intake and be developed as a treatment for obesity remains to be investigated.

General introduction - Part II

Progesterone and synthetic progestins used for oestrus prevention in the bitch and their systemic effects

1. Progesterone and synthetic progestins

Natural progesterone is biosynthesized and secreted by partial luteinized granulosa cells during the preovulatory LH peak and consequently the corpus luteum during the luteal phase of the oestrous cycle and serves to prepare the genital tract for the reception and development of the fertilized ovum. The name progesterone comes from *pro*, *ges*tation, *ster*ol, and *one*, indicating this first recognized function of the steroid hormone. However, apart from the genital tract and ovaries, also brain, bone, and especially the mammary gland (Lantinga-van Leeuwen et al., 2000c) are important target organs for progesterone action. The terms progestin, progestagen, and progestogen are used interchangeably to refer to any of the manufactured steroids with progestational activity and derived from progesterone or related steroids. Progestins are widely used in companion animal medicine and the main use involves the control of the reproductive cycle (Briggs, 1983; Romagnoli and Concannon, 2003).

A progestational agent, capable of maintaining pregnancy in ovariectomized animals and of causing endometrial gland secretion, was identified, isolated, and eventually synthesized in the first decades of the 20th century. In the 1930s, it was discovered that progesterone administered by intramuscular injection was capable of blocking ovulation in rabbits. A quest for progesterone-like substances that could control reproduction ensued, leading to the synthesis and characterization of a number of progestins with potential contraceptive application. These included medroxyprogesterone acetate (MPA), melengestrol acetate, megestrol acetate, and others. In the 1960s, some progestins being evaluated for use in humans were found to induce mammary tumours in Beagle bitches during toxicity studies (Frank et al., 1979; Gräf and El Etreby, 1979; Edgren, 1994). Thereafter, long-term animal studies became a requirement by the Food and Drug Administration and the World Health Organization for any progestin to be marketed for human use (Johnson, 1989; Jordan, 1994). In veterinary medicine, depot-injectable MPA rapidly became, and remained for a few

decades, the most widely used progestin in Europe. In the United States, however, it was quickly withdrawn from the veterinary market because of a high incidence of uterine disease reported in dogs administered MPA. Other progestins that were developed as potential human contraceptives have also been marketed for contraceptive use in dogs and/or cats e.g. oral megestrol acetate, oral MPA, oral delmadinone acetate, oral clormadinone acetate, and depot-injectable proligestone (Romagnoli and Concannon, 2003). In a variety of *in vivo* and *in vitro* assays in various species, such compounds have been examined for relative biopotency, bioavailability, oestrogenic activity, and androgenic activity. Often, however, different compounds have not been evaluated in the same way, and therefore relative differences are not clear-cut. Species differences in potency and efficacy are also known to exist.

2. Mechanism of action of progestins

In most if not in all target tissues, progesterone and synthetic progestins diffuse through the cell membrane, and bind to intracellular receptors. In most mammalian species two progesterone receptor (PR) variants are known, the 51-94 kDa PR-A and the larger 116-120 kDa PR-B form. PR-A and PR-B share the same hormone- and DNA-binding domains and differ only in the length of the amino terminus (Graham and Clarke, 1997). After binding of progesterone, the progesterone-PR complex binds to a progesterone-response-element in the nuclear genome, resulting in suppression or activation of transcription and eventual translation of specific gene sequences regulated by progesterone. The translation products include structural and secretory proteins, enzymes, and other regulatory proteins. It is also likely that in some tissues, progesterone, similar to oestradiol, can bind to membrane receptors and has cellular effects in response to binding to membrane receptors (Graham and Clarke, 1997; Romagnoli and Concannon, 2003).

3. Reproductive effects of progestins

In addition to its progestational activity in maintaining pregnancy, progesterone was initially characterized and assayed based on its ability to increase uterine weight (acting synergistically with oestrogen) and its ability to increase endometrial glandularity and endometrial secretory activity (Nelson et al., 1982). Numerous studies on progesterone and progestins have demonstrated that their administration can have actions that can be classified

as progestational, anti-oestrogenic, anti-androgenic, anti-gonadotrophic, and/or contraceptive (Romagnoli and Concannon, 2003).

The pregnancy supporting or progestational actions of progesterone and synthetic progestins include the following: stimulation of endometrial gland development and secretion, promotion of cervical closure, suppression of uterine motility by depressing myometrial sensitivity and contractility directly and by decreasing the availability of myometrial oxytocin receptors, and stimulating proliferation of mammary tissue, especially lobulo-alveolar tissue (Nelson et al., 1982; Mol et al., 1995a,b). Prior progestin administration reduces or prevents oestrogen-induced phenomena including vaginal bleeding, oestrus behaviour, and ciliation of the oviduct by suppressing the synthesis of oestrogen receptors normally stimulated by exposure to oestrogens. Progestin administration reduces, inhibits, or reverses some effects of androgens, including libido, possibly by interfering with androgen or other steroid receptors' reponses to their own hormone ligands. Progestin administration can also suppress follicle development and prevent ovulation. The exact mechanism of the contraceptive activity of progestins is still unclear. In many species there is evidence that contraceptive progestins reduce serum concentrations of gonadotrophins. However, there is little information about the effects of progestins on gonadotrophin secretion in dogs. In one study high doses of MPA administered to Beagle bitches for several months did not reduce the increased concentrations of LH in ovariectomized bitches nor did it lower LH concentrations in intact bitches (McCann et al., 1987). In another study high doses of megestrol acetate did not suppress basal gonadotrophin secretion during anoestrus, nor was the pituitary hypersecretion of LH and FSH in ovariectomized bitches suppressed (Colon et al., 1993). The contraceptive activity of progestins may involve the prevention of increases in gonadotrophin secretion above basal values. In addition, there may be a direct negative effect on follicle development in the ovary (Colon et al., 1993).

The progestins most frequently used for oestrus prevention in the dog are proligestone and MPA. Because the drug cannot be rapidly withdrawn after injection of the depot progestin, care must be taken to use the lowest possible effective dosing regimen. The single injection dosage recommended by the manufacturer for proligestone ranges from 10 mg/kg for a dog of about 60 kg, to 30 mg/kg for one of 3 kg, s.c., and for MPA the single injection dose is 2 mg/kg (maximum 60 mg), s.c. (Schaefers-Okkens, 1996). They should be administered during anoestrus about one month before the expected follicular phase. The first oestrus after the use of proligestone in the majority of bitches can be expected within 9-12 months; after MPA administration it may be up to 2-3 years. Medroxyprogesterone acetate

can also be administered orally, 5 mg once daily (10 mg for large dogs during the first 5 days) for as long as oestrus prevention is wanted or for a maximum of 21 days. The recurrence of oestrus may vary from 2-9 months (Schaefers-Okkens, 1996). In the United States the advise dosage for megestrol acetate, a progestin which probably has a stronger progestagenic effect than MPA, is 0.5 mg/kg orally once daily for 32 days starting during anoestrus, or 2 mg/kg for 8 days starting at the onset of pro-oestrus (Schaefers-Okkens, 1996).

4. Additional effects of progestins

Side effects have been observed after prolonged (6-12 months or longer), chronic use of progestins such as during the course of chronic toxicity studies. They are also reported with varying severity and varying or unknown frequency during the course of treatment with recommended contraceptive doses.

4.1 Induction of mammary growth hormone secretion

In the 1970s acromegalic features were reported to occur in some dogs used in toxicological studies on long-term treatment with progestins (Tucker, 1971; Sloan et al., 1975). Initially it was denied that GH might be involved in the development of the physical features reminiscent of acromegaly (Hansel et al., 1977). In 1980 confirmation that progestin administration can lead to increased circulating GH concentrations was obtained (Concannon et al., 1980). The elevated plasma GH levels declined after cessation of progestin administration (Rijnberk et al., 1980). This phenomenon appeared not to be confined to exogenous progestins, as an excess of GH was also found in bitches during the luteal phase of the oestrous cycle (Concannon et al., 1980; Eigenmann et al., 1983; Rutteman et al., 1987). The absence of a pulsatile pattern in plasma GH concentrations after progestin treatment pointed to autonomous GH production, which only could be inhibited by treatment with the antiprogestin RU 38486 (Watson et al., 1987). This autonomous release was further substantiated by the fact that GHRH did not stimulate and SS did not inhibit GH expression and release (Selman et al., 1991). The progestin-induced increase of plasma GH concentration was associated with elevated plasma concentrations of IGF-I (Selman et al., 1994a).

Because the GH overproduction could not be attributed to a pituitary tumour or an ectopic neoplastic production of GHRH, as in other species, and because cessation of progestin administration resulted in gradual normalization of the circulating GH levels, an extra-pituitary site of GH production was looked for. Measurement of GH concentrations in

tissue homogenates revealed that the progestin-induced GH excess in the dog originated from foci of hyperplastic ductular epithelium of the mammary gland (Selman et al., 1994a; van Garderen et al., 1997). Further evidence came from the arterio-venous gradient over the mammary gland and the fast decrease of plasma GH concentrations after complete mastectomy (Selman et al., 1994a). RT-PCR analysis revealed the expression of the gene encoding GH in normal mammary tissue and in benign and malignant mammary tumours (Mol et al., 1995b). The expression of the GH gene was also documented for feline and human mammary tissue, indicating that the phenomenon of mammary GH expression is not unique for the dog (Mol et al., 1995a; Mol et al., 1996). Immunohistochemical analysis and in situ hybridization revealed that both immunoreactive GH and GH mRNA were present in normal and tumourous mammary epithelial cells. Growth hormone-containing secretory granules could also be demonstrated in epithelial cells by immunoelectron microscopy (van Garderen et al., 1997). Sequence analysis has revealed that the gene encoding GH in the mammary gland is identical to the pituitary GH gene (Mol et al., 1995a; Mol et al., 1995b). The regulation of extra-pituitary GH gene expression is largely unknown. There are at least two differences between the regulation of GH expression in the mammary gland and that in the pituitary gland. First of all, the synthesis and release of mammary GH are highly dependent upon progesterone (Selman et al., 1994a; Lantinga-Van Leeuwen et al., 1999). Secondly, expression of mammary GH is independent of the transcription factor Pit-1, as is GH expression in bone marrow (Lantinga-Van Leeuwen et al., 1999).

The co-localization of GH and the progesterone receptor in the canine mammary gland supports the concept that ligand-activated progesterone receptors may play a direct role in GH gene promotor activation (Lantinga-van Leeuwen, 2000c). The presence of progesterone receptors in mammary gland tissue of dogs opens possibilities for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH overproduction.

4.2 Increased incidence of mammary tumours

The role of progestins in the pathogenesis of human breast cancer has been highly debated (van Leeuwen 1991; Pike et al., 1993). Several epidemiologic studies have linked the use of contraceptive agents during adolescence or before a full-term pregnancy to a higher risk of developing breast cancer at a young age (Pike et al., 1981; van Leeuwen et al., 1991). The question of whether this increased risk is attributable to the oestrogen or progestin content of the contraceptives has not been answered satisfactorily, but there is good reason to

assume that this increased risk is due to the progestin component (Lee et al., 1987; Kay and Hannaford, 1988). This assumption is supported by observations that the highest proliferation rates of mammary epithelium are found in the progesterone-dominated luteal phase of the menstrual cycle and in women receiving progestin-only formulations of contraceptives, indicating a strong mitogenic action of progestins upon mammary epithelium (Horwitz, 1991; Clarke and Sutherland, 1990).

In the dog, prolonged administration of oestrogens does not increase the incidence of mammary tumours (Rutteman, 1992), but treatment of female dogs with progestins at high dosages induces a dose-dependent mammary tumour development (Casey et al., 1979; Misdorp 1991; Rutteman, 1992). Also, endogenous ovarian steroids appear to promote mammary tumourigenesis in dogs, as ovariectomy, even performed at an advanced age, protects against mammary tumour formation (Misdorp 1988). As mentioned above, GH gene expression has been demonstrated in neoplastic mammary tissue of the dog (Mol et al., 1995b). The expression in normal tissue is stimulated by progestins and might mediate the progestin-stimulated development of canine mammary tumours. Progestin-induced GH probably participates in the cyclic development of the mammary gland but may promote mammary tumourigenesis by stimulating proliferation of susceptible, and sometimes transformed, mammary epithelial cells. Progestin-induced mammary GH might function as an autocrine or paracrine growth factor, in view of the presence of the GH receptor that has been demonstrated in the normal canine mammary gland and in mammary tumours of dogs (van Garderen and Schalken, 2002).

In young queens exogenous progestins, but also endogenous progesterone, may cause extensive proliferation of mammary duct epithelium and stroma, leading to the so-called fibroadenomatous hyperplasia (Hayden and Johnson, 1986). Growth hormone mRNA has been demonstrated in mammary tissues of cats with progestin-induced fibroadenomatous changes (Mol et al., 1995b).

4.3 Increased incidence of uterine pathology

Teunissen (1952) reported that progestins may induce the development of cystic endometrial hyperplasia (CEH) in bitches. In line with this observation CEH is frequently seen in bitches treated repeatedly with progestins for prevention of oestrus (Sokolowski and Zimbelman 1974; Goyings et al., 1977). Cystic endometrial hyperplasia may also develop spontaneously in the luteal phase of the oestrous cycle of middle-aged or elderly bitches, i.e., bitches that have gone through several luteal phases (Dow, 1958). The luteal phase of the

oestrous cycle of the bitch differs from that of most other mammals because it is characterized by a prolonged increase of plasma concentrations of progesterone, irrespective of pregnancy (Concannon et al., 1975).

Uterine pathology involves proliferation of the glandular endometrium and cystic dilatation of the endometrial glands with endometrial fluid accumulated in their lumen. Cystic endometrial hyperplasia may be an incidental finding and the natural incidence of CEH is not known. Cystic endometrial hyperplasia predisposes the uterus to infection and can result in pyometra.

From studies in which CEH was induced experimentally in the dog (Dow, 1959), it has become clear that metoestrus or progesterone influence in general seems to be required or at least predisposes to development of CEH. This is remarkable because in women, cows, ewes, mares, and sows endometrial hyperplasia is associated only with excess stimulation by oestrogen from cystic follicles, oestrogenic implants or granulosa cell tumours (Potter et al., 1991). In women it is well known that progesterone induces endometrial atrophy (Seidman et al., 1997), while in dogs progestin treatment has been associated with endometrial hyperplasia (Anderson et al., 1965; Fidler, 1966). The exposure of captive wild felids to progestins is a strong risk factor for development of uterine carcinoma (Harrenstien et al., 1996). In contrast, endometrial carcinomas in dogs, even under progestin treatment, are rare (Kennedy, 1993). This suggests that the canine endometrium responds in a different way to progesterone compared to other species.

As mentioned before, locally produced mammary GH most likely plays a autocrine and/or paracrine role in the progestin-induced proliferation of mammary epithelium (Mol et al., 1995b). Because of the similarity of the progestin-induced epithelial changes in both the mammary gland and the uterus, it is reasonable to assume that GH is also involved in the development of progestin-induced CEH. Although immunoreactive GH was found in the uterine epithelial cells of progestin treated dogs, the absence of mRNA encoding GH in uterine tissue as shown by RT-PCR suggests that this immunoreactive GH does not originate in the uterus (Kooistra et al., 1997). This finding refutes the hypothesis that local production of GH is involved in the pathogenesis of progestin-induced CEH. However these findings do not exclude the possibility that GH plays a role in the pathogenesis of progestin-induced CEH, whereby progestins promote the expression of membrane-bound GH receptors of uterine epithelial cells. In combination with the increase in progestin-induced circulating mammary-derived GH this would explain the presence of GH in uterine epithelial cells. The presence of mRNA encoding the GH receptor has already been demonstrated in the human

uterus (Sharara and Nieman, 1995). The possible role of progestin-induced mammary-derived GH in the pathogenesis of CEH warrants further investigation.

4.4 Prolonged pregnancy

This occurs if progestins are administered subcutaneously at the onset of the follicular phase and the bitch or queen is mated. The gestation will be prolonged and a caesarean section may be needed, unless a progesterone receptor antagonist, such as mifepristone or aglépristone, is given (Schaefers-Okkens, 1996).

4.5 Insulin resistance and diabetes mellitus

The progestin-induced GH excess may give rise to glucose intolerance, which may lead to "exhaustion" of the pancreatic β-cells and subsequently diabetes mellitus (Eigenmann et al., 1983). Diabetes mellitus is a common finding in acromegaly in humans, cats and dogs and can be explained by the diabetogenic properties of GH, leading to insulin resistance (Eigenmann and Rijnberk, 1981).

The occurrence of the afore mentioned side-effects is, with the exception of 'prolonged pregnancy', largely dependent upon total progestin exposure. With the advised dosage regimens the exposure may be higher with MPA and megestrol acetate than with proligestone, the latter being a rather weak progestagen (Schaefers-Okkens, 1996).

4.6 Suppression of the hypothalamic-pituitary-adrenal axis

Besides an effect on the progesterone receptor, MPA also has intrinsic glucocorticoid properties due to the relatively high affinity of MPA for the glucocorticoid receptor (Selman et al., 1996). Suppression of the hypothalamic-pituitary-adrenocortical axis by MPA has been reported in both humans (Willemse et al., 1990) and dogs (Selman et al., 1994b; Selman et al., 1996). In man the use of high doses of MPA may even lead to Cushing's syndrome (Simononski et al., 1989).

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Chapter 3

Pulsatile secretion pattern of growth hormone in dogs with pituitary-dependent hyperadrenocorticism

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Abstract

The amplitude and frequency of growth hormone (GH) secretory pulses are influenced by a variety of hormonal signals, among which glucocorticoids play an important role. The aim of this study was to investigate the pulsatile secretion pattern of GH in dogs in which the endogenous secretion of glucocorticoids is persistently elevated, i.e., in dogs with pituitary-dependent hyperadrenocorticism (PDH). Blood samples for the determination of the pulsatile secretion pattern of GH were collected at 10-min intervals between 0800h and 1400h in sixteen dogs with PDH and in six healthy control dogs of comparable age. The pulsatile secretion patterns of GH were analyzed using the Pulsar programme.

Growth hormone was secreted in a pulsatile fashion in both dogs with PDH and control dogs. There was no statistical difference between the mean (± SEM) basal GH level in dogs with PDH (0.7 ± 0.1 µg/l) and the control dogs (0.6 ± 0.1 µg/l). The mean area under the curve above the zero-level (AUC_0) for GH in dogs with PDH (4.6 ± 0.6 µg/lx6h) was significantly lower than that in the control dogs (7.3 ± 1.0 µg/lx6h). Likewise, the mean area under the curve above the baseline (AUC_{base}) for GH in dogs with PDH (0.6 ± 0.1 µg/lx6h) was significantly lower than that in the control dogs (3.7 ± 1.0 µg/lx6h). The median GH pulse frequency in the dogs with PDH (2 pulses/6h, range 0 to 7 pulses/6h) was significantly lower ($P = 0.04$) than that (5 pulses/6h, range 3 to 9 pulses/6h) in the control group

The results of this study demonstrate that PDH in dogs is associated with less GH secreted in pulses than in control dogs, whereas the basal plasma GH concentrations were similarly low in both groups. It is discussed that the impaired pulsatile GH secretion in dogs with PDH is the result of alterations in function of pituitary somatotrophs and changes in supra-pituitary regulation.

Introduction

Like the other hormones of the canine pituitary anterior lobe, growth hormone (GH) secretion is pulsatile in nature (Takahashi et al., 1981; Kooistra et al., 2000; Lee et al., 2001). Pituitary GH secretion is regulated predominantly by the opposing actions of the stimulatory hypothalamic peptide GH-releasing hormone (GHRH) and the inhibitory hypothalamic peptide somatostatin (SS). Each GH secretory episode seems to be initiated by a burst of GHRH into the hypophyseal portal system, preceded by a reduction of somatostatinergic input to the pituitary (Plotsky and Vale, 1985). In addition to these hypothalamic hormones, a recently identified GH-releasing peptide, called ghrelin, is likely to play a role in the regulation of pituitary GH secretion (Kojima et al., 1999). The amplitude and frequency of GH secretory pulses are influenced by a variety of hormonal signals, among which glucocorticoids play an important role (Devesa et al., 1992).

Glucocorticoids are important physiological regulators of GH synthesis and secretion. In humans and rats, glucocorticoids enhance GH gene transcription (Evans et al., 1982; Karin et al., 1990) and increase pituitary GHRH receptor numbers (Seifert et al., 1985; Miller and Mayo, 1997; Ohyama et al., 1998). Consequently, both spontaneous and GHRH-induced GH secretion are stimulated by acute administration of dexamethasone (Wehrenberg et al., 1983; Casanueva et al., 1990; Veldhuis et al., 1992). A minimum level of cortisol is essential for normal GH production. Individuals with hypoadrenocorticism (Addison's disease) may be GH-deficient because of poor GH synthesis (Allen, 1996). In line with this observation, humans with idiopathic adrenocorticotrophic hormone (ACTH) deficiency need appropriate glucocorticoid replacement to re-establish the normal pattern of GH response to stimulatory tests (Giustina et al., 1989). However, when in humans the endogenous secretion of glucocorticoids is persistently elevated, as in Cushing's disease, the spontaneous and stimulated GH secretion are blunted (Takahashi et al., 1992; Magiakou et al., 1994; Borges et al., 1997).

In dogs, pituitary-dependent hyperadrenocorticism (PDH) is one of the most common endocrine diseases and has many similarities with Cushing's disease in humans (Kemppainen and Peterson, 1994). Consequently, changes in pituitary GH secretion may also be expected in dogs with PDH. Indeed, it has been reported that basal plasma GH levels are lower and stimulated GH secretion is blunted in dogs with PDH (Peterson and Altszuler, 1981; Regnier and Garnier, 1995; Meij et al., 1997). However, information on changes in the spontaneous pulsatile secretion pattern of GH in dogs with PDH is lacking. Therefore, we investigated the

6-h pulsatile secretion pattern of GH in sixteen dogs with PDH and compared the characteristics of these secretory profiles with those of six healthy control dogs of comparable age.

Materials and methods

Dogs

A total of six female and ten male dogs with PDH and six healthy Beagle bitches were used in this study. The mean (± SEM) age (8.3 ± 0.5 years; median 8 years, range 5-12 years) and the mean (± SEM) body weight (15.7 ± 1.7 kg, median 14 kg, range 7-30 kg) of the dogs with PDH did not differ significantly from those of the control dogs (7.8 ± 0.2 years, median 8 years, range 7-8.5 years, and 14.7 ± 0.6 kg, median 15 kg, range 12-17 kg, respectively).

The suspicion of hyperadrenocorticism was based upon medical history, physical examination, and results of routine haematological and biochemical testing. Hyperadrenocorticism was diagnosed when the mean corticoid/creatinine (C/C) ratio in two consecutive morning urine sample exceeded 10 x 10^{-6} (Rijnberk et al., 1988). Differentiation between PDH and hyperadrenocorticism due to an adrenocortical tumour was accomplished by administering, after collection of the second urine sample, three oral doses of dexamethasone 0.1 mg/kg body weight at 8-h intervals. When the C/C ratio in the third urine sample was less than 50 % of the mean of the first two samples, the dog was categorized as being responsive to dexamethasone and PDH was diagnosed (Rijnberk et al., 1988). In the dogs with less suppression of the third urinary C/C ratio, pituitary dependency was established by the finding of non-suppressed plasma ACTH levels (≥10 ng/l). The latter is justified because ectopic ACTH- or CRH-producing tumours have not been reported in dogs. In addition to these biochemical function tests, the diagnosis of PDH was supported by visualization of the adrenals by ultrasonography (Voorhout et al., 1990) and computed tomography of the pituitary gland (Kooistra et al., 1997; Meij et al., 1998).

Blood sample collection

Blood samples for the determination of the plasma concentration of GH were collected at 10-min intervals between 0800h and 1400h. Blood samples were collected by jugular venipuncture and immediately placed in chilled EDTA-coated tubes, and centrifuged. Plasma was stored at -20° C until assayed.

Hormone determination

Plasma ACTH concentrations were measured using a commercially available two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands). The intra- and interassay coefficients of variation were 3.2 % and 7.8 %, respectively, and the sensitivity was 1 ng/l. There was no cross-reaction between the antiserum and α-melanocyte-stimulating hormone or ACTH precursors (Findling and England, 1990).

Urinary corticoid concentrations were measured by radioimmunoassay (RIA) as described previously (Rijnberk et al., 1988). The intra- and interassay coefficients of variation were 6 % and 8 %, respectively, and the sensitivity was 1 nmol/l. The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) and the C/C ratio was calculated (Stolp et al., 1983; Rijnberk et al., 1988).

Plasma GH concentrations were measured by a homologous RIA as described by Eigenmann and Eigenmann (1981). The intra- and interassay coefficients of variation were 3.8 % and 7.2 %, respectively. The sensitivity of the assay was 0.3 μg/l.

Statistical analysis

The 6-h secretion patterns of GH were analyzed using the Pulsar programme developed by Merriam and Wachter (1982). The programme identifies secretory peaks by height and duration from a smoothed baseline, using the assay standard deviation (SD) as a scale factor. The cut-off parameters G1-G5 of the Pulsar programme were set at 3.98, 2.4, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively, resulting in a false-positive error rate of less than 5 %. The smoothing time, a window used to calculate a running mean value, was set at 5h. The weight assigned to peaks was 0.05. The A, B, and C values of the Pulsar programme used to calculate the variance of the assay, were set at A=0, B=7.2, and C=5. The values extracted from the Pulsar analyses included the overall mean of the smoothed baseline, the number of peaks, the area under the curve above the zero level (AUC_0), and the AUC above the baseline (AUC_{base}).

Differences in parameters between control dogs and dogs with PDH were evaluated by the unpaired Student's t-test (two-tailed). Since the data were not assumed to be normally distributed, differences in GH pulse frequency were determined by non-parametric analysis, using Wilcoxon-Mann-Whitney test. Values are expressed as mean ± SEM or range or as median and range. $P < 0.05$ was considered significant.

Ethics of the study

The experiments in this study were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University.

Results

The mean basal urinary C/C ratio ranged from 23 to 301 x 10^{-6} in the dogs with PDH. In thirteen of the sixteen dogs with PDH oral dexamethasone suppressed the urinary C/C ratio to less than 50 % of the mean basal urinary C/C ratio of the first two days (range 3 to 33 %). In the remaining three dogs the urinary C/C ratios after oral dexamethasone were 57 %, 85 %, and 105 % of the mean basal urinary C/C ratio of the first two days. The plasma ACTH concentrations in these three dogs were 90 ng/l, 111 ng/l, and 129 ng/l, respectively.

Growth hormone was secreted in a pulsatile fashion in both dogs with PDH and control dogs (Figure 1). There was no statistical difference ($P = 0.57$) between the mean (± SEM) basal GH level in dogs with PDH (0.7 ± 0.1 µg/l) and the control group (0.6 ± 0.1 µg/l). The mean AUC_0 for GH in dogs with PDH (4.6 ± 0.6 µg/lx6h) was significantly lower ($P < 0.05$) than that in the control group (7.3 ± 1.0 µg/lx6h). Likewise, the mean AUC_{base} for GH in dogs with PDH (0.6 ± 0.1 µg/lx6h) was significantly lower ($P = 0.03$) than that in the control group (3.7 ± 1.0 µg/lx6h). The median GH pulse frequency in the dogs with PDH (2 pulses/6h, range 0 to 7 pulses/6h) was significantly lower ($P = 0.04$) than that (5 pulses/6h, range 3 to 9 pulses/6h) in the control group (Table 1).

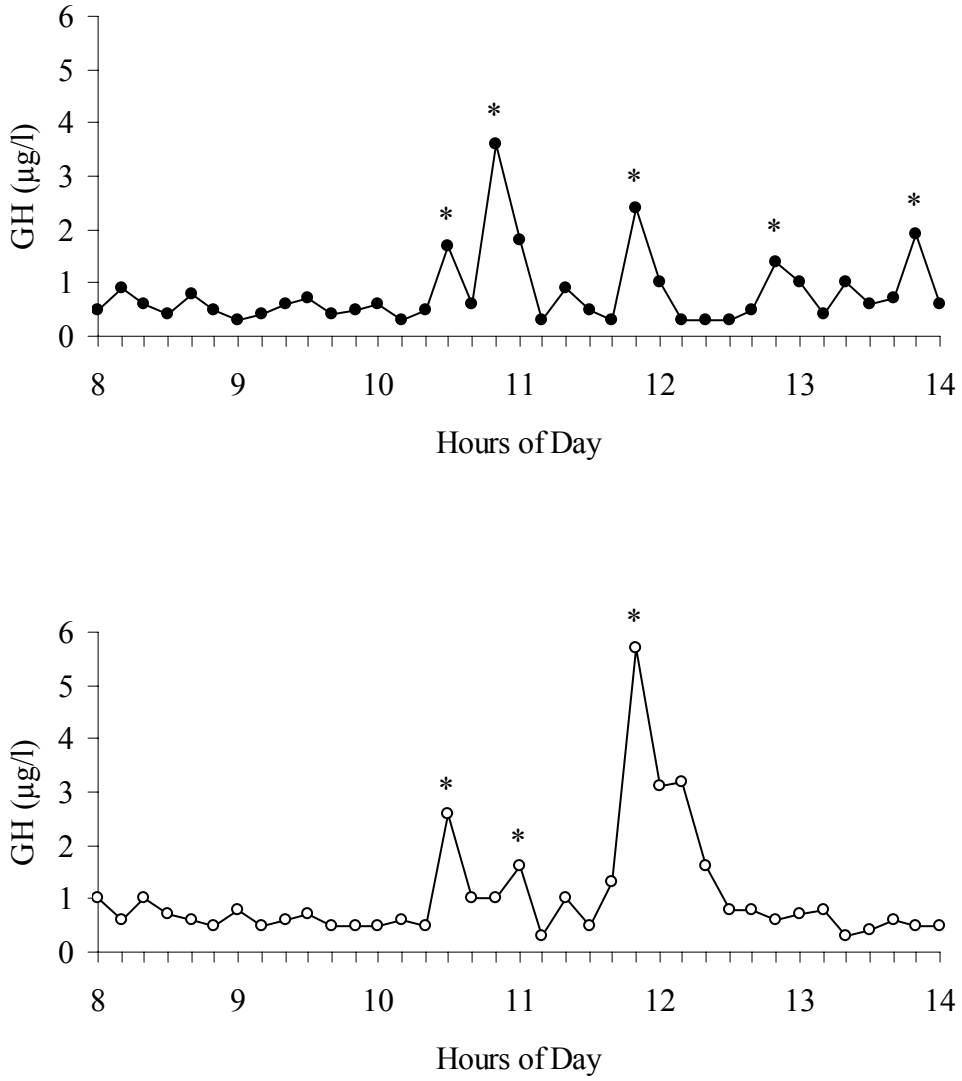

Figure 1. The 6-h secretory profile of GH in a healthy Beagle dog (●) and a dog with pituitary-dependent hyperadrenocorticism (○). Significant pulses, calculated by the Pulsar programme, are indicated with an asterisk.

Table 1. Characteristics of the 6-h secretory profiles of GH in sixteen dogs with pituitary-dependent hyperadrenocorticism and in six control dogs of comparable age. Basal GH = mean basal plasma GH level; AUC_0 = area under the curve above the zero-level for GH; AUC_{base} = area under the curve above the baseline for GH; Frequency = number of GH pulses per 6h.

	Basal GH (µg/l)	AUC_0 (µg/lx6h)	AUC_{base} (µg/lx6h)	Frequency (pulses/6h)
Dog with PDH				
Dog 1	1.2	7.5	0.2	0
Dog 2	0.5	4.1	1.2	5
Dog 3	0.3	3.0	1.1	6
Dog 4	0.3	2.2	0.4	2
Dog 5	0.5	2.9	0.1	0
Dog 6	0.5	3.4	0.3	2
Dog 7	0.6	3.6	0.4	2
Dog 8	0.4	4.2	1.8	7
Dog 9	1.2	7.9	0.6	3
Dog 10	0.8	5.6	0.8	5
Dog 11	0.3	2.0	0.2	1
Dog 12	0.5	4.9	1.8	5
Dog 13	0.5	3.3	0.4	2
Dog 14	1.2	7.2	0.2	0
Dog 15	0.3	2.5	0.6	3
Dog 16	1.6	9.8	0.2	0
Control dogs				
Dog 1	0.5	7.5	4.6	5
Dog 2	0.5	3.2	0.4	3
Dog 3	0.6	6.3	2.7	3
Dog 4	0.8	7.5	2.6	9
Dog 5	0.7	8.7	4.3	7
Dog 6	0.5	10.5	7.7	5

Discussion

The results of this study demonstrate that GH is secreted in a pulsatile fashion in both healthy dogs and dogs with PDH. However, the low AUC for GH, both above the zero-level and above the baseline, and the low GH pulse frequency compared with healthy dogs indicate that less GH is secreted in pulses in dogs with PDH. Spontaneous pulsatile GH secretion has also been reported to be suppressed in humans with PDH and rats treated for four weeks with high doses of glucocorticoids (Magiakou et al., 1994; Wajchenberg et al., 1996; Ohyama et al., 1997). The impaired pulsatile GH secretion may be the result of alterations in supra-pituitary regulation and changes at the level of the somatotroph.

The inhibitory effect of chronic hypercorticism on pituitary GH secretion involves, at least in part, enhancement of hypothalamic SS release (Wehrenberg et al., 1990; Wajchenberg et al., 1996; Terzolo et al., 2000). Support for the concept of the enhancement of SS tone by glucocorticoids comes from observations in rats that the hypothalamic content of immunoreactive SS (Nakagawa et al., 1987) and hypothalamic SS mRNA levels (Nakagawa et al., 1992) are increased following chronic dexamethasone administration. Further evidence for a regulatory role of glucocorticoids on GH secretion acting at the hypothalamic level is derived from *in vivo* studies in rats after passive immunization with anti-SS antibodies. This immunization reverses the inhibitory effect of high levels of circulating glucocorticoids on stimulated GH response (Wehrenberg et al., 1990; Mallo et al., 1993). However, pyridostigmine, which activates cholinergic synapses and thus suppresses hypothalamic SS release, does not modify plasma GH levels in humans with Cushing's syndrome (Leal-Cerro et al., 1990). Thus enhancement of hypothalamic SS release is not the only factor suppressing pituitary GH secretion in chronic hypercorticism. Inhibition of pituitary GH secretion may also be explained by a decrease in hypothalamic GHRH synthesis and secretion. Hypothalamic GHRH mRNA levels were indeed reduced in rats treated with high doses of glucocorticoids (Miell et al., 1991; Senaris et al., 1996; Ohyama et al., 1997).

In addition to their effect at the hypothalamic level, glucocorticoids may also influence GH secretion by acting directly at the pituitary level. Studies in humans, rats, and dogs have demonstrated that chronic glucocorticoid excess inhibits the GH response to GH-releasing stimuli, such as GHRH (Peterson and Altszuler, 1981; Wehrenberg et al., 1983; Hotta et al., 1988; Burguera et al., 1990; Voltz et al., 1995; Ohyama et al., 1997; Meij et al., 1997; Watson et al., 2000). In humans with Cushing's syndrome, blunted GH responses were also found to a synthetic hexapeptide (GHRP-6) which releases GH by a direct effect at the pituitary level through receptors other than GHRH receptors (Leal-Cerro et al., 1994). These observations suggest that chronic hypercorticism may also have a direct effect on pituitary somatotrophs, although the impaired response might also be the result of the persisting inhibitory effect of SS.

Although the GH response to GHRH is markedly impaired, an increase in the number of pituitary GHRH receptors has been reported in rats treated for four weeks with high doses of dexamethasone (Ohyama et al., 1997). The increase in the number of pituitary GHRH receptors may be caused by decreased GHRH secretion, since Miki et al. (1996) reported an increase in pituitary GHRH receptor mRNA levels in rats after immunoneutralization for GHRH. Therefore it may be hypothesized that post GHRH receptor signalling is impaired in

somatotrophs exposed to high doses of dexamethasone for long periods (Ohyama et al., 1997). The decrease in hypothalamic GHRH secretion may result in a lack of priming of the somatotrophs and, subsequently, in reduced GH synthesis and secretion and decreased responsiveness to exogenously administered GHRH (Thakore and Dinan, 1994). This concept is supported by the observation that there was a clear GH response to GHRH plus pyridostigmine in humans with Cushing's disease treated for one week with GHRH (Leal-Cerro et al., 1993). This suggests that blunted GH secretion in patients with Cushing's syndrome is at least partially mediated by decreased priming of the somatotrophs with endogenous GHRH. In addition, it has been demonstrated in young rats that administration of cortisone acetate decreases the number of somatotrophs in the pituitary gland (Niimi et al., 1993).

The suppressed pulsatile GH secretion in dogs with PDH may also be ascribed to obesity. Under the influence of glucocorticoid excess energy derived from protein catabolism is increased and the contribution from lipid oxidation is decreased. This effect leads to characteristic changes in body habitus that are frequently associated with glucocorticoid excess. Indeed, one of the cardinal physical features of dogs with hyperadrenocorticism is centripetal obesity (Rijnberk, 1996). Like chronic hypercorticism, obesity is associated with insulin-like growth factor-I (IGF-I)-mediated GH suppression (Magiakou et al., 1994) and blocked GH response to GH-releasing stimuli (Cordido et al., 1993). Elevated plasma free fatty acid levels (Leal-Cerro et al., 1997) and changes in circulating leptin levels (Pombo et al., 1999) may be contributing factors to the deranged GH secretion observed in humans with Cushing's syndrome. Leal-Cerro et al. (1998) demonstrated that in humans with Cushing's syndrome hyporesponsiveness of the somatotrophic cells to GHRH is improved after a short-term hypocaloric diet. However, in contrast to chronic hypercorticism (Leal-Cerro et al., 1994) intravenous administration of a combination of GHRH and GHRP-6 resulted in an elevated GH response in obese humans (Cordido et al., 1993), indicating that obesity is not the only explanation for the blocked GH response in chronic hypercorticism.

In contrast to observations of Meij et al. (1997), in the present study the basal plasma GH concentrations were not different in the dogs with PDH compared to the control dogs. However, the basal plasma GH levels in the study of Meij et al. (1997) were derived from only three blood samples collected at 15-min intervals. Therefore, it is possible that pulses may have contributed to the higher basal plasma GH levels in the control dogs in the study of Meij et al. (1997). In addition, in the study of Meij et al. (1997) the basal plasma GH levels in dogs with PDH (median age 10 years) were compared with those of control dogs with a

median age of two years. The significantly lower basal plasma GH levels in dogs with PDH may therefore have been caused by the effects of ageing. Indeed, it has been reported that GH secretion is decreased in elderly humans (Finkelstein et al., 1972; Zadik et al., 1985; Wilshire et al., 1995) and aged rats (Sonntag et al., 1980; De Gennaro Colonna et al., 1994). Also in the dog there are indications that ageing is associated with impaired GH secretion. The basal plasma GH levels in the control dogs in the present study were lower than those in young adult bitches (Kooistra et al., 2000; Lee et al., 2001) employing the same GH assay. The decreased GH secretion in elderly humans has been ascribed to the blunted response of GH to GHRH (Shetty and Duthie, 1995) and feedback disruption of the regulatory GH-IGF-I system (Veldhuis, 1997). The decreased GH secretion in elderly rats is associated with decreased pituitary GH content (Sonntag et al., 1980), reduced pituitary GH mRNA (Takahashi et al., 1990), and reduction of hypothalamic GHRH mRNA levels (De Gennaro Colonna et al., 1994).

With regard to the similar basal plasma GH levels in dogs with PDH and control dogs, it is important to notice that these values were close to the sensitivity of the GH assay used in the present study. Although the results of this study indicate that the basal plasma GH levels were similarly low in both groups, an ultrasensitive GH assay may be needed to confirm this observation.

In conclusion, the results of this study demonstrate that PDH in dogs is associated with less GH secreted in pulses, whereas the basal plasma GH concentrations were similarly low in both groups. The impaired pulsatile GH secretion in dogs with PDH may be the result of alterations in function of pituitary somatotrophs and changes in supra-pituitary regulation.

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Chapter 4

Effects of growth hormone-releasing peptides on the release of adenohypophyseal hormones in healthy dogs and in dogs with pituitary-dependent hyperadrenocorticism

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Abstract

The aim of this study was to investigate the effects of ghrelin and growth hormone-releasing peptide-6 (GHRP-6) on the release of growth hormone (GH), adrenocorticotrophic hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and prolactin (PRL) in dogs with pituitary-dependent hyperadrenocorticism (PDH) and in healthy dogs of comparable age. In eight healthy dogs, the responses to ghrelin and GHRP-6 were compared to those of GH-releasing hormone (GHRH) and NaCl 0.9 % (control). In seven dogs with PDH, the effects of ghrelin and GHRP-6 were compared with their effects in healthy dogs.

In the healthy dogs, GHRH, GHRP-6, and ghrelin caused a significant rise in plasma GH concentrations. Administration of GHRH elicited significantly higher plasma GH concentrations than administration of ghrelin and GHRP-6. In the dogs with PDH, the GHRP-6-induced release of GH was significantly lower than in healthy dogs. Administration of ghrelin elicited a GH release that did not differ significantly between dogs with PDH and healthy dogs. Ghrelin and GHRP-6 did not cause a significant rise in plasma ACTH, cortisol, TSH, LH, and PRL concentrations in either the healthy dogs or the dogs with PDH.

It is concluded that in comparison with GHRH, GHRP-6 and ghrelin have a low GH-releasing potency in healthy dogs. In dogs with PDH, the GH release in response to GHRP-6 is impaired. Neither GHRP-6 nor ghrelin activates the pituitary-adrenocortical axis or stimulates TSH, LH, and PRL release in healthy elderly dogs and dogs with PDH.

Introduction

For many years it was thought that the pulsatile secretion of growth hormone (GH) by pituitary somatotrophs was controlled by the two antagonistic hypothalamic peptides GH-releasing hormone (GHRH) and somatostatin (SS) (Plotsky and Vale, 1985). However, GH release can also be elicited by synthetic GH secretagogues (GHSs) (Bowers et al., 1977, Momany et al., 1981). Synthetic GHSs exert their effect on GH release by acting through receptors different from those for GHRH (Casanueva and Dieguez, 1999). Growth hormone-releasing peptide-6 (GHRP-6) was the first powerful GHS used in humans and rats. Nowadays, GHRP-6 is the gold standard against which all so-called non-classic GHSs are compared (Korbonits and Grossman, 1995). After the introduction of GHRP-6, a new generation of peptidyl and non-peptidyl GHSs was developed (Casanueva and Dieguez, 1999). Interestingly, most of the GHSs were constructed well before the isolation of GHRH in 1982 (Guillemin et al., 1982, Rivier et al., 1982). In 1996, the GHS-receptor (GHS-R), a specific G-protein-coupled seven-transmembrane receptor, was identified by Pong et al. The GHS-R is present in various tissues (e.g. pituitary, hypothalamus, heart, lung, pancreas, intestine, adipose tissue). The demonstration of a hypothalamic and pituitary localization of the human GHS-R is consistent with its role in regulating GH release. The expression of this receptor in other central and peripheral regions may imply that it is involved in additional yet undefined physiological functions (Guan et al., 1997; Papotti et al., 2000; Kojima et al., 2001).

In 1999, Kojima et al. purified and characterized the endogenous ligand for the GHS-R from rat and human stomach and called it ‘ghrelin’. Ghrelin has since been identified in the fundus of the stomach of dogs and appears to be highly conserved (Tomasetto et al., 2001). Unlike the digestive enzymes, ghrelin is not secreted into the gastrointestinal tract but released into the bloodstream to act - among other possible functions - on the pituitary to release GH. Ghrelin is a peptide of 28 amino acids, in which the hydroxyl group of the serine 3 residue is esterified by *n*-octanoic acid. This octanoylation is essential for its GH-releasing activity (Kojima et al., 1999).

In healthy humans, the endocrine effects of GHSs are not specific for GH. Administration of synthetic GHSs also significantly increases the secretion of prolactin (PRL), adrenocorticotrophic hormone (ACTH), and cortisol (Massoud et al., 1996, Arvat et al., 2001). According to Kojima et al. (1999), ghrelin specifically stimulates GH release and does not affect the secretion of other adenohypophyseal hormones in rats. In contrast, Arvat et

al. (2001) demonstrated that intravenous administration of ghrelin, apart from stimulating GH secretion, also increases circulating PRL, ACTH, and cortisol levels in healthy humans.

Several pathological (e.g. obesity and chronic hypercortisolism) and non-pathological (e.g. ageing) states in humans are characterized by a reduction in pituitary GH secretion (Leal-Cerro et al., 1994). Chronic hypercortisolism is not only associated with reduced pituitary GH release (Hartog et al., 1964) but also with an impaired GH response to various stimuli (Casanueva, 1992). Even a combination of GHRH and GHRP-6, which is a very powerful GH-releasing stimulus, is unable to induce significant GH release in humans with Cushing's syndrome (Leal-Cerro et al., 1994). So far, there are no reports on the effects of ghrelin on GH release in patients with hyperadrenocorticism.

The aim of this study was to investigate the effects of ghrelin and GHRP-6 on the release of GH, ACTH, cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and PRL in dogs with pituitary-dependent hyperadrenocorticism (PDH) and in healthy dogs of comparable age.

Materials and methods

Dogs

For the first study, eight healthy Beagle dogs (four males and four females) with ages ranging from 7 to 12 years (median age: 10 years) were used. In the second study, seven dogs of different breeds (seven males, two intact females and one spayed female), aged 9 to 13 years (median age: 10 years), with PDH were studied.

Diagnosis of hyperadrenocorticism was based upon elevated ($> 10 \times 10^{-6}$) corticoid/creatinine (C/C) ratios in two consecutive morning urine samples (Rijnberk et al., 1988). After collection of the second urine sample, the dogs received three oral doses of dexamethasone (0.1 mg/kg body weight) at 8-h intervals. Then a third urine sample was collected. If the C/C ratio in this third urine sample was less than 50 % of the mean of the first two samples, PDH was diagnosed (Galac et al., 1997). In one dog in which the urinary C/C ratio was suppressed less than 50 %, the diagnosis of PDH was confirmed by an elevated plasma ACTH concentration, bilaterally enlarged adrenal glands on ultrasonographic examination, and an abnormal pituitary gland on computed tomography (Voorhout, 1990, Voorhout et al., 1990).

Study design and blood sample collection

The first study was a randomized clinical trial in which the eight healthy Beagle dogs were randomly allocated to four groups. According to a Latin-square design, the dogs received an intravenous injection of either human GHRH (2 µg per kg body weight), GHRP-6 [(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)[3]] (2 µg per kg body weight), human ghrelin (2 µg per kg body weight) (Peninsula Laboratories Inc. Belmont, CA, USA), or 0.9 % NaCl (control). In order to avoid carry-over effects, washout periods of at least 4 days were included in the protocol.

In the second study, the seven dogs with PDH received an intravenous injection of either GHRP-6 (2 µg per kg body weight) or ghrelin (2 µg per kg body weight). One week later, the other compound was administered.

Blood samples for the determination of plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL were collected by jugular venipuncture at -15 and 0, 5, 10, 20, 30, and 45 min and immediately transferred to ice-chilled EDTA-coated tubes (GH, ACTH, cortisol, LH and PRL) or heparin-coated tubes (TSH). Samples were centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determination

The urinary corticoid concentrations were measured with a non-commercial radioimmunoassay (RIA). The urinary corticoid concentration was related to the urinary creatinine concentration and the corticoid/creatinine ratio was calculated (Stolp et al., 1983; Rijnberk et al., 1988).

Plasma GH concentrations were determined with a homologous RIA (Eigenmann and Eigenmann, 1981). The intra- and interassay coefficients of variation were 3.8 % and 7.2 % respectively, and the sensitivity of the assay was 0.3 µg/l. The degree of cross-reaction with canine PRL was 2 %.

Plasma ACTH concentrations were measured with an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The interassay coefficient of variation was 7.8 % and the sensitivity was 0.2 pmol/l.

Plasma cortisol concentrations were measured with a commercially available RIA (Diagnostic Products Corporation, Los Angeles, CA, USA), validated for the dog. The intra- and interassay coefficients of variation ranged from 3.0 % to 5.1 % and from 4.0 % to 6.4 %, respectively. The sensitivity of the assay was 5.5 nmol/l.

Plasma TSH concentrations were determined with a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite canine TSH, Diagnostic Products Corporation [DPC]) according to the instructions of the manufacturer. The intra-assay coefficients of variation were 5.0 %, 4.0 %, and 3.8 % at TSH levels of 0.20, 0.50, and 2.60 µg/l, respectively. The interassay coefficients of variation were 6.3 % and 8.2 % at TSH levels of 0.16 and 2.80 µg/l, respectively. The sensitivity of the assay was 0.03 µg/l. Cross-reactivity with FSH and LH was negligible.

Plasma LH concentrations were determined with a heterologous RIA as described previously by Nett et al. (1975). A rabbit antiserum raised against ovine LH (CSU-204; kindly supplied by G.D. Niswender, Colorado State University), radioiodinated NIAMDD-bLH-4, and canine pituitary standard LER 1985-1 (a gift from Dr. L.E. Reichert, Albany Medical College, NY) were used in this assay. The intra- and interassay coefficients of variation for values higher than 0.5 µg/l were 2.3 % and 10.5 % respectively. The sensitivity of the assay was 0.3 µg/l.

Plasma concentrations of PRL were determined with a previously validated heterologous RIA (Okkens et al., 1985). The intra-assay and interassay coefficients of variation were 3.5 % and 11.5 %, respectively. The sensitivity of the assay was 0.8 µg/l.

Statistical analysis

The plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL at -15 and 0 min were averaged per dog and used in all analyses as the basal plasma hormone concentration (basal).

Statistical analyses were performed using the MIXED procedure (SAS Version 8.02, SAS Institute Inc., Cary, NC, USA) with dog within group as the unit of analysis. Dog was considered a random effect. A first-order autoregressive covariance structure was used to model the autocorrelation in the repeated measures of the response variables. All data were log-transformed for further analysis to correct for non-normality.

For the first study, models were fitted for GH, ACTH, cortisol, TSH, LH, and PRL respectively, with compound (ghrelin, GHRP-6, GHRH, and 0.9 % NaCl), group of dogs (1 to 4), day of compound administration (day 1 to 4), and time (repeated measurement of hormones starting with the basal concentration), included as fixed effects. It was assumed there were no interactions between effects. For the second study, models were fitted separately for the responses of GH, ACTH, cortisol, TSH, LH, and PRL respectively, after ghrelin or GHRP-6 administration. Group (healthy dogs versus dogs with PDH), time

(repeated measurement of hormones starting with the basal concentration), and the interaction between group and time, allowing different slopes for both groups over time, were included as fixed effects. Values are expressed as mean ± SEM concentrations. Statistical significance was defined at $P \leq 0.05$.

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

Results

No side effects were observed during or after administration of the GH-releasing agents. In the healthy dogs, administration of ghrelin, GHRP-6, and GHRH resulted in a mean plasma GH concentration of 1.8 ± 0.5 µg/l, 2.7 ± 1.0 µg/l, and 5.5 ± 1.0 µg/l, respectively, which was significantly higher than that after administration of NaCl 0.9 % (0.9 ± 0.2 µg/l). GHRH administration caused a significantly higher mean plasma GH concentration (5.5 ± 1.0 µg/l) than administration of ghrelin (1.8 ± 0.5 µg/l) or GHRP-6 (2.7 ± 1.0 µg/l) (Table 1 and Figure 1).

Administration of the GH secretagogues did not cause stimulation of the pituitary-adrenocortical axis. Administration of ghrelin (25 ± 5 ng/l), GHRP-6 (30 ± 6 ng/l), and GHRH (21 ± 4 ng/l) resulted in mean plasma ACTH concentrations that did not differ significantly from those measured after administration of NaCl 0.9 % (25 ± 5 ng/l). Furthermore, administration of ghrelin (64 ± 19 nmol/l), GHRP-6 (80 ± 24 nmol/l), or GHRH (55 ± 8 nmol/l) resulted in mean plasma cortisol concentrations that were not significantly higher than those measured after administration of NaCl 0.9 % (73 ± 13 nmol/l) (Table 1 and Figure 1).

In the healthy dogs, the mean plasma TSH, LH, and PRL concentrations did not differ significantly when treated with the GH-releasing agents or NaCl 0.9 % (Table 1 and Figure 1).

Table 1. Mean (± SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL in eight healthy dogs treated with different GH secretagogues (GHSs) (ghrelin, GHRP-6, GHRH) and NaCl 0.9 % (control).

	Ghrelin	GHRP-6	GHRH	Control
GH (µg/l)	1.8 ± 0.5 **a,b**	2.7 ± 1.0 **a,c**	5.5 ± 1.0 **a,b,c**	0.9 ± 0.2
ACTH (ng/l)	25 ± 5	30 ± 6 **b**	21 ± 4 **b**	25 ± 5
Cortisol (nmol/l)	64 ± 19 **a**	80 ± 24 **b**	55 ± 8 **a,b**	73 ± 13
TSH (µg/l)	0.26 ± 0.07	0.24 ± 0.06	0.28 ± 0.09	0.23 ± 0.05
LH (µg/l)	3.8 ± 1.2	5.5 ± 1.5	3.5 ± 1.0	4.1 ± 1.6
PRL (µg/l)	3.4 ± 0.9	2.5 ± 0.5	2.8 ± 0.6	3.0 ± 0.4

a: significant difference in hormone concentrations between GHS and control group.

b, c: significant difference in hormone concentrations between indicated GHSs.

Administration of GHRP-6 to the healthy dogs elicited a mean plasma GH concentration of 2.7 ± 1.0 µg/l, which was significantly higher than the level in the dogs with PDH (0.8 ± 0.2 µg/l). Administration of ghrelin (1.8 ± 0.5 µg/l) led to a mean plasma GH concentration in the healthy dogs that did not differ significantly from that in the dogs with PDH (1.5 ± 0.7 µg/l). Neither GHRP-6 nor ghrelin caused a significant rise in plasma ACTH and cortisol concentrations in dogs with PDH. (Table 2, Figures 2 and 3). The mean plasma TSH, LH, and PRL concentrations in the healthy dogs after GHRP-6 or ghrelin administration did not differ significantly from that in the dogs with PDH and these hormone concentrations were not stimulated by the GHSs (Table 2, Figures 2 and 3).

Table 2. Mean (± SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) and eight healthy dogs treated with GHRP-6 and ghrelin.

	GHRP-6		Ghrelin	
	PDH	Healthy	PDH	Healthy
GH (µg/l)	0.8 ± 0.2 **a**	2.7 ± 1.0 **a**	1.5 ± 0.7	1.8 ± 0.5
ACTH (ng/l)	120 ± 30 **a**	30 ± 6 **a**	114 ± 24 **a**	25 ± 5 **a**
Cortisol (nmol/l)	246 ± 53 **a**	80 ± 24 **a**	174 ± 26 **a**	64 ± 19 **a**
TSH (µg/l)	0.21 ± 0.06	0.24 ± 0.06	0.24 ± 0.05	0.26 ± 0.07
LH (µg/l)	6.3 ± 2.6	5.5 ± 1.5	7.3 ± 2.7	3.8 ± 1.2
PRL (µg/l)	3.6 ± 0.9	2.5 ± 0.5	4.1 ± 1.3	3.4 ± 0.9

a: significant difference in hormone concentrations between dogs with PDH and healthy dogs

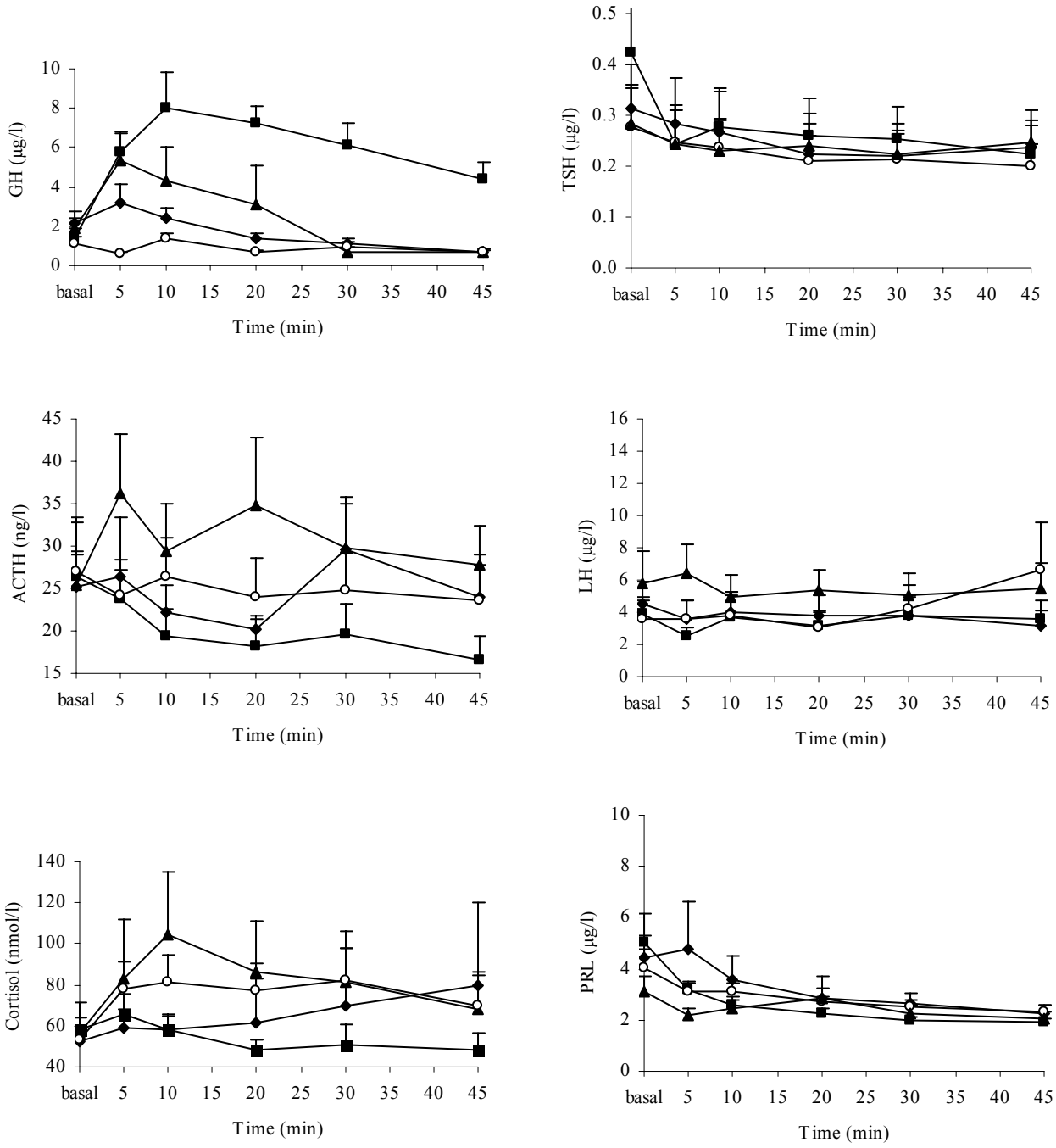

Figure 1. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after intravenous administration of ghrelin (♦), GHRP-6 (▲), GHRH (■), or NaCl 0.9 % (○) in eight healthy dogs.

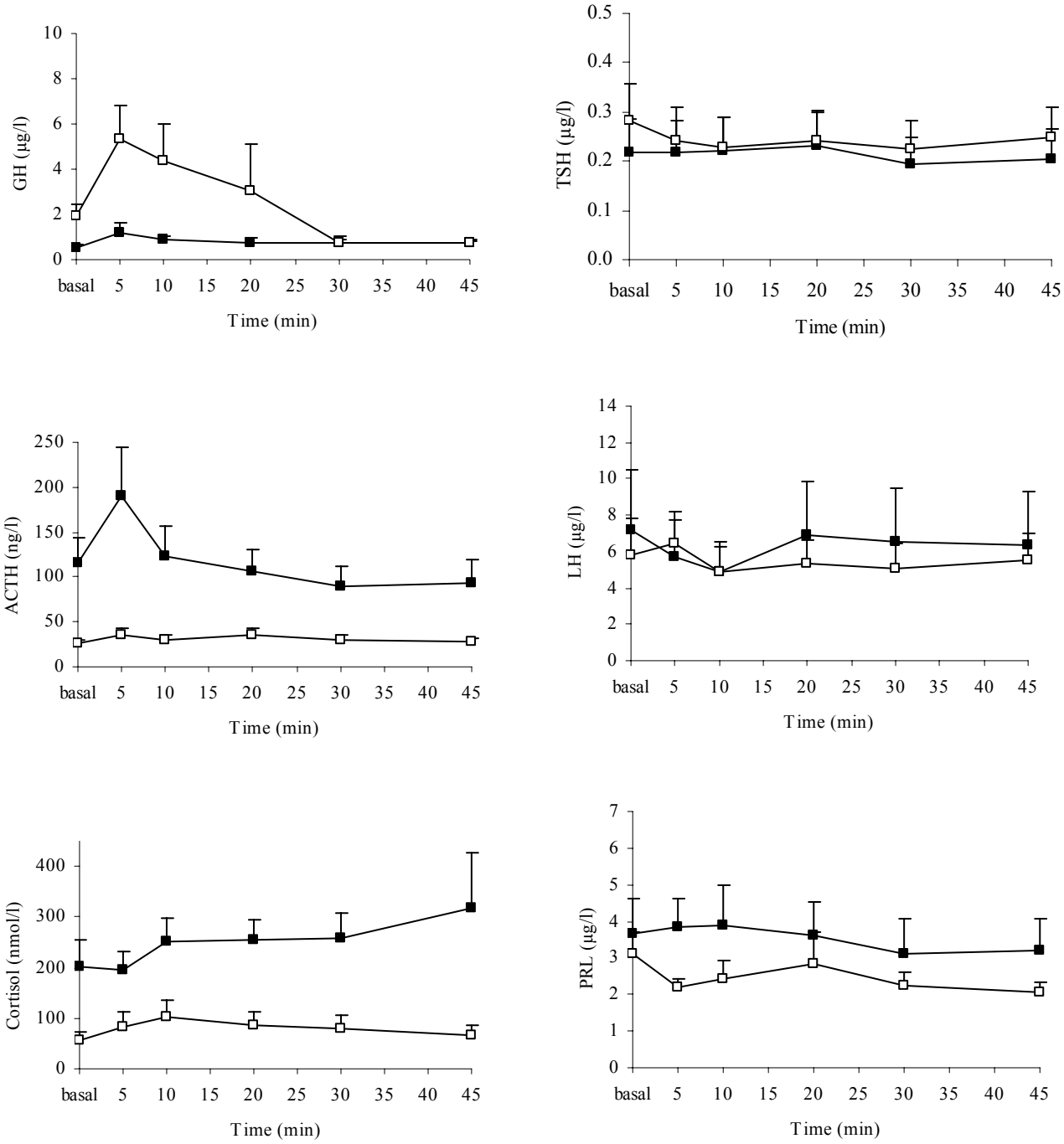

Figure 2. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after administration of GHRP-6 in eight healthy dogs (□) and in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) (■).

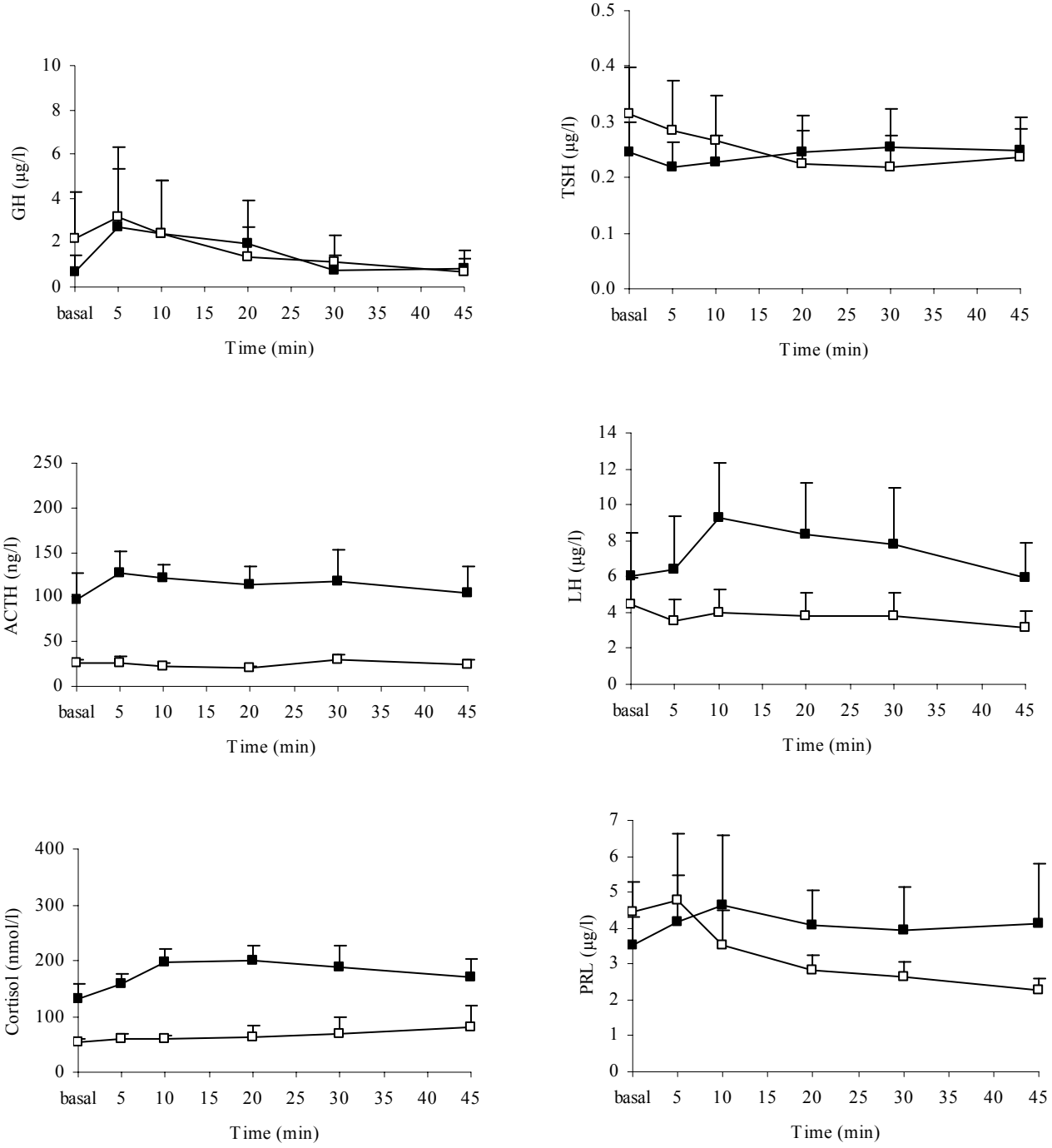

Figure 3. Mean (+ SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL after intravenous administration of ghrelin in eight healthy dogs (□) and in seven dogs with pituitary-dependent hyperadrenocorticism (PDH) (■).

Discussion

The results of this study demonstrate that both the natural and a synthetic ligand of the GHS-R (ghrelin and GHRP-6, respectively) stimulate GH release in healthy dogs. The results also underline the existence of intriguing species differences with regard to the GH-releasing potency of GHSs. In rats, the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999), whereas in humans, ghrelin is a more potent stimulus of GH secretion than GHRH or the synthetic GHS hexarelin (Arvat et al., 2001). The results of the present study indicate that, compared with the GH-releasing effect of GHRH, ghrelin and GHRP-6 are not very potent stimulators of GH release in elderly dogs.

The GH response to stimulation with GHRH in the healthy dogs was considerably lower than that reported previously in healthy Beagle dogs (Meij et al., 1996). This may at least partly be due to the effects of ageing, because the healthy Beagle dogs in the study of Meij et al. (1996) had a median age of two years. It has been reported that GH secretion decreases in humans with increasing age (Finkelstein et al., 1972; Zadik et al., 1985; Wilshire et al., 1995) and in aged rats (Sonntag et al., 1980; De Gennaro Colonna et al., 1994). The decreased GH secretion in elderly humans has been ascribed to a blunted response of GH to GHRH (Shetty and Duthie, 1995) and feedback disruption of the regulatory GH-insulin-like growth factor-I system (Veldhuis, 1997). The decreased GH secretion in elderly rats is associated with a decreased pituitary GH content (Sonntag et al., 1980), reduced pituitary GH mRNA (Takahashi et al., 1992), and reduction of hypothalamic GHRH mRNA levels (De Gennaro Colonna et al., 1994). Also in the dog there are indications that ageing is associated with an impaired GH secretion. Cella et al. (1995) have demonstrated that the GH responsiveness to hexarelin, a synthetic GHS, decreases with ageing in the dog. This was previously shown for GH-releasing peptides in humans (Bowers et al., 1992) and rats (Walker et al., 1990). Data for aged dogs reinforce the idea that the pituitary mechanisms subserving GH secretion are, at least partially, preserved during ageing. Thus, the reduced GH secretion is mainly due to inadequate hypothalamic stimulation rather than a primary pituitary hypofunction (Franchimont et al., 1989; Walker et al., 1991; Cella et al., 1993). Because of the effects of ageing on GH secretion, in the present study dogs with PDH were compared with healthy dogs of comparable age.

Chronic hypercortisolism is associated with impaired somatic growth, reduced GH release (Hartog et al., 1964), and a blocked GH response to various GH stimuli (Casanueva, 1992). Even a combination of GHRH and GHRP-6, which is a very powerful GH-releasing

stimulus, is unable to induce significant GH release in humans with Cushing's syndrome (Leal-Cerro et al., 1994). The results of the present study demonstrate that administration of GHRP-6 also results in a blunted GH response in dogs with PDH. The GH response after administration of ghrelin to dogs with PDH and healthy dogs was low and not significantly different. The mechanism by which chronic glucocorticoid excess inhibits GH secretion is not yet fully understood. Several hypotheses have been proposed, the most important of which are GHRH hyposecretion, enhancement of hypothalamic SS release, or a combination of these (Leal-Cerro et al., 1998). In addition to the effect at the hypothalamic level, chronic glucocorticoid excess may also influence GH secretion by acting directly at the pituitary level (Leal-Cerro et al., 1994).

Polyphagia, polyuria, polydipsia, thin coat, alopecia, and muscular atrophy are the classical clinical manifestations of PDH in dogs (Rijnberk et al., 1968). Another cardinal physical feature is centripetal obesity with abdominal enlargement. As not only chronic hypercortisolism but also obesity is associated with an impaired GH response to GH-releasing stimuli (Bowers, 1993), it can be hypothesized that the suppressed GH release in Cushing's syndrome is related to obesity as well. Indeed, Leal-Cerro et al. (1998) demonstrated that in humans with Cushing's disease the hyporesponsiveness of somatotrophs to GHRH is improved after a short-term (3 days) hypocaloric diet. Indeed, GH secretion is increased after fasting in healthy humans (Ho et al., 1988) and healthy dogs (Arce et al., 1991), indicating that caloric restriction exerts an important stimulatory effect on GH secretion. This may be explained by two concurrently operating mechanisms, an increase in hypothalamic GHRH and a decrease in hypothalamic somatostatin, leading to increased plasma GH levels (Ho et al., 1988). However, in contrast to the situation of chronic hypercortisolism (Leal-Cerro et al., 1994), intravenous administration of the combination of GHRH and GHRP-6 resulted in an elevated GH response in obese humans (Bowers, 1993). This indicates that the impaired GH response in individuals with Cushing's syndrome cannot be explained solely by obesity.

Administration of ghrelin and GHRP-6 did not cause a stimulation of the pituitary-adrenocortical axis and did not stimulate TSH, LH, and PRL release in either the healthy dogs or the dogs with PDH. These results are in line with observations for anaesthetized rats reported by Kojima et al. (1999), who found that intravenous administration of ghrelin specifically stimulated GH release but did not affect other adenohypophyseal hormones. In contrast, Arvat et al. (2001) demonstrated that intravenous administration of ghrelin, apart from stimulating GH release, also increased circulating levels of PRL, ACTH, and cortisol in

healthy humans. So far, there is no explanation for this discrepancy other than species differences.

In conclusion, the results of this study demonstrate that, in comparison with GHRH, GHRP-6 and ghrelin have a low GH-releasing potency in healthy dogs. In dogs with PDH, the GH response to GHRP-6 is impaired. Neither GHRP-6 nor ghrelin activates the pituitary-adrenocortical axis or stimulates TSH, LH, and PRL release in healthy elderly dogs and dogs with PDH.

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Chapter 5

Effects of growth hormone secretagogues on the release of adenohypophyseal hormones in young and old healthy dogs

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Abstract

The effects of three growth hormone secretagogues (GHSs), ghrelin, growth hormone-releasing peptide-6 (GHRP-6), and growth hormone-releasing hormone (GHRH), on the release of adenohypophyseal hormones, growth hormone (GH), adrenocorticotrophic hormone (ACTH), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), prolactin (PRL) and on cortisol were investigated in young and old healthy Beagle dogs.

Ghrelin proved to be the most potent GHS in young dogs, whereas in old dogs, GHRH administration was associated with the highest plasma GH concentrations. The mean plasma GH response after administration of ghrelin was significantly lower in the old dogs compared with the young dogs. The mean plasma GH concentration after GHRH and GHRP-6 administration was lower in the old dogs compared with the young dogs, but this difference did not reach statistical significance. In both age groups, the GHSs were specific for GH release as they did not cause significant elevations in the plasma concentrations of ACTH, cortisol, TSH, LH, and PRL.

It is concluded that in young dogs, ghrelin is a more powerful stimulator of GH release than either GHRH or GHRP-6. Ageing is associated with a decrease in GH-releasing capacity of ghrelin, whereas this decline is considerably lower for GHRH or GHRP-6.

Introduction

In 1977, Bowers and co-workers reported the growth hormone (GH)-releasing properties of enkephalin-derived peptides (Bowers et al., 1977). Among these synthetic peptides, GH-releasing peptide-6 (GHRP-6) proved to be a potent releaser of GH, both *in vitro* and *in vivo*, in several species (Bowers et al., 1984; Casanueva and Dieguez, 1999). After the synthesis of GHRP-6, new peptydil (e.g. hexarelin) and non-peptydil (e.g. MK-0677) GH secretagogues (GHSs) with a higher bioavailability and a longer life span were produced (Ghigo et al., 1994; Patchett et al., 1995). These synthetic GHSs have a potent GH-releasing activity in humans, mice, rats (Bowers et al., 1977; Bowers et al., 1984; Ghigo et al., 1994; Casanueva and Dieguez, 1999), swine, goats, and cows (Hayashida et al., 2001).

In humans, nearly all synthetic GHSs are more powerful than growth hormone-releasing hormone (GHRH) in terms of GH release (Casanueva and Dieguez, 1999). Studies in dogs have shown that orally (e.g. MK-0677, capromorelin) and intravenously (e.g. hexarelin) administered GHSs are effective GH releasers as well (Cella et al., 1995; Jacks et al., 1996; Rigamonti et al., 1999; Carpino et al., 2003). However, the action of the synthetic GHSs is not always confined to the promotion of GH release (Smith et al., 1997; Casanueva and Dieguez, 1999; Lamberts, 1999). For instance, in man, synthetic GHSs such as GHRP-6 stimulate the secretion of PRL, ACTH, and cortisol as well (Massoud et al., 1996; Smith et al., 1997; Casanueva and Dieguez, 1999; Lamberts, 1999; Arvat et al., 2001). Additionally in rats, GHRP-6 activates the pituitary-adrenocortical axis (Thomas et al., 1997).

GHSs stimulate GH release from the pituitary somatotrophs by acting on receptors different from those for GHRH (Momany et al., 1981). In the mid-nineties, the GHS-receptor (GHS-R), a G-protein-coupled seven-transmembrane receptor was first detected in the anterior pituitary and hypothalamus of rats and humans (Pong et al., 1996). In 1999, Kojima et al. purified and characterized the endogenous ligand for the GHS-R in rats and humans. The 28-amino-acid peptide with an *n*-octanoyl modification at its third Serine residue was called ‘ghrelin’. Surprisingly, its expression was found to be much higher in the stomach than in any other tissue. Tomasetto and coworkers (2001) identified cDNA encoding ghrelin from the fundus of the canine stomach as well, and found that it is highly conserved with man, mouse and rat. Thus, structural heterogeneity of ghrelin among species seems minor, and we could probably expect a rather functional homogeneity in various mammalian species. Ghrelin’s expression is restricted to the X/A- like cells, or Ghr-cells, of the oxyntic gland (Date et al., 2000; Rindi et al., 2002).

The GH-releasing activity of ghrelin is more marked in humans than in animals (Smith et al., 1997; Kojima et al., 1999; Seoane et al., 2000; Takaya et al., 2000). In humans, the GH response to ghrelin is considerably greater than that observed following administration of GHRH or synthetic GHSs (Seoane et al., 2000; Takaya et al., 2000; Arvat et al., 2001; Bowers, 2001), whereas in rats, the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999). In both isolated pituitary cells and intact (anaesthetized) rats, ghrelin solely stimulates GH release and does not affect the secretion of other adenohypophyseal hormones (Kojima et al., 1999). However, in humans, ghrelin significantly increases circulating concentrations of adrenocorticotrophic hormone (ACTH), cortisol, and prolactin (PRL), without affecting the release of luteinizing hormone (LH), follicle-stimulating hormone (FSH) or thyroid-stimulating hormone (TSH) (Massoud et al., 1996; Arvat et al., 2001).

The ageing process of organisms may be regarded as a progressive fall in bodily functions associated with a diminished ability to maintain homeostasis (Everitt and Meites, 1989). Both basal and stimulated GH secretion and circulating insulin-like growth factor-I (IGF-I) concentrations decline with age in several mammalian species (Finkelstein et al., 1972; Rudman, 1985; Zadik et al., 1985; Corpas et al., 1992; Wilshire et al., 1995; Muller et al., 2002). Also in dogs, the GH responsiveness to GH stimuli, such as GHRH and hexarelin, decreases with ageing (Cella et al., 1989; Cella et al., 1995). Until now, the effect of ageing on the GH responsiveness to ghrelin has not been studied in dogs. Preliminary results on the effects of GHSs on GH, ACTH, and cortisol concentrations in old dogs have been reported earlier (Bhatti et al., 2002).

The aim of this study was to compare the effects of ghrelin, GHRP-6, and GHRH on the release of GH, ACTH, cortisol, TSH, LH, and PRL in both young and old healthy dogs.

Materials and methods

Dogs

Four young female and four young male Beagle dogs aged between 13-17 months (median 15 months) and four old female and four old male Beagle dogs aged between 7-12 years (median 10 years) were used. The mean body weight of the young dogs (11.8 kg) was significantly lower ($P = 0.01$, Mann-Whitney test) than that of the old dogs (17.9 kg). The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples. They were housed in pairs in indoor-outdoor runs, had free access to tap water

and were fed on a commercial dog food. They were healthy and had no history of illnesses or treatments. All studies were carried out in conscious animals after an overnight fast. The bitches were in anoestrus during the study.

Study design and blood sample collection

Two 4x4 cross-over studies (young and old dogs, respectively) were conducted at different times. Each dog received four treatments sequentially on four different days (day 1 to 4) with a washout period of at least four days in between. The dogs were two by two randomly assigned to one of the four treatment sequences. The four treatments consisted of an intravenous injection of either human ghrelin (MW 3370.9) in a dose of 2 µg/kg body weight (Peninsula Laboratories Inc), GHRP-6 [(His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)^3] (MW 872.4) in a dose of 2 µg/kg body weight, human GHRH (MW 5036.6) in a dose of 2 µg/kg body weight (hGHRF; Peninsula Laboratories Inc), or NaCl 0.9 % (control).

Blood samples for the determination of plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL were collected by jugular venipuncture at 15 min before and 0, 5, 10, 20, 30, and 45 min after intravenous administration of the treatments and immediately transferred to ice-chilled EDTA-coated tubes (GH, ACTH, cortisol, LH, and PRL) or heparin-coated tubes (TSH). Samples were centrifuged at 4^o C for 10 min. Plasma was stored at -25^o C until assayed.

Hormone determination

Plasma GH concentrations were determined with a homologous radioimmunoassay (RIA) (Eigenmann and Eigenmann, 1981). The intra- and interassay coefficients of variation were 3.8 % and 7.2 %, respectively, and the sensitivity of the assay was 0.3 µg/l. The degree of cross-reaction with canine PRL was 2 %.

Plasma ACTH concentrations were determined with an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The inter-assay coefficient of variation was 7.8 % and the sensitivity was 0.2 pmol/l.

Plasma cortisol concentrations were determined with a commercially available RIA (Diagnostic Products Corporation), validated for the dog. The intra- and interassay coefficients of variation ranged from 3.0 to 5.1 % and from 4.0 to 6.4 %, respectively. The sensitivity of the assay was 5.5 nmol/l.

Plasma TSH concentrations were determined with a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite canine TSH, Diagnostic Products Corporation [DPC]) according to the instructions of the manufacturer. The intra-assay coefficients of variation were 5.0 %, 4.0 %, and 3.8 % at TSH levels of 0.20, 0.50, and 2.60 µg/l, respectively. The interassay coefficients of variation were 6.3 % and 8.2 % at TSH levels of 0.16 and 2.80 µg/l, respectively. The sensitivity of the assay was 0.03 µg/l. Cross-reactivity with FSH and LH was negligible.

Plasma LH concentrations were determined with a heterologous RIA as described previously by Nett et al. (1975). A rabbit antiserum raised against ovine LH (CSU-204; kindly supplied by G.D. Niswender, Colorado State University), radioiodinated NIAMDD-bLH-4, and canine pituitary standard LER 1985-1 (a gift from Dr. L.E. Reichert, Albany Medical College, NY) were used in this assay. The intra- and interassay coefficients of variation for values higher than 0.5 µg/l were 2.3 % and 10.5 % respectively. The sensitivity of the assay was 0.3 µg/l.

Plasma concentrations of PRL were determined with a previously validated heterologous RIA (Okkens et al., 1985). The intra- and interassay coefficients of variation were 3.5 % and 11.5 %, respectively. The sensitivity of the assay was 0.8 µg/l.

Statistical analysis

1. Cross-over studies

Mixed models with dog as random effect were fitted to study the association between treatment (ghrelin, GHRP-6, GHRH, and NaCl 0.9 %) and the plasma hormone concentrations (GH, ACTH, cortisol, TSH, LH, and PRL) in the young and the old dogs. Treatment, day of treatment (day 1-4), and time (repeated measures of adenohypophyseal hormones and cortisol starting with the basal concentration) were included as fixed effects. The four treatments were compared pair wise adjusting for multiple comparisons (Tukey's correction). In addition, difference between the plasma hormone concentration (GH, ACTH, cortisol, TSH, LH, and PRL) just before (0 min) injection of the GHSs or NaCl 0.9 % and the maximal plasma hormone concentration after injection was calculated (referred to as the maximal increment) and used to capture the effect of treatment. Therefore, a mixed model was fitted to the maximal increments as response variables with dog as random effect and day of treatment and treatment as fixed effects. The four treatments were compared pair wise (Tukey's correction).

2) Comparison between young and old dogs

The basal plasma hormone concentrations (mean of -15 and 0 min, collected in the cross-over studies) of the young and the old dogs were compared using an independent samples t-test to study differences between age groups before treatment. Additionally, if the analyses based on the cross-over studies indicated a significant treatment effect in one or both age groups, a repeated measures analysis was performed in order to compare the respective hormone response between old and young dogs. Therefore, models were fitted including dog as random effect, and age (young versus old dogs), time (-15, 0, 5, 10, 20, 30, and 45 min), and the interaction between age and time as categorical fixed effects. A first-order autoregressive covariance structure was used to model the correlation in the repeated measures of the response variables.

Statistical significance was defined at $P \leq 0.05$. Values are expressed as mean concentrations of all hormone measurements ± SEM. Analyses were performed with SAS version 8.02 (SAS Institute Inc) and S-Plus version 6.1 for Windows (Insightful Corp).

Ethics of the study

This study was approved by the Ethical Committees of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands, and the Faculty of Veterinary Medicine, Ghent University, Belgium.

Results

No side effects were observed during or after administration of the GH-releasing agents.

The mean plasma GH concentration in the young dogs when treated with ghrelin was 15.0 ± 5.1 µg/l, which was significantly higher than the concentration in the dogs when treated with GHRP-6 (2.7 ± 1.2 µg/l) or NaCl 0.9 % (1.8 ± 1.1 µg/l). The mean plasma GH concentration in the young dogs when treated with ghrelin did not differ significantly from this concentration in dogs when treated with GHRH (7.5 ± 3.8 µg/l). The mean GH concentration in the young dogs when treated with GHRH did not differ significantly from that in dogs when treated with GHRP-6 (Figure 1a). Analysis of the maximal increment indicated that the mean plasma GH response was significantly higher after treatment with ghrelin when compared with this response after GHRP-6 ($P = 0.01$) or NaCl 0.9 % ($P =$

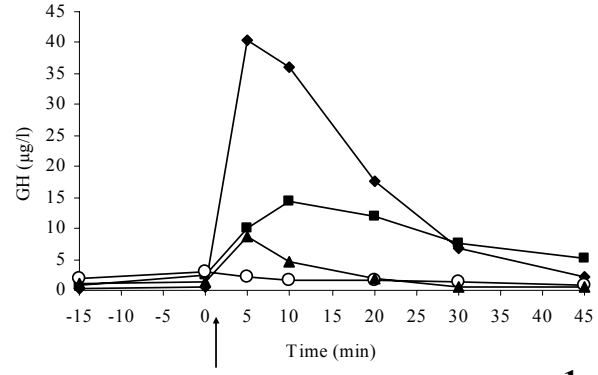
0.007) injection, and tended to be higher than the response induced by administration of GHRH (P = 0.06) (Figure 2a).

The mean plasma ACTH concentration in the young dogs when treated with ghrelin, GHRP-6, GHRH, and NaCl 0.9 % was 68 ± 22 ng/l, 61 ± 17 ng/l, 33 ± 7 ng/l, and 38 ± 9 ng/l, respectively. Although plasma ACTH concentrations were higher when the dogs were injected with ghrelin or GHRP-6, the concentrations were not significantly different from these measured when GHRH or NaCl 0.9 % were administered (Figure 1b). A treatment effect could not be demonstrated based on comparison of the maximal increments (Figure 2b).

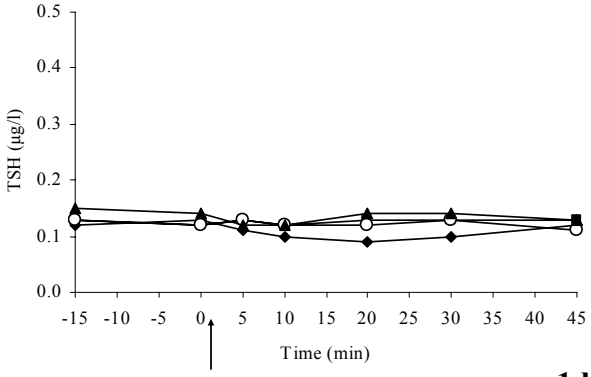
The mean plasma cortisol concentration in the young dogs when treated with ghrelin, GHRP-6, and GHRH was 102 ± 23 nmol/l, 109 ± 22 nmol/l, and 87 ± 21 nmol/l, respectively. These levels were not significantly different from that obtained when NaCl 0.9 % was administered (84 ± 21 nmol/l) (Figure 1c). No treatment effect was present based on comparison of the maximal increments (Figure 2c).

In the young dogs, the mean plasma TSH, LH, and PRL concentrations did not differ significantly when treated with the GH-releasing agents or NaCl 0.9 % (Figures 1d-f, respectively), and no treatment effects were present based on comparison of the maximal increments (Figures 2d-f, respectively).

The mean plasma GH concentration in the old dogs when treated with ghrelin, GHRP-6, and GHRH was 1.8 ± 0.5 µg/l, 2.7 ± 1.0 µg/l, and 5.5 ± 1.0 µg/l, respectively, which was significantly higher than the concentration in the dogs when treated with NaCl 0.9 % (0.9 ± 0.2 µg/l). The mean plasma GH concentration in dogs when treated with GHRH was significantly higher than in dogs when treated with ghrelin or GHRP-6 (Figure 3a). Moreover, analysis of the maximal increment indicated that the plasma GH response was significantly higher after treatment with GHRH than the response after administration of ghrelin or NaCl 0.9 % (Figure 4a).



1a



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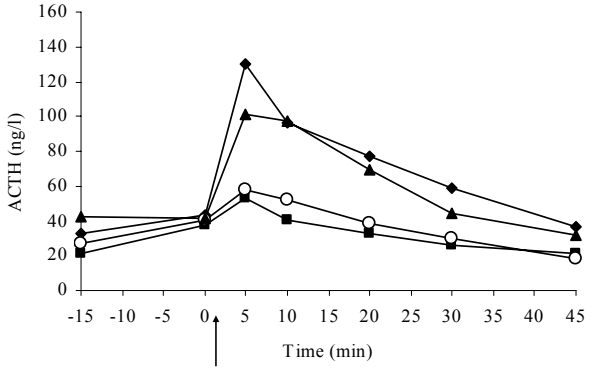
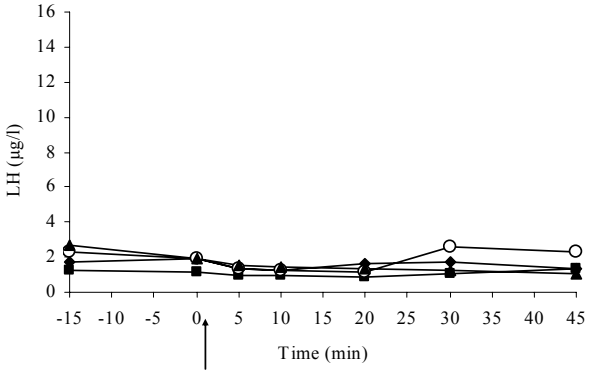


1b



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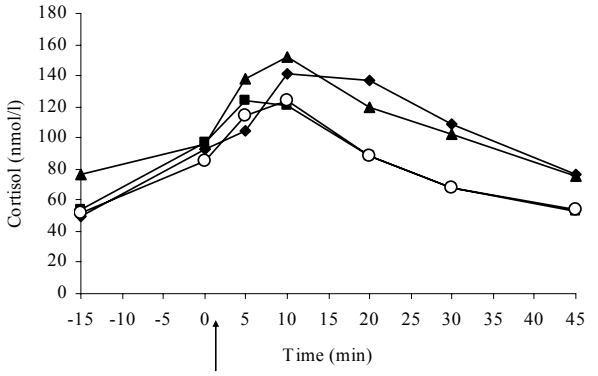

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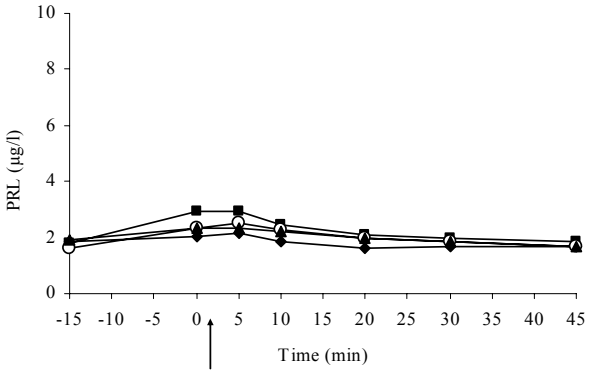
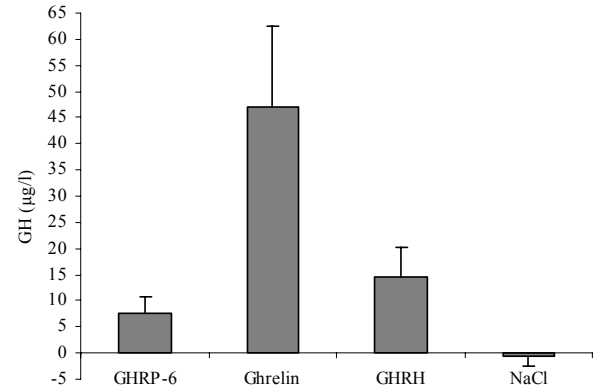
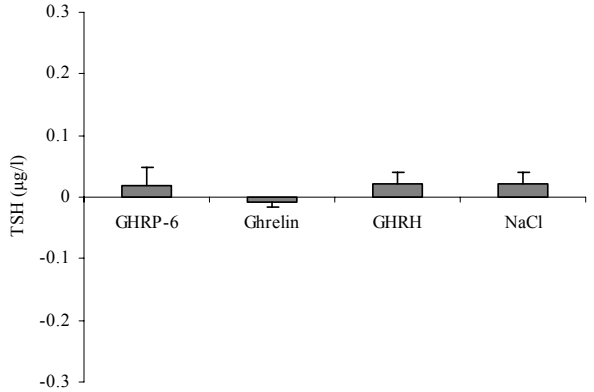


1f

Figure 1. (a-f) Mean plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL before and after intravenous administration of ghrelin (♦), GHRP-6 (▲), GHRH (■), or NaCl 0.9 % (○) in eight healthy young dogs. The arrows indicate the intravenous administration of the treatment.



2a



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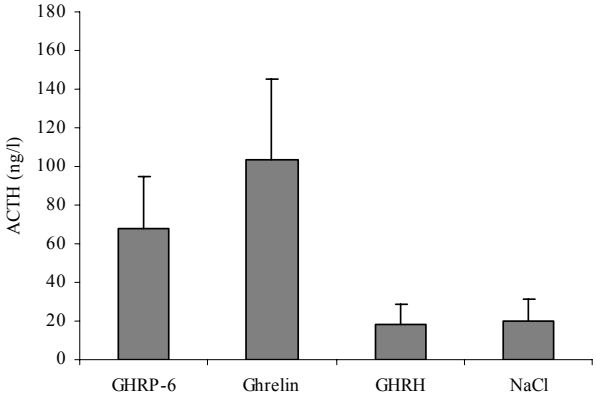

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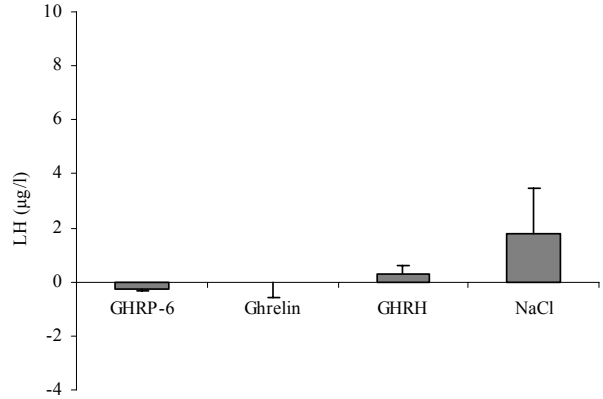

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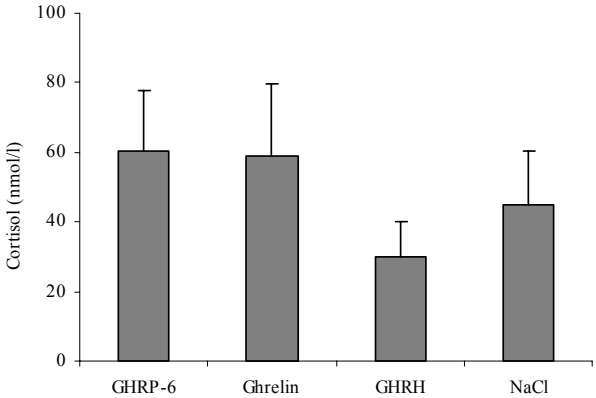

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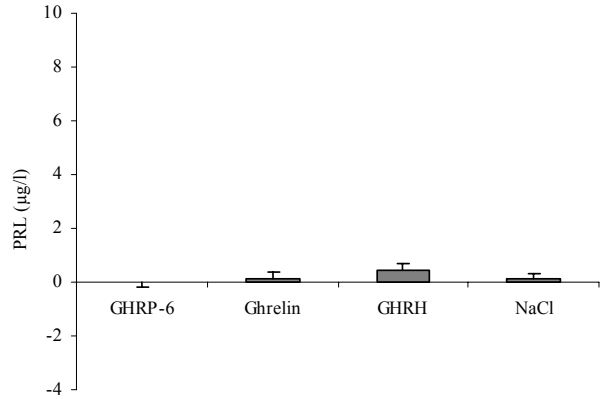


2f

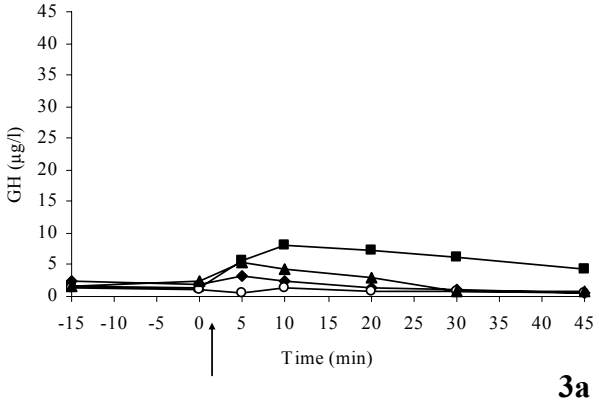
Figure 2. (a-f) The mean (+SEM) difference in plasma concentrations of GH, ACTH, cortisol, TSH, LH and PRL between 0 min and the maximal increment after administration of the GHSs and NaCl 0.9 % in eight healthy young dogs.

The mean plasma ACTH concentration in the old dogs when treated with ghrelin (25 ± 5 ng/l), GHRP-6 (30 ± 6 ng/l), and GHRH (21 ± 4 ng/l) was not significantly different from that in the dogs when treated with NaCl 0.9 % (25 ± 5 ng/l) (Figure 3b). Also, the mean plasma cortisol concentration in the old dogs when treated with ghrelin (64 ± 19 nmol/l), GHRP-6 (80 ± 24 nmol/l), or GHRH (55 ± 8 nmol/l) did not differ significantly from that in the dogs when treated with NaCl 0.9 % (73 ± 13 nmol/l) (Figure 3c). Additionally, no treatment effects were present based on comparison of the maximal increments (Figures 4b and 4c, respectively).

In the old dogs, the mean plasma TSH, LH, and PRL concentrations did not differ significantly when treated with the GH-releasing agents or NaCl 0.9 % (Figures 3d-f, respectively), and no treatment effects were present based on comparison of the maximal increments (Figures 4d-f, respectively).

The mean basal plasma GH concentration in the young dogs (1.4 ± 0.5 µg/l) did not differ significantly from that in the old dogs (1.6 ± 0.3 µg/l). The mean basal plasma concentrations of ACTH and cortisol were significantly lower in the old dogs (26 ± 3 ng/l and 55 ± 4 nmol/l, respectively) than in the young dogs (36 ± 4 ng/l and 76 ± 7 nmol/l, respectively). The mean basal plasma concentrations of TSH, LH, and PRL were significantly higher in the old dogs (0.32 ± 0.05 µg/l, 4.4 ± 0.7 µg/l, 4.2 ± 0.4 µg/l, respectively) than in the young dogs (0.13 ± 0.01 µg/l, 1.9 ± 0.2 µg/l and 2.1 ± 0.2 µg/l, respectively).

The mean plasma GH response after treatment with ghrelin, GHRP-6, and GHRH, respectively, was compared between young and old dogs. The mean plasma GH response after administration of ghrelin was significantly lower in the old dogs compared with the young dogs. In addition, this plasma GH response evolved significantly different over time between both groups (Figure 1a versus Figure 3a). The mean plasma GH response after treatment with GHRP-6 or GHRH was lower in the old dogs when compared with the young dogs, but this was not statistically significant. Additionally, this plasma GH response did not evolve significantly different over time when both groups were compared (Figure 1a versus Figure 3a).



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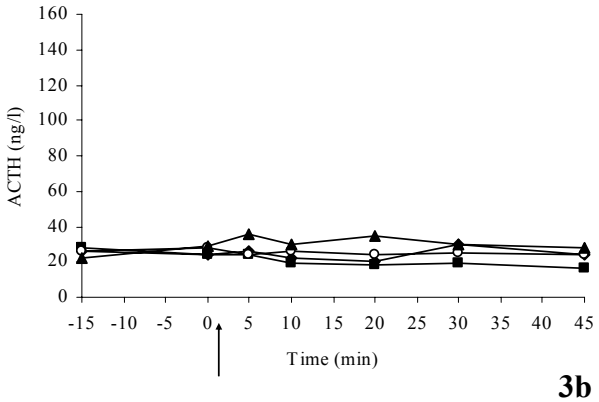

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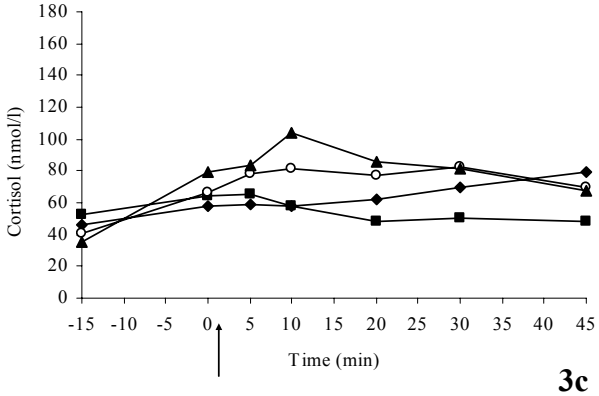

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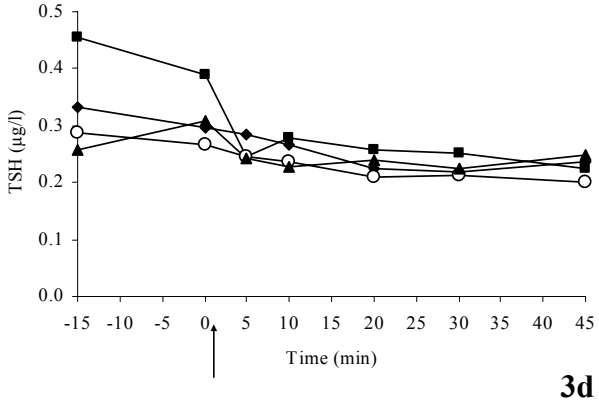

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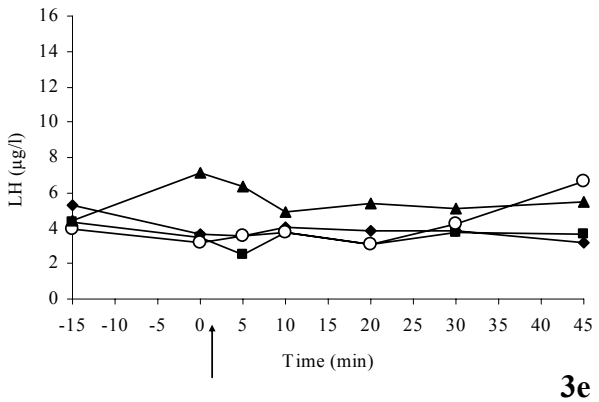

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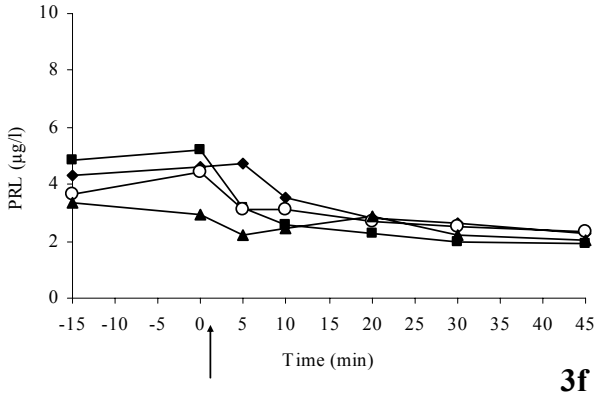
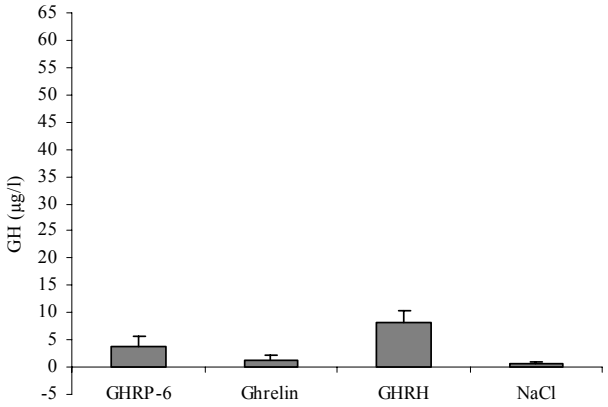


3f

Figure 3. (a-f) Mean plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL before and after intravenous administration of ghrelin (♦), GHRP-6 (▲), GHRH (■), or NaCl 0.9 % (○) in eight healthy old dogs. The arrows indicate the intravenous administration of the treatment.



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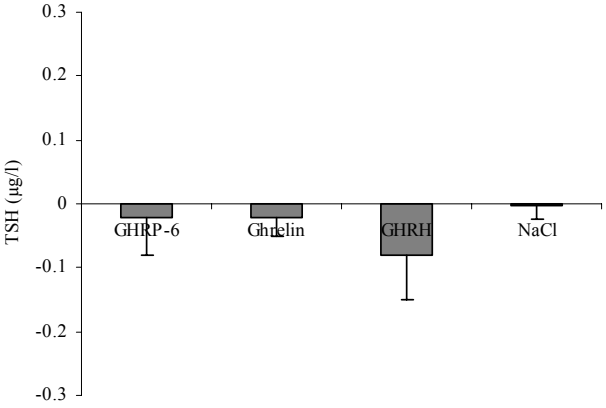

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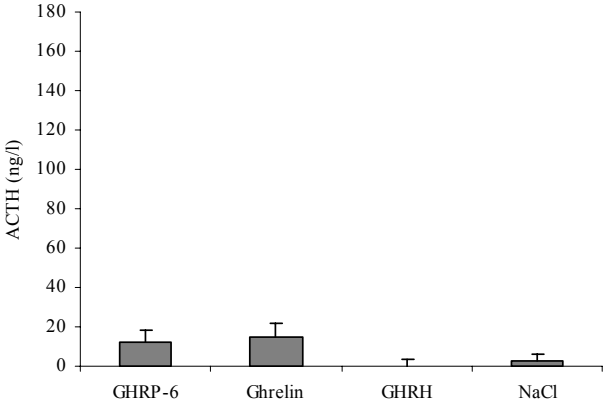

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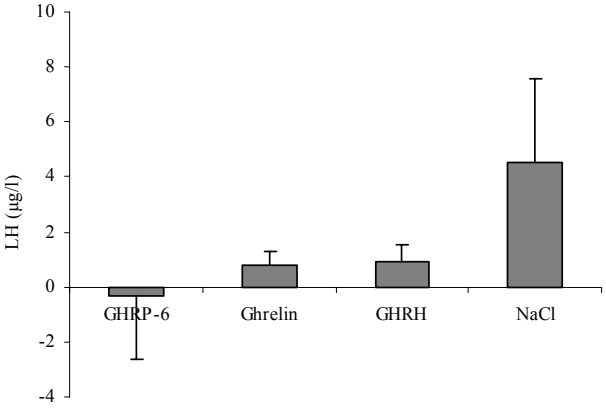

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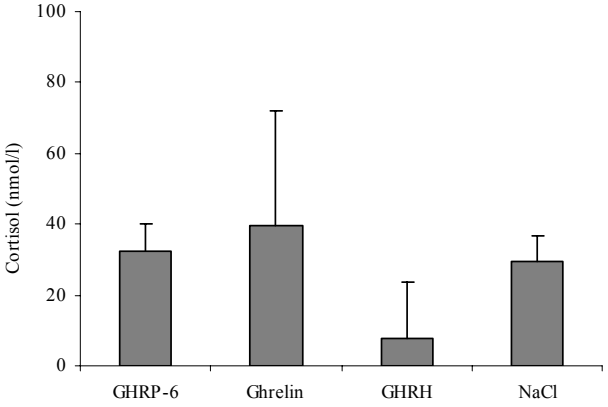

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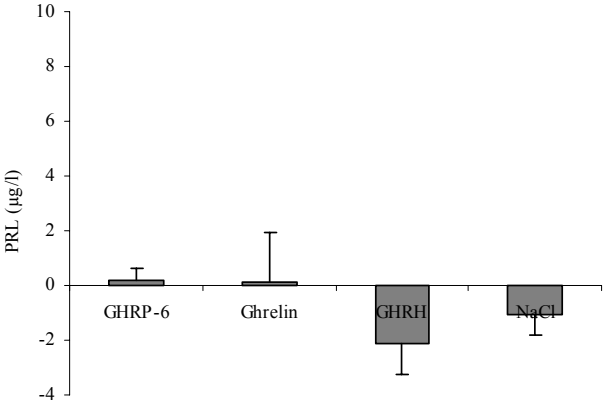

4f

Figure 4. (a-f) The mean (+SEM) difference in plasma concentrations of GH, ACTH, cortisol, TSH, LH and PRL between 0 min and the maximal increment after administration of the GHSs and NaCl 0.9 % in eight healthy old dogs.

Discussion

The results of the present study demonstrate that the natural ligand of the GHS-R, ghrelin, causes a significant rise in plasma GH levels in healthy young and old dogs. In the young dogs, ghrelin is a potent releaser of GH when compared with the other stimulants used in our study. However, in the old dogs, GHRH elicited higher plasma GH levels than ghrelin, GHRP-6, or NaCl 0.9 % administration. With regard to the GH-releasing potency of GHSs, these findings further substantiate the existence of remarkable species-related differences. In rats, the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999), whereas in humans ghrelin is a more potent stimulus of GH secretion than GHRH or the synthetic GHS hexarelin (Takaya et al., 2000; Arvat et al., 2001).

Our findings also indicate the existence of age-related differences in the GH-releasing potency of GHSs. The ghrelin-induced GH response was much lower at old age than at young age. In addition, the GHRP-6- and GHRH-induced release of GH was lower in the old versus the young dogs, although this difference was not statistically significant. These observations are compatible with findings in humans, showing that not only the GH-releasing effect of ghrelin (Broglio et al., 2003) but also that of GHRH and peptidyl or nonpeptidyl synthetic GHSs undergoes an age-related decrease (Bowers et al., 1992; Aloi et al., 1994; Chapman et al., 1996; Muccioli et al., 2002; Broglio et al., 2003). In old rats, the GH response to synthetic GHSs is impaired as well (Ceda et al., 1986; Walker et al., 1990). Also in old dogs, the GH responsiveness to the synthetic GHS hexarelin has been reported to be low (Cella et al., 1995).

The GH-releasing activity of ghrelin and synthetic GHSs depends on the functional integrity of the hypothalamus-pituitary unit (Muccioli et al., 2002). In humans, it has been demonstrated that the age-related reduction of both spontaneous and stimulated GH secretion reflects age-related changes in the neural control of somatotrope function (Giustina and Veldhuis; 1998; Ghigo et al., 1999). These changes include a concomitant reduction in the secretion of GHRH and enhancement in somatostatin release (Kelijman, 1991; Giustina and Veldhuis; 1998; Ghigo et al., 1999; Muller et al., 1999). It seems that an impairment of pituitary function does not play a major role (Muller et al., 1999). Indeed, repeated GHRH injections in elderly subjects, combined administration of GHRH and clonidine in old dogs, or GHRH + GHRP-6 injection in aged rats (Walker et al., 1991) significantly increases circulating GH levels (Cella et al., 1993; Nicolas et al., 1994). Additionally, the age-related decrease of the GH response to ghrelin and synthetic GHSs agrees with the well-known *in*

vitro hyporesponsiveness of the aged somatotroph cells to the majority of provocative stimuli, including GHRH, despite the availability of a remarkable GH-releasable pool (Giustina and Veldhuis, 1998; Ghigo et al., 1999; Muccioli et al., 2002)

These observations support the idea that the somatopause is driven primarily by the hypothalamus and that the pituitary somatotrophs retain their capacity to synthesize and secrete adequate levels of GH (Franchimont et al., 1989; Walker et al., 1990; Corpas et al., 1992; Cella et al., 1993; Muller et al., 1999; Muccioli et al., 2002).

A decline of GHS-Rs in the ageing brain (Arvat et al., 1998; Muccioli et al., 2002) could further explain the reduced GH response to ghrelin/GHSs in elderly humans. Based on the relative great age-related decline in the GH response to ghrelin compared with that to GHRH in dogs, it may be hypothesized that also in dogs GHS-R expression decreases considerably with age.

In the present study, the old dogs were significantly heavier than the young dogs. Obesity, a condition commonly observed in adulthood, is associated with an impaired GH response to GH-releasing stimuli (Daughaday and Rotwein, 1989; Bowers, 1993; Arvat et al., 1998). This may have played a contributing role in the age-associated decline of the GH response. A longitudinal study, in which the GHS-induced hormone responses in dogs in function of age are investigated over several years while maintaining a constant body weight in all dogs, could lead to more reliable conclusions.

Also with regard to the effects of GHSs on the release of adenohypophyseal hormones other than GH, there are interesting species-related differences. In this study, the action of ghrelin and GHRP-6 appeared to be GH-specific in dogs, i.e., the stimulants did not induce a significant rise in plasma concentrations of ACTH, cortisol, TSH, LH, and PRL in either the young dogs or the old dogs. The absence of a TSH and LH response to GHSs is compatible with the results of previous studies of the hormone-releasing effects of ghrelin in humans and rats (Arvat et al., 1997; Kojima et al., 1999). In contrast, the stimulatory effect of ghrelin and synthetic GHSs on PRL secretion varies with the species studied.

In humans, ghrelin and synthetic GHSs induce a significant release of PRL which is independent of both gender and age and probably results from direct stimulation of somatomammotroph cells (Renner et al., 1994; Arvat et al., 1997; Takaya et al., 2000; Muccioli et al., 2002). In dogs (Hickey et al., 1994) and rats (Kojima et al., 1999), synthetic GHSs do not stimulate PRL release. This species-related difference may be explained by different numbers of somatomammotrophs in various species (Raun et al., 1998), with humans having a high proportion of these cells (Frawley and Boockfor, 1991).

Furthermore, administration of the GHSs did not elicit a significant activation of the pituitary-adrenocortical axis in the dogs. In contrast, intravenous administration of ghrelin or synthetic GHSs, such as hexarelin, considerably increases circulating levels of ACTH and cortisol in healthy humans (Massoud et al., 1996; Arvat et al., 1997; Takaya et al., 2000). Kojima et al. (1999) reported that intravenously administered ghrelin specifically stimulates GH release in anaesthetized rats and in isolated rat pituitary cells, but does not affect the release of other adenohypophyseal hormones. However, Thomas et al. (1997) have shown that GHRP-6 mediates the release of ACTH and cortisol in conscious rats. The mechanism by which ghrelin and synthetic GHSs stimulate the pituitary-adrenocortical axis is still unknown, but seems to be mediated via the hypothalamus as it is lost after cutting the pituitary stalk (Loche et al., 1995). They may interact with hypothalamic peptides (e.g. corticotrophin-releasing hormone, arginine vasopressin, and neuropeptide Y) controlling ACTH release (Dickson and Luckman, 1997; Thomas et al., 1997; Korbonits et al., 1999; Broglio et al., 2003), most probably primarily via arginine vasopressin (Korbonits et al., 1999).

In swine, some recently developed selective GHSs, such as ipamorelin, induce massive GH secretion without any elevation in ACTH, cortisol, or PRL release (Raun et al., 1998), whereas GHRP-6 and GHRP-2 administration in this species cause a strong activation of the pituitary-adrenocortical axis. This suggests the existence of subtypes of GHS-Rs with differential effects on GH, ACTH, and PRL release. Furthermore, intravenous bolus administration of ghrelin or synthetic GHSs results in high blood levels and reflects a pharmacological rather than a physiological action of the peptides. It is possible that, at physiological concentrations, these GHSs do not increase ACTH, cortisol, or PRL concentrations (Svensson et al., 1998).

Most basal plasma hormone concentrations did differ significantly when the young and old dogs were compared. Although it is generally accepted that basal GH secretion decreases in humans and animals with increasing age (Finkelstein et al., 1972; Sonntag et al., 1980; Zadik et al., 1985; Borst et al., 1994; De Gennaro Colonna et al., 1994; Wilshire et al., 1995), the basal plasma GH concentration in our study was not significantly different in the young and the old dogs. For identifying age-related differences in GH secretion, determination of the pulsatile secretion pattern of GH is much more sensitive. Indeed, the pulsatile secretion pattern of GH is significantly lower in old humans, rats, and dogs than in young individuals (Zadik et al., 1985; Cella et al., 1989; Borst et al., 1994). The higher basal plasma concentrations of ACTH and cortisol in the young dogs may indicate that the stress of handling caused a stronger activation of the pituitary-adrenocortical axis in these young dogs

than in the old dogs. In some species, ageing is associated with a decrease in stress-induced activation of adrenocortical function (Van Eekelen et al., 1995).

In conclusion, the results of this study demonstrate the existence of age-related differences with regard to the GH-releasing activity of GHSs. Ghrelin is, compared to GHRH and GHRP-6, the most potent stimulator of GH release in young dogs. In old dogs, GHRH administration is associated with the highest elevations in plasma GH levels when compared to administration of GHRP-6 or ghrelin. The GH-releasing capacity of ghrelin decreases with age whereas this decline is considerably lower for GHRP-6 or GHRH. Furthermore, ghrelin and GHRP-6 are specific releasers of GH and do not stimulate the pituitary-adrenocortical axis or the release of TSH, LH, or PRL in dogs.

Acknowledgements

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Chapter 6

Ghrelin-stimulation test in the diagnosis of canine pituitary dwarfism

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Abstract

This study investigated whether ghrelin, a potent releaser of growth hormone (GH) secretion, is a valuable tool in the diagnosis of canine pituitary dwarfism. The effect of intravenous administration of ghrelin on the release of GH and other adenohypophyseal hormones was investigated in German shepherd dogs with congenital combined pituitary hormone deficiency and in healthy Beagles.

Analysis of the maximal increment (i.e. difference between pre- and maximal post-ghrelin plasma hormone concentration) indicated that the GH response was significantly lower in the dwarf dogs compared with the healthy dogs. In none of the pituitary dwarfs, the ghrelin-induced plasma GH concentration exceeded 5 µg/l at any time. However, this was also true for three healthy dogs. In all dogs, ghrelin administration did not affect the plasma concentrations of adrenocorticotrophic hormone (ACTH), cortisol, thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and prolactin (PRL) .

Thus, while a ghrelin-induced plasma GH concentration above 5 µg/l excludes GH deficiency, false-negative results may occur.

Introduction

The secretion of growth hormone (GH) is primarily under control of the central nervous system and is mainly regulated by the opposing effects of the hypothalamic hormones GH-releasing hormone (GHRH) and somatostatin (Plotsky and Vale, 1985). The existence of another, unknown factor involved in the control of somatotroph function has been hypothesized for several years. This was based on the observation that synthetic peptidyl and non-peptidyl compounds, named GH secretagogues (GHSs) or GH-releasing peptides (GHRPs), showed a strong GH-releasing effect (Bowers et al., 1977; Casanueva and Dieguez 1999) and acted on a specific GHS-receptor (GHS-R), i.e. the GHS-R type 1a (Howard et al., 1996; McKee et al., 1997). The endogenous ligand of the GHS-R type 1a has recently been purified from rat and human stomach (Kojima et al., 1999) and has also been identified in the fundus of the canine stomach (Tomasetto et al., 2001). This 28-amino-acid peptide, ghrelin, has a unique structure with an *n*-octanoyl ester at its third Serine residue which is crucial for its biological activity, at least in terms of GH release (Kojima et al., 1999; Bednarek et al., 2000, Date et al., 2000; Hayashida et al., 2001).

Ghrelin is a potent stimulator of GH release in several species such as humans (Arvat et al., 2000; Peino et al., 2000; Takaya et al., 2000), rats (Kojima et al., 1999) and dogs (Bhatti et al., 2002; Bhatti et al., 2006 in press). In rats, the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999), whereas in humans, ghrelin releases more GH than GHRH and the synthetic GHS hexarelin (Ghigo et al., 2001). In young dogs, ghrelin is a more potent stimulator of GH secretion than GHRH or GHRP-6 (Bhatti et al., 2006 in press). However, the action of ghrelin and the synthetic GHSs is not exclusively confined to GH release. For example, in humans these substances also stimulate the secretion of prolactin (PRL), adrenocorticotrophic hormone (ACTH), and cortisol (Massoud et al., 1996; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001). In rats, GHRP-6 activates the hypothalamic-pituitary-adrenal axis (Thomas et al., 1997). In contrast, ghrelin and GHRP-6 specifically release GH in young dogs (Bhatti et al., 2006 in press).

Pituitary dwarfism or congenital GH deficiency in dogs is encountered most often in German shepherd dogs. In this breed, pituitary dwarfism is due to a simple, autosomal recessive inherited abnormality (Andresen and Willeberg, 1976). In contrast to disproportionate dwarfism caused by for example congenital hypothyroidism, the condition of congenital GH deficiency is characterized by profound proportionate dwarfism with retention of puppy coat or secondary hairs and lack of primary or guard hairs, and symmetrical

alopecia. Intrapituitary cysts are often detected at a young age and gradually enlarge with time (Kooistra et al., 1998). Functionally, German shepherd dwarf dogs have a combined pituitary hormone deficiency. An absolute deficiency of GH, PRL and thyroid-stimulating hormone (TSH) is associated with an impaired release of gonadotrophins, whereas ACTH secretion is preserved (Hamann et al., 1999; Kooistra et al., 2000). The abnormality in these dwarfs is most likely caused by a mutation in a developmental transcription factor that precludes effective expansion of a pituitary stem cell after the differentiation of the corticotroph cells (Kooistra et al., 2000). To date, sequence analysis of genomic DNA from German shepherd dwarfs has not revealed causative mutations in candidate genes (Lantinga-van Leeuwen et al., 2000a; Lantinga-van Leeuwen et al., 2000b; van Oost et al., 2002).

As ghrelin is a more potent stimulator of GH release than GHRH in young dogs, it may be useful for diagnostic purposes. We therefore investigated the effects of intravenous administration of ghrelin on the plasma concentration of GH in German shepherd dogs with pituitary dwarfism. Additionally, the plasma concentrations of ACTH, cortisol, TSH, PRL, and luteinizing hormone (LH) before and after ghrelin administration were measured in these dogs.

Materials and methods

Dogs

Six German shepherd dwarfs from different litters (four males and two females) referred to the Department of Clinical Sciences of Companion Animals, Utrecht University, because of an abnormal hair coat and proportionate growth retardation were used in this study. The mean age at presentation was 12 months (range 9 to 21 months) and the mean body weight was 9.6 kg (range 7.5 to 14.0 kg).

The results of the pituitary dwarfs were compared with data available from a previously conducted study in eight Beagle dogs (four males and four females) with a mean age of 13 months (range 10 to 17 months) and a mean body weight of 11.8 kg (range 10.0 to 13.5 kg) (Bhatti et al., 2006 in press). The dogs had free access to tap water and were fed a commercial dog food. They had no history of illness and had not received any medications.

The mean age of the healthy dogs did not differ significantly ($P = 0.5$, independent samples t-test) from that in the pituitary dwarfs. The mean body weight of the healthy dogs was slightly higher ($P = 0.04$, independent samples t-test) than in the dwarfs.

All experiments were carried out in conscious animals after an overnight fast. In the female healthy dogs, blood samples were obtained in anoestrus.

Study design and blood sample collection

In all dogs, human ghrelin (MW 3370.9) (Peninsula Laboratories Inc. Belmont, CA, USA), was administered intravenously in a dose of 2 µg/kg body weight through an intravenous catheter. The peptide was dissolved according to the Peninsula laboratory requirements and was stored at -20° C. Ghrelin, in a concentration of 10 µg/ml, was thawed before administration. For each dog a fresh vial was applied. The mean volume to be injected was 1.9 ml for the pituitary dwarfs and 2.4 ml for the healthy dogs.

Blood samples for the determination of plasma concentrations of GH, ACTH, cortisol, TSH, LH and PRL were collected by jugular venipuncture at 15 min before and 0, 5, 10, 20, 30, and 45 min after ghrelin injection. Blood samples for the determination of plasma concentrations of insulin-like growth factor-1 (IGF-I) were collected by jugular venipuncture at -15 min and 0 min. The samples were immediately transferred to ice-chilled EDTA-coated tubes (GH, IGF-I, ACTH, cortisol, LH and PRL) or heparin-coated tubes (TSH) and centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determination

Plasma GH concentrations were determined with a homologous radioimmunoassay (RIA) (Eigenmann and Eigenmann, 1981). The intra- and interassay coefficients of variation were 3.8 % and 7.2 % respectively, and the sensitivity of the assay was 0.3 µg/l. The degree of cross-reaction with canine PRL was 2 %.

Plasma ACTH concentrations were determined with an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The interassay coefficient of variation was 7.8 % and the sensitivity was 0.2 pmol/l.

Plasma cortisol concentrations were determined with a commercially available RIA (Diagnostic Products Corporation (DPC), Los Angeles, CA, USA), validated for dogs. The intra-assay and inter-assay coefficients of variation ranged from 3.0 % to 5.1 % and from 4.0 % to 6.4 %, respectively. The sensitivity of the assay was 5.5 nmol/l.

Plasma TSH concentrations were determined with a homologous solid-phase, two-site chemiluminiscent enzyme immunometric assay (Immulite® canine TSH, DPC, Los Angeles, CA, USA) according to the instructions of the manufacturer. The intra-assay coefficients of variation were 5.0 %, 4.0 %, and 3.8 % at TSH levels of 0.20, 0.50, and 2.60 µg/l,

respectively. The interassay coefficients of variation were 6.3 % and 8.2 % at TSH levels of 0.16 and 2.80 µg/l, respectively. The sensitivity of the assay was 0.03 µg/l. Cross-reactivity with FSH and LH was negligible.

Plasma LH concentrations were determined with a heterologous RIA as described previously by Nett et al., (1975). A rabbit antiserum raised against ovine LH (CSU-204), radioiodinated NIAMDD-bLH-4, and canine pituitary standard LER 1985-1 were used in this assay. The intra- and interassay coefficients of variation for concentrations higher than 0.5 µg/l were 2.3 % and 10.5 %, respectively. The sensitivity of the assay was 0.3 µg/l.

Plasma concentrations of PRL were determined with a previously validated heterologous RIA (Okkens et al., 1985). The intra- and inter-assay coefficients of variation were 3.5 % and 11.5 %, respectively. The sensitivity of the assay was 0.8 µg/l.

Plasma IGF-I concentrations were determined with a heterologous RIA validated for the dog (Nap et al., 1993). The intra-assay and interassay coefficients of variation were 4.7 % and 15.6 %, respectively, and the sensitivity of the assay was 3 µg/l.

Statistical analysis

Basal plasma concentrations (mean of -15 and 0 min) of GH, IGF-I, ACTH, cortisol, TSH, LH and PRL in the dogs with pituitary dwarfism and the healthy dogs were compared using an independent samples t-test to study differences between both dog groups before treatment.

Additionally, the difference between the plasma hormone concentration just before (0 min) injection of ghrelin and the maximal plasma hormone concentration after injection was calculated for each dog (referred to as the maximal increment) and used to capture the effect of treatment. Therefore, an independent samples t-test was used to study the differences in maximal increments between both dog groups.

Statistical significance was defined at $P \leq 0.05$. Values are expressed as mean concentration ± SEM. Statistical analysis was performed with SPSS version 12.0 for windows (SPSS Inc., Chicago, Illinois, USA).

Ethics of the study

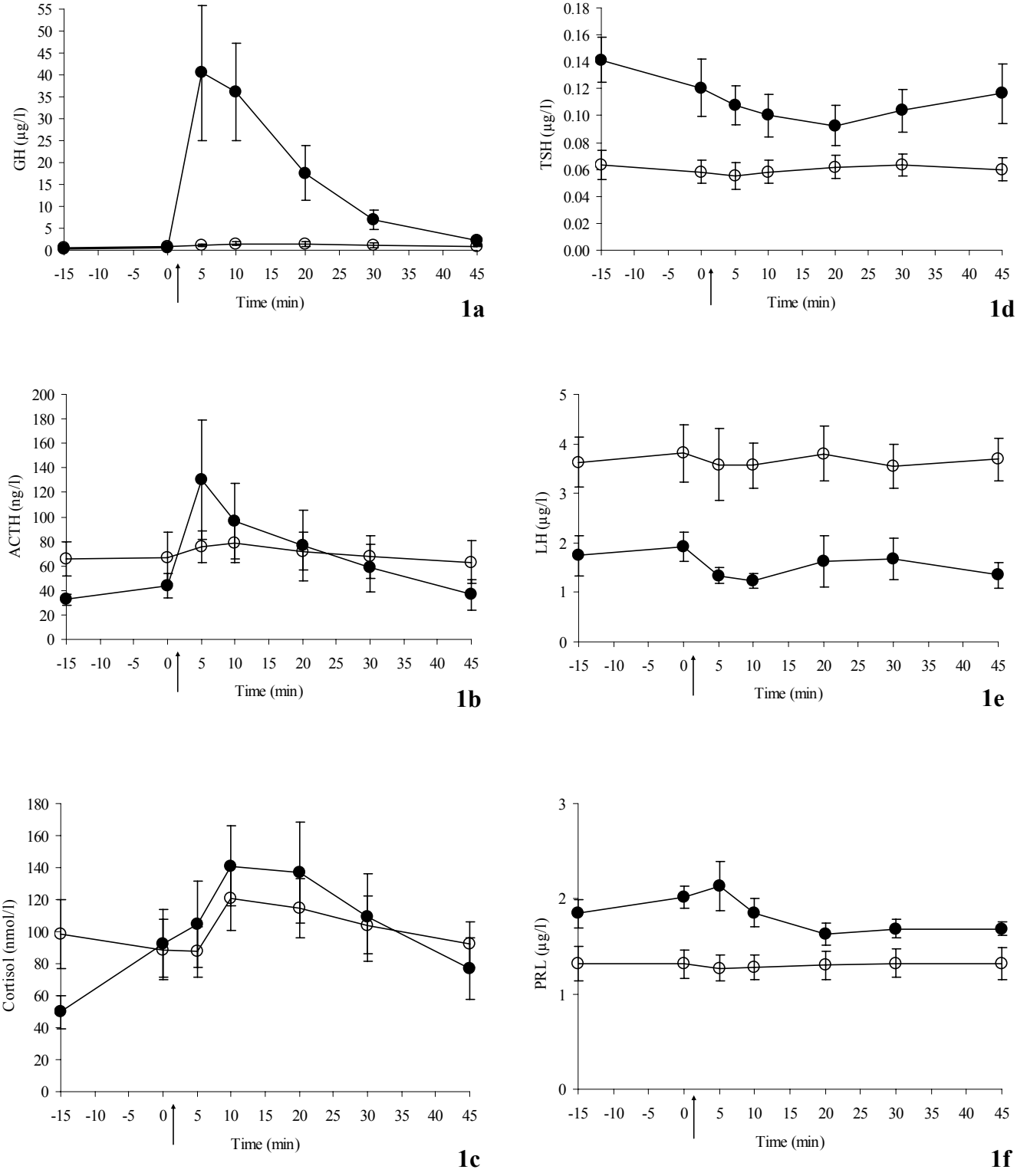
This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

Results

No side effects were observed during or after intravenous administration of ghrelin, neither in the German shepherd dwarfs nor in the healthy dogs.

The mean basal plasma GH concentration was not significantly different in the dogs with pituitary dwarfism (0.7 ± 0.1 µg/l) and in the healthy dogs (0.5 ± 0.1 µg/l) and neither was the mean basal plasma IGF-I concentration (55 ± 14 µg/l versus 88 ± 13 µg/l, respectively) (Table 1). The mean basal plasma concentrations of ACTH and cortisol in the dogs with pituitary dwarfism (67 ± 17 ng/l and 94 ± 20 nmol/l, respectively) did not differ significantly from those in the healthy dogs (38 ± 6 ng/l and 71 ± 15 nmol/l, respectively). However, the mean basal plasma concentration of TSH and PRL in the dogs with pituitary dwarfism (0.06 ± 0.01 µg/l and 1.3 ± 0.2 µg/l, respectively) were significantly lower compared with those in the healthy dogs (0.12 ± 0.02 µg/l and 2.2 ± 0.2 µg/l, respectively). The mean basal plasma concentration of LH in the pituitary dwarfs (3.7 ± 0.6 µg/l) was significantly higher than in the healthy dogs (1.8 ± 0.3 µg/l).

Analysis of the maximal increment indicated that the mean plasma GH response after ghrelin injection was significantly lower in the dwarf dogs (1.4 ± 0.4 µg/l) compared with that in the healthy dogs (45.3 ± 14.8 µg/l) (Figure 1a). In none of the pituitary dwarfs, the ghrelin-induced plasma GH concentration exceeded 5 µg/l at any time (Table 1). However, this was also true for 3 healthy dogs. Administration of ghrelin did not affect the plasma concentrations of ACTH, cortisol, TSH, LH and PRL in either the dogs with pituitary dwarfism or in the healthy dogs (Figures 1b-f, respectively) .



Figures 1. (a-f) Mean (± SEM) plasma concentrations of GH, ACTH, cortisol, TSH, LH, and PRL respectively, before and after intravenous administration of 2 µg/kg ghrelin (arrow) in six German shepherd dogs with pituitary dwarfism (○) and in eight healthy Beagle dogs (●).

Table 1. Mean basal (-15 and 0 min) plasma IGF-I (Basal IGF-I) and GH (Basal GH) concentration and maximal plasma GH concentration after intravenous administration of ghrelin (GH max) in eight healthy Beagle dogs and in six German shepherd dogs with pituitary dwarfism.

		Basal IGF-I (µg/l)	Basal GH (µg/l)	GH max (µg/l)
Healthy dogs	*Dog 1*	125	0.8	1.7
	Dog 2	124	0.2	76
	Dog 3	72	0.2	124
	Dog 4	123	0.2	0.2
	Dog 5	51	0.6	4.8
	Dog 6	43	1.0	47
	Dog 7	63	0.9	57
	Dog 8	58	0.5	69
Pituitary dwarfs	*Dog 1*	52	0.4	4.1
	Dog 2	3	0.4	0.7
	Dog 3	106	0.6	1.9
	Dog 4	78	1.1	1.9
	Dog 5	45	1.2	2.2
	Dog 6	47	0.5	1.2

Discussion

Assessment of random basal plasma GH concentrations is inadequate for documentation of hyposomatotropism, because circulating GH concentrations in healthy dogs and pituitary dwarfs may overlap (Kooistra et al., 2000). Indeed, the results of this study illustrate the absence of a significant difference in basal plasma GH concentration between healthy dogs and German shepherd dogs with pituitary dwarfism. Although the plasma IGF-I concentration represents the secretion of GH, this concentration cannot be used to irrefutably diagnose hyposomatotropism. In GH-deficient human adults, circulating IGF-I levels are within the reference range in about 60% of cases (Gill et al., 1998). In accordance with previous canine studies (Eigenmann et al., 1984; Rijnberk et al., 1993; Kooistra et al., 1998; Kooistra et al., 2000), the mean basal plasma IGF-I concentration in the pituitary dwarfs was lower than that in the healthy dogs, but due to a considerable overlap this difference did not reach statistical significance.

Thus, because of comparable plasma concentrations of GH and IGF-I between pituitary dwarfs and healthy individuals, the diagnosis of GH deficiency has to be based upon the results of a stimulation test. In dogs, most α-adrenergic drugs (such as clonidine and xylazine) or GHRH are commonly used to test the GH secretory capability of the pituitary

somatotrophs. In pituitary dwarfs, there is no significant increase in plasma GH concentration after administration of one of the aforementioned GHSs (Eigenmann et al., 1984). Adverse reactions, such as sedation, bradycardia, hypotension, and collapse, may preclude the use of xylazine and clonidine, especially at high doses (Eigenmann and Eigenmann, 1981; Lothrop 1988). Since ghrelin is a more potent GH-releaser than GHRH in young dogs, (Bhatti et al., 2006 in press), we tested whether ghrelin can be used to diagnose canine pituitary dwarfism. In none of the dwarf dogs ghrelin administration resulted in a rise of the plasma GH concentration above 5 μg/l. This finding corresponds with observations in humans with isolated childhood-onset GH deficiency, in whom the GH response to ghrelin is also markedly reduced (Aimaretti et al., 2002). However, in some of the healthy dogs the plasma GH concentration remained low after ghrelin administration as well. Thus while a ghrelin-induced plasma GH concentration higher than 5 μg/l seems to exclude GH deficiency, false-negative results may be encountered. The reason for the blunted ghrelin-induced GH response in healthy dogs remains unclear. In other species such as man, mice and rats, a similar atypical hormone response to ghrelin has not been mentioned in literature. Stress may decrease the responsiveness of the pituitary somatotrophs to GH stimulation tests in humans (Lazarus, 1984) and may have played a role in our study.

In line with earlier findings in German shepherd dwarf dogs (Hamann et al., 1999; Kooistra et al., 2000), the basal plasma TSH and PRL concentrations in these dogs were significantly lower than those in healthy dogs. In addition, ACTH and consequently cortisol secretion seemed to be preserved in the dwarf dogs. In contrast with the study of Kooistra et al., (2000), we found that the basal plasma LH concentration was significantly higher in the dogs with pituitary dwarfism. This unexpected finding warrants further investigation of basal LH secretion in German shepherd dwarfs with combined pituitary hormone deficiency.

Treatment of this form of combined pituitary hormone deficiency in German shepherd dwarfs consists of restoring the two most important hormone deficits, i.e., TSH and GH deficiency. The secondary hypothyroid state can easily be treated by oral supplementation of synthetic l-thyroxine. Growth hormone substitution therapy in these dwarfs is less straightforward. Canine GH is not yet available for therapeutic use. Moreover, antibody formation precludes the use of biosynthetic human GH (van Herpen et al., 1994). The amino acid sequence of canine GH is identical to that of porcine GH (Ascacio-Martinez and Barrera-Saldana, 1994), but the results of treatment of pituitary dwarf dogs with porcine GH are not satisfactory (Eigenmann and Eigenmann, 1981). In addition, treatment with heterologous GH is expensive. At present, the only available treatment option consists of administration of

medroxyprogesterone acetate (Kooistra et al., 1998) or proligestone (Knottenbelt and Herrtage, 2002). The GH production induced by progestins originates from foci of hyperplastic ductular epithelium in the mammary gland (Selman et al., 1994; Mol et al., 1995; van Garderen et al., 1997). In dogs, this mammary-derived GH reaches the systemic circulation (Selman et al., 1994). Unfortunately, treatment with progestins may result in undesirable side-effects, such as diabetes mellitus, cystic endometrial hyperplasia, recurrent pyoderma, and associated pruritis (Kooistra et al., 1998; Knottenbelt and Herrtage, 2002; Romagnoli and Concannon, 2003).

Based on the strong and reproducible GH-releasing effect of GHSs, these molecules, particularly if orally active, could theoretically have therapeutic usefulness in hyposomatotropism. The GHS-R type 1a is present in the pituitary and hypothalamus of several mammalian species and is distinct from the GHRH receptor (Howard et al., 1996; McKee et al., 1997; Pong et al., 1996). As GHRH is not able to stimulate the release of GH from the pituitary somatotrophs in German shepherd dogs with pituitary dwarfism (Kooistra et al., 2000), theoretically the ghrelin-activated pathway offers another treatment option. However, in line with the absence of pituitary somatotrophs, no GH response occurred and consequently ghrelin is not a candidate for the treatment of this disorder. Again theoretically, the possibility that long-term treatment with ghrelin would restore circulating GH levels in dogs with hyposomatotropism can not be ruled out. Though, long-term administration of MK-0677, a synthetic non-peptidyl GHS, was not very effective in increasing growth velocity in children with isolated GH deficiency, however they did benefit from recombinant human GH replacement therapy (Yu et al., 1998).

In humans and rats, the endocrine activity of both ghrelin and synthetic GHSs is not specific for GH. In healthy humans, intravenous administration of GHRP-6 or ghrelin also increases circulating concentrations of PRL, ACTH, and cortisol (Massoud et al., 1996; Casanueva and Dieguez, 1999; Takaya et al., 2000; Arvat et al., 2001; Ghigo et al., 2001). In rats, GHRP-6 activates the hypothalamic-pituitary-adrenal axis (Thomas et al., 1997). In contrast, in the present study ghrelin did not cause significant elevations in plasma concentrations of ACTH, cortisol, TSH, LH and PRL, neither in the healthy dogs nor in the German shepherd dwarfs.

The data of the ghrelin-stimulation test in German shepherd dwarfs were compared with those in healthy Beagle dogs. This may raise the question whether the differences in the results might be attributable to differences in breed. However, in a study of Meij et al., (1997), no breed-related variation in the response of GH to stimulation with GHRH was

noticed in dogs with pituitary-dependent hyperadrenocorticism. Therefore the lack of response of the pituitary somatotrophs to stimulation with ghrelin can be considered abnormal in the German shepherd dwarfs. Comparison with healthy, normal-sized German shepherd dogs of a comparable age as the dwarf dogs might have been more appropriate, but these dogs were not available for endocrine testing. In addition, these dogs would have weighed considerably more than the German shepherd dwarfs and this discrepancy could also have influenced the plasma GH levels. For these reasons, healthy Beagle dogs of a comparable age and body weight were used as a control group.

It can be concluded that administration of the natural ligand of the GHS-R type 1a, i.e., ghrelin, is not able to induce a considerable rise in plasma GH concentration in German shepherd dogs with pituitary dwarfism. While a ghrelin-induced plasma GH concentration higher than 5 µg/l seems to exclude GH deficiency, false-negative results may be encountered.

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Chapter 7

Effects of food intake and fasting on the plasma ghrelin concentration in healthy dogs

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Abstract

The growth hormone (GH)-releasing peptide ghrelin, primarily secreted by the stomach, causes weight gain by increasing food intake and reducing fat utilization and seems to play a role in meal initiation in several mammalian species. In this study, blood samples were collected from 8.30 a.m. to 5 p.m. in nine healthy Beagle dogs when food was administered as usual at 10 a.m., after a 1-day fast, after a 3-day fast, and after re-feeding at 10 a.m. the next day. Circulating concentrations of acylated ghrelin, GH, insulin-like growth factor-I (IGF-I), glucose, and insulin were determined.

The overall mean plasma ghrelin concentrations were significantly lower when food was administered than after fasting. Shortly after feeding, the time-specific mean plasma ghrelin concentrations decreased, but this decline did not reach statistical significance. The overall mean plasma GH and IGF-I concentrations did not differ significantly between the 4 different periods. The time-specific mean plasma GH concentration differed significantly over time with a maximum concentration at 0 min. This concentration did not change differently over time in the 4 different periods. Circulating overall mean glucose and insulin concentrations were significantly higher after re-feeding the day after the 3-day fast compared with the 3 other periods. The time-specific mean plasma glucose and serum insulin concentrations differed significantly over time. In addition, the course of these concentrations over time differed significantly in the 4 sampling periods.

In conclusion, in dogs fasting and food intake are associated with higher and lower circulating ghrelin concentrations, respectively, suggesting that also in this species ghrelin participates in the control of feeding behavior and energy homeostasis. The changes in plasma ghrelin concentrations are not associated with similar changes in plasma GH concentrations, whereas insulin and glucose concentrations appear to change reciprocally with the ghrelin concentrations.

Introduction

In 1999, Kojima and co-workers reported the isolation of ghrelin from the rat stomach (Kojima et al., 1999). The presence of ghrelin in the stomach has been confirmed in humans, cows, pigs, dogs, and horses (Date et al., 2000; Hayashida et al., 2001; Tomasetto et al., 2001). Ghrelin acts as the ligand for the growth hormone secretagogue receptor type 1a (GHS-R 1a) and is able to stimulate growth hormone (GH) secretion from pituitary cells in many species such as rats, humans, goats, dogs, and fish (Kojima et al., 1999, Date et al., 2000; Hayashida et al., 2001, Bhatti et al., 2002; Kaiya et al., 2003; Bhatti et al., 2006 in press). The mature ghrelin molecule contains 28 amino acids and its third Serine residue is acylated by *n*-octanoic acid which is essential for binding to the GHS-R 1a, for the GH-releasing capacity of ghrelin, and most likely for its other endocrine actions (Kojima et al., 1999; Bednarek et al., 2000; Muccioli et al., 2001).

Through activation of pathways distinct from those leading to GH secretion, ghrelin also acts as a potent orexigenic peptide (Kamegai et al., 2000; Tschop et al., 2000; Wren et al., 2000; Wren et al., 2001). In rodents, ghrelin causes weight gain by increasing food intake and reducing fat utilization (Kamegai et al., 2000; Wren et al., 2000; Nakazato et al., 2001). Ghrelin is the only known circulating orexigen, and has a potency similar to that of neuropeptide Y (NPY). This appetite-stimulating effect appears to be mediated, at least in part, through activation of NPY/agouti gene-related peptide (AGRP) neurons in the hypothalamic arcuate nucleus, 94 % of which express the GHS-R (Willesen et al., 1999). NPY and AGRP are thought to be mediators of the ghrelin-induced increased food intake because antagonism of either NPY or AGRP signalling in the brain attenuates the orexigenic potency of injected ghrelin (Dickson et al., 1997; Kamegai et al., 2000; Asakawa et al., 2001; Shintani et al., 2001).

Ghrelin may also play a role in meal initiation in humans, since the concentration of this peptide increases immediately prior to a meal and decreases after eating (Cummings et al., 2001). Also in sheep and cows it has been demonstrated that preprandial ghrelin surges occur as many times per day as meals are provided (Sugino et al., 2002a,b; Miura et al., 2004). These results indicate that ghrelin secretion may be a trigger for endogenous appetite signals.

Consistent with this, circulating ghrelin concentrations are increased in anorexia and cachexia and reduced in obesity (Shiiya et al., 2002; Cummings and Shannon, 2003; Tolle et al., 2003). These changes are opposite to those induced by leptin, an adipocyte-derived

hormone that reduces appetite and increases energy expenditure in animal models. Thus both ghrelin and leptin reflect the metabolic balance and may drive neuroendocrine and metabolic responses to changes in nutritional status (Inui et al., 2001; Yoshihara et al., 2002; Cummings and Foster, 2003).

Ghrelin production in the stomach is not only stimulated by fasting but is also related to glucose and insulin metabolism. In humans, oral and intravenous glucose administration decreases plasma ghrelin concentrations, whereas lipids or high-fat diets suppress the postprandial ghrelin concentrations less effectively (Mohlig et al, 2002; Monteleone et al., 2003; Weigle et al., 2003). Moreover, Blom et al. (2005) have shown in humans that the ghrelin response to a high-carbohydrate meal is related to insulin secretion.

So far there are only a few reports (Jeusette et al., 2005a,b; Yokoyama et al., 2005) on the role of ghrelin in dogs, a carnivorous animal with an evolutionary background of being able to cope with long periods of starvation. However, nowadays the domestic dog does not need to endure starvation but rather food surplus, that often leads to obesity.

In this study we investigated the physiological endocrine effects of food intake and fasting in healthy Beagle dogs. Therefore, the circulating concentrations of ghrelin, GH, insulin-like growth factor-I (IGF-I), glucose, and insulin were measured when food was administered at the usual time, after a 1-day fast, after a 3-day fast, and after re-feeding the next day.

Materials and methods

Dogs

Nine normal-weight ovariohysterectomized Beagle dogs, 5 years of age, were used. They received a commercial dog food (Hill's Science Plan - Adult - Canine Maintenance®) at 10 a.m. each day and were given water *ad libitum*. The mean body weight of the dogs was 12 kg (range 9.5-14.0 kg). The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples.

Study design and blood sample collection

The dogs were randomly assigned to three groups. For the second and third group the study started one and two days, respectively, after that of the first group. All dogs were put on a food-fast regimen for five consecutive days: on day 1, food was given as usual at 10 a.m.; on days 2, 3, and 4, food was withheld; and on day 5, the dogs were re-fed at 10 a.m.

Blood samples were collected by jugular venipuncture on days 1 (= period 1), 2 (= period 2), 4 (= period 3), and 5 (= period 4) at 8.30 a.m., 10 a.m., 10.30 a.m., 11 a.m., 11.30 a.m., 12.30 p.m., 1.30 p.m., 3 p.m., and 5 p.m. In these blood samples the concentrations of acylated ghrelin, GH, glucose, and insulin were determined. Plasma IGF-I concentrations were determined only in the blood samples collected at 8.30 a.m. and 10 a.m.

For the determination of plasma ghrelin concentrations, the collection procedures and storage were carried out in accordance with the protocol supplied by the manufacturer (Ghrelin (active) radioimmunoassay (RIA) kit, Linco Research, St Louis, MO, USA). Blood samples were immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4° C for 10 min. Per one ml plasma, 50 µl of 1 N HCl and 10 µl phenylmethylsulfonyl fluoride was added. Plasma was immediately stored at -25° C until analysis.

For the determination of plasma GH and IGF-I concentrations, the blood samples were immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Blood samples for the measurement of plasma glucose concentrations were collected in sodium-fluoride tubes, and those for the measurement of serum insulin concentrations were collected in tubes with cloth activator.

Hormone determination

Plasma ghrelin concentrations were measured with a (human) ghrelin RIA kit (Linco Research, St Louis, MO, USA). This assay specifically measures biologically active ghrelin (Janssen et al., 2001). The RIA technique detects human ghrelin with equal accuracy in the dog (Yokoyama et al., 2005). Values of ghrelin measured with the human ghrelin RIA kit after serial dilutions of various dog samples showed good linearity, indicating that in this assay canine ghrelin behaves immunochemically similar to that of the human ghrelin standards. The sensitivity of the assay was 10 ng/l.

Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS). The intra-assay coefficient of variation value was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l.

Total plasma IGF-I concentrations were measured after acid-ethanol extraction to remove interfering IGF binding proteins. Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room

temperature. After centrifugation for 30 min at 5500 g at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7.4), 13 mM Na_2EDTA, and 0.25 % (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7 %. Plasma IGF-I concentrations were measured with a heterologous RIA validated for the dog (Favier et al., 2001). The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Programme (Harbor-UCLA Medical Center, Torrance CA).

Serum immunoreactive insulin concentrations were measured by immunoradiometric assay (IRMA). The two-site IRMA method (INS-Irma®: BioSource Europe S.A., Nivelles, Belgium) had an intra-assay and interassay coefficient of variation of 4.5 % and 4.7 %, respectively. According to information supplied by the manufacturer, there is 0 % cross-reactivity with human pro-insulin. Serial dilutions of canine serum were parallel to the standard curve of human insulin. The sensitivity of the assay was 7 pmol/l.

Statistical analysis

Changes in circulating concentrations of ghrelin, GH, glucose and insulin were analyzed based on a mixed model with dog as random effect (repeated measures ANOVA with compound symmetry structure) and period (1 to 4), blood sampling times (8.30 a.m., 10 a.m., 10.30 a.m., 11 a.m., 11.30 a.m., 12.30 p.m., 1.30 p.m., 3 p.m., and 5 p.m.), and their interaction as categorical fixed effects. In the analysis both mean concentrations over the different sampling points (denoted by overall mean concentration) and concentrations at different sampling points (denoted by time-specific mean concentration) were compared, with mean referring to the mean over the different dogs. The four periods were compared pair wise using Tukey's multiple comparisons technique at a global significance level of 5 %. Plasma IGF-I concentrations between the four periods were compared with one-way ANOVA. All values are expressed as mean ± SEM.

Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Results

The overall mean plasma ghrelin concentration when food was given at day 1 (152 ± 34 ng/l) was significantly (P = 0.005) lower than after a 1-day fast (181 ± 42 ng/l). The overall mean plasma ghrelin concentration after re-feeding the day after the 3-day fast (143 ± 32 ng/l) was significantly lower than after a 1-day fast (181 ± 42 ng/l, P = 0.001) and after a 3-day fast (183 ± 53 ng/l, P = 0.009) (Figure 1a and Figure 1b).

The time-specific mean plasma ghrelin concentrations decreased shortly after feeding, but this decline did not reach statistical significance. Additionally, the time-specific mean plasma ghrelin response did not change differently over time when the 4 periods were compared (Figure 1a). There was a considerable interindividual variation in the pre-food and post-food changes of the time-specific plasma ghrelin concentration (Figure 1c).

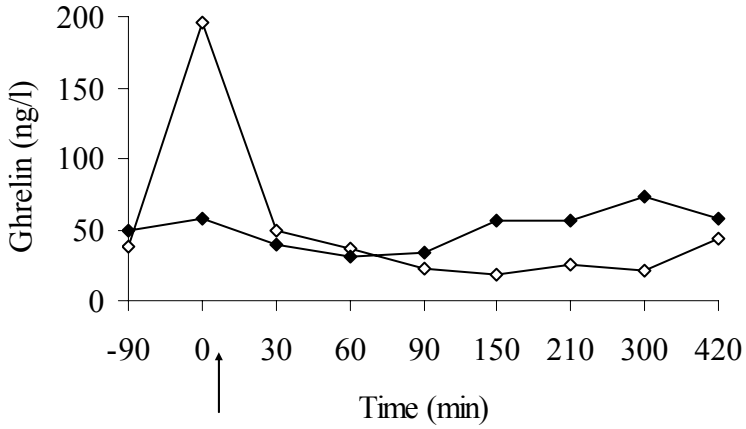

Figure 1c. Plasma ghrelin profiles in two Beagle dogs before (-90 and 0 min) and after (30, 60, 90, 150, 210, 300, 420 min) ingestion of a meal at 10 a.m (arrow). Note the heterogeneity in the rise before and the decline after food intake.

The overall mean plasma GH concentrations in the dogs when food was given at day 1, after a 1-day fast, after a 3-day fast, and after re-feeding the next day (1.7 ± 0.3 µg/l, 1.5 ± 0.2 µg/l, 1.4 ± 0.3 µg/l, and 1.5 ± 0.3, respectively) did not differ significantly (Figure 2). The time-specific mean plasma GH concentration differed significantly over time (P<0.0001) with a maximum concentration at 0 min. The time-specific mean plasma GH concentration did not change differently over time in the 4 different periods (Figure 2). No significant differences were found in the overall mean plasma IGF-I concentrations when the dogs were given food at day 1 (63 ± 9 µg/l), after a 1-day fast (60 ± 8 µg/l), after a 3-day fast (53 ± 7 µg/l), and after re-feeding the next day (47 ± 6 µg/l).

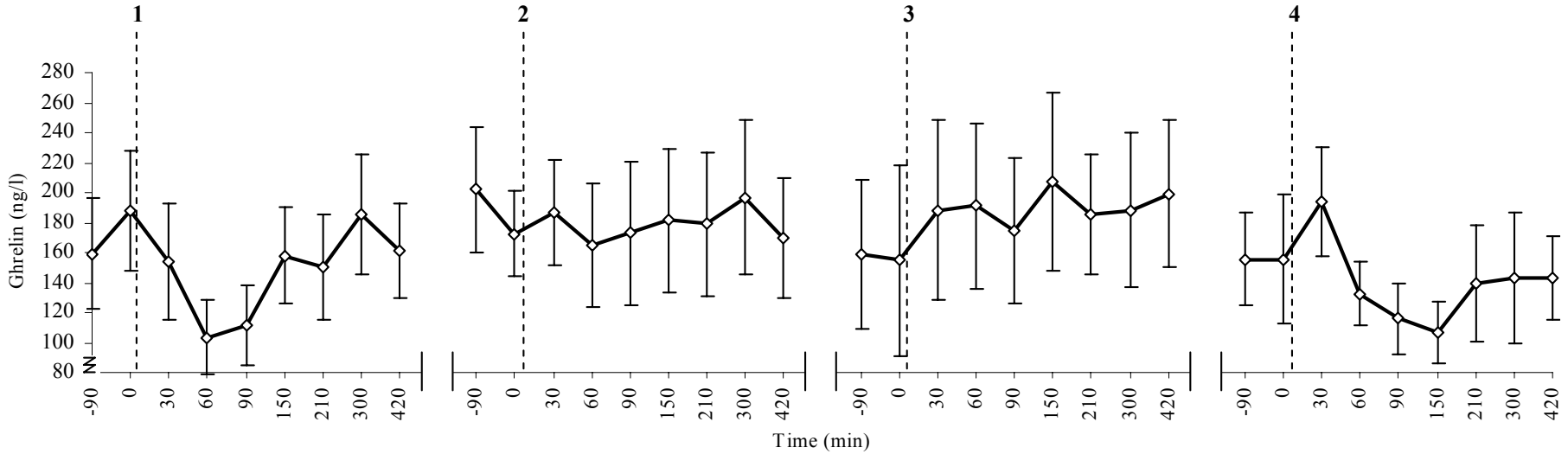

Figure 1a. Mean (± SEM) plasma ghrelin concentrations in 9 Beagle dogs before (-90 and 0 min) and after (30, 60, 90, 150, 210, 300, 420 min) ingestion of a meal at 10 a.m. **(1)**, after a consecutive 1-day fast **(2)**, after a 3-day fast **(3)**, and before and after ingestion of a meal at 10 a.m. the day after the 3-day fasting period **(4)**.

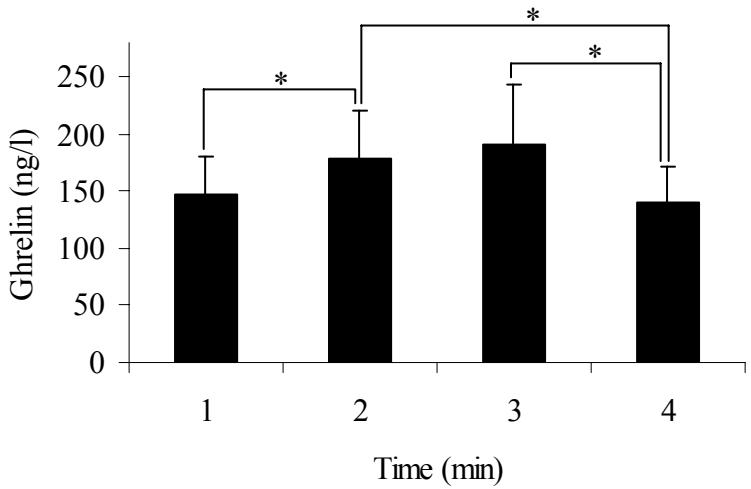

Figure 1b. Mean (+ SEM) plasma ghrelin concentration when a meal was given at 10 a.m. **(1)**, after a 1-day fast **(2)**, after a 3-day fast **(3)**, and when a meal was given again at 10 a.m. the day after the 3-day fasting period **(4)**. Significant differences between periods are indicated with an asterisk.

The overall mean plasma glucose concentrations when food was given at day 1 (4.5 ± 0.1 mmol/l), after a 1-day fast (4.6 ± 0.1 mmol/l), and after a 3-day fast (4.5 ± 0.1 mmol/l) were significantly lower ($P < 0.0001$) compared with this concentration after re-feeding the day after the 3-day fast (5.0 ± 0.2 mmol/l) (Figure 3).

The overall mean serum insulin concentrations when food was given at day 1 (28 ± 5 IU/l) and after re-feeding the day after the 3-day fast (27 ± 4 IU/l) were significantly higher ($P < 0.0001$) than after a 1-day fast (14 ± 2 IU/l) and after a 3-day fast (12 ± 2 IU/l) (Figure 4).

The time-specific mean plasma glucose and serum insulin concentrations differed significantly ($P<0.0001$) over time (Figures 3 and 4, respectively). In addition, the course of these concentrations over time differed significantly ($P<0.0001$) in the 4 sampling periods (Figures 3 and 4, respectively).

Discussion

The results of the present study demonstrate a significant difference in overall mean plasma ghrelin concentrations between food intake and fasting, characterized by lower plasma ghrelin concentrations when a meal was administered and higher plasma ghrelin concentrations during fasting. These findings are consistent with those for other species. For example, in rodents, circulating ghrelin concentrations increase with fasting and are suppressed within min by re-feeding or enteral infusions of nutrients but not water (Tschop et al., 2000; Asakawa et al., 2001). These observations suggest that the gastric peptide ghrelin may play an important role in controlling feeding behaviour and energy homeostasis.

The higher plasma ghrelin concentrations during fasting may be consistent with a physiological role for this hormone in increasing appetite and the initiation of food intake. Similar to the situation in rodents, circulating ghrelin concentrations in humans are rapidly suppressed by food intake, and 24-h plasma ghrelin profiles reveal marked preprandial increases and postprandial decreases associated with every meal (Cummings et al., 2001). Although meal-time hunger is a common, daily experience in humans and animals, the molecular determinants of this sensation remain incompletely understood. Several observations, mostly from animal studies, indicate that ghrelin contributes to the sensation of hunger and participates in meal initiation.

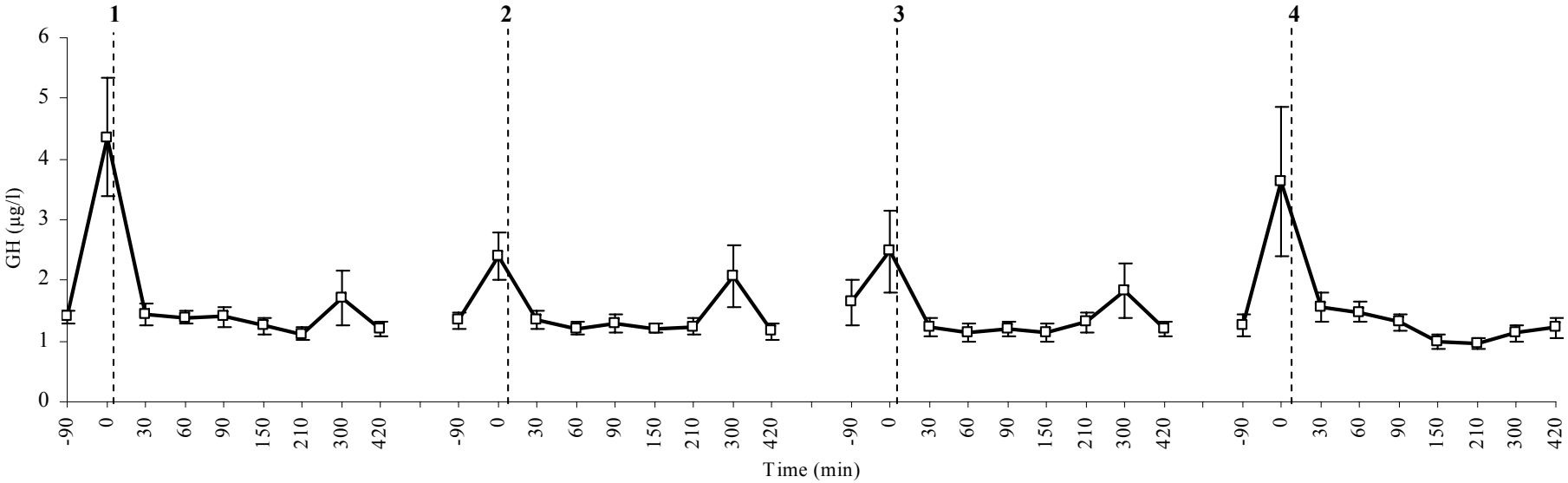

Figure 2. Mean (± SEM) plasma GH concentrations in 9 Beagle dogs before (-90 and 0 min) and after (30, 60, 90, 150, 210, 300, 420 min) ingestion of a meal at 10 a.m. **(1)**, after a consecutive 1-day fast **(2)**, after a 3-day fast **(3)**, and before and after ingestion of a meal at 10 a.m. the day after the 3-day fasting period **(4)**.

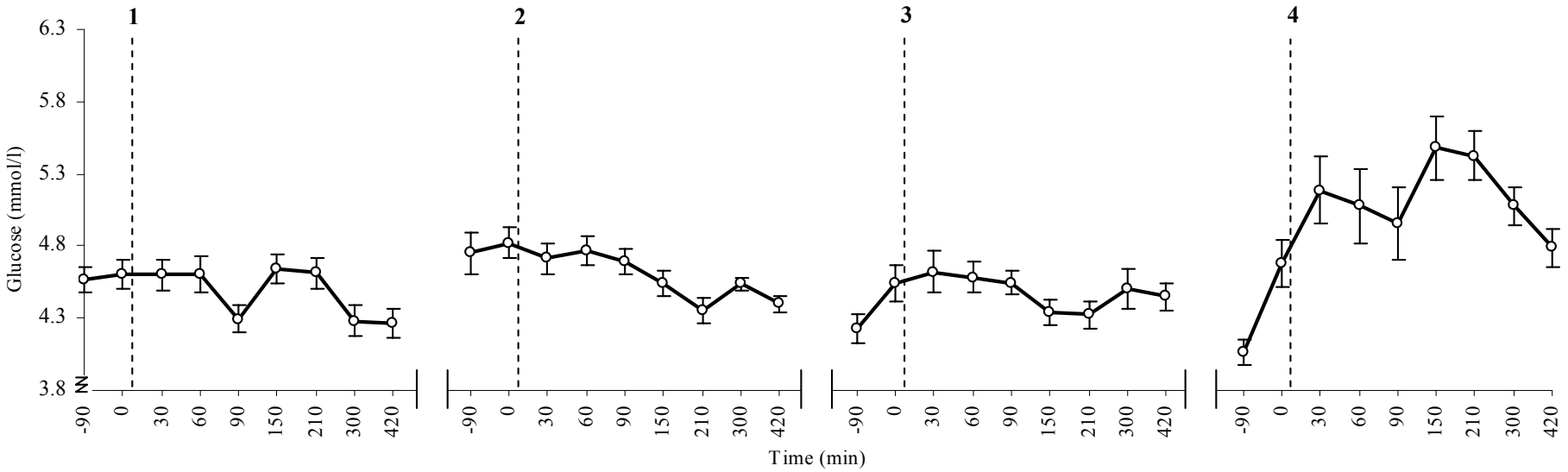

Figure 3. Mean (± SEM) plasma concentrations of glucose in 9 Beagle dogs before (-90 and 0 min) and after (30, 60, 90, 150, 210, 300, 420 min) ingestion of a meal at 10 a.m. **(1)**, after a consecutive 1-day fast **(2)**, after a 3-day fast **(3)**, and before and after ingestion of a meal at 10 a.m. the day after the 3-day fasting period **(4)**.

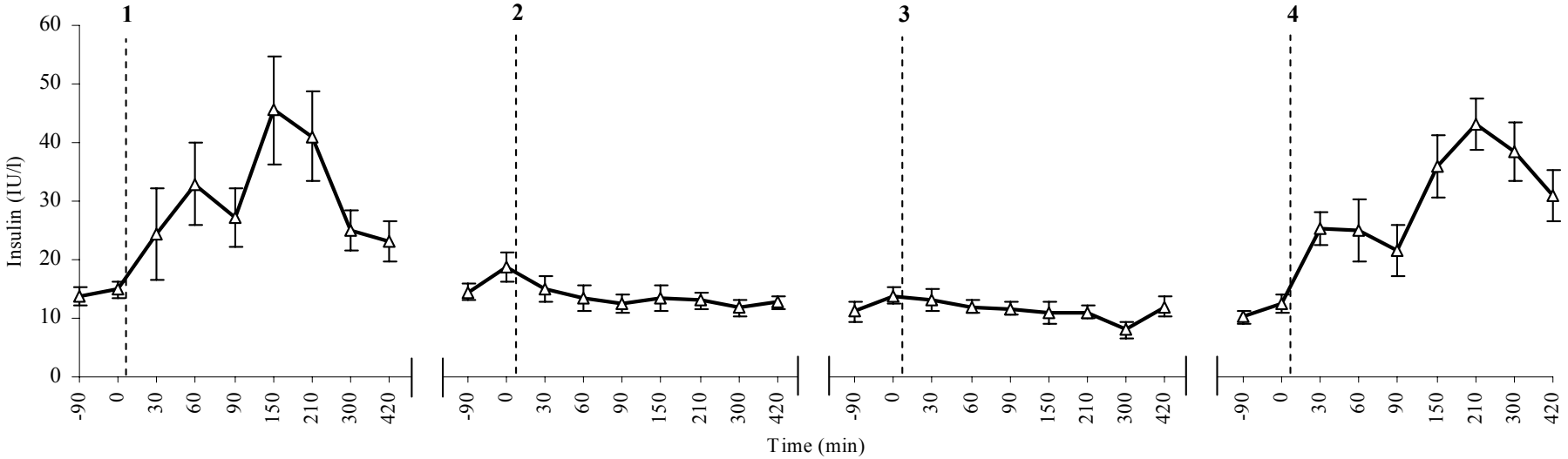

Figure 4. Mean (± SEM) serum concentrations of insulin in 9 Beagle dogs before (-90 and 0 min) and after (30, 60, 90, 150, 210, 300, 420 min) ingestion of a meal at 10 a.m. **(1)**, after a consecutive 1-day fast **(2)**, after a 3-day fast **(3)**, and before and after ingestion of a meal at 10 a.m. the day after the 3-day fasting period **(4)**.

Firstly, the majority of circulating ghrelin is produced by the stomach and duodenum, organs well positioned to detect recently ingested food (Kojima et al., 1999; Gnanapavan et al., 2002; Date et al., 2000). Secondly, despite being produced peripherally, ghrelin acts centrally to stimulate food intake (Tschop et al., 2000; Nakazato et al., 2001; Wren et al., 2000). Thirdly, the orexigenic actions of ghrelin are rapid and short-lived, increasing both food intake (Asakawa et al., 2001) and gastric acid secretion (Masuda et al., 2000) within 20 min after intraperitoneal injection, a time course that is consistent with a role in meal initiation. Fourthly, exogenous ghrelin triggers eating when administered at times of minimal spontaneous food intake (Nakazato et al., 2001; Wren et al., 2000). Finally, the most clearly documented targets of ghrelin action in the brain are the hypothalamic neurons that co-secrete the well-known orexigens, NPY and AGRP (Cummings and Shannon, 2003). These neuropeptides are implicated in the central regulation of meal initiation as their expression increases at times of maximal spontaneous food intake in rodents, whereas that of other neuropeptides involved in energy balance remain relatively constant throughout the day (Lu et al., 2002).

With regard to the plasma ghrelin concentration in the present study, neither a significant time effect, nor a significant interaction between time and the four different periods was observed. Nevertheless, at day 1 the highest time-specific plasma ghrelin concentrations were observed immediately before feeding. It is possible that this insignificant preprandial rise occurred as an anticipatory response to feeding because the dogs had been fed at the same time of the day for several years. That a psychological factor might have played a role in the present study is further supported by the observation that, after a few days of fasting, the highest time-specific plasma ghrelin level was observed not before food administration but immediately after the "unexpected" administration of food on day 5. In line with this assumption, Sugino et al. (2002a) demonstrated that psychological factors, i.e. an expectation of food administration, may stimulate ghrelin secretion just before feeding in sheep. This transient increase in ghrelin secretion just before feeding may be part of a conditioned emotional response. It is well known that secretion of saliva and gastric acid preceding food intake is induced by a conditioned emotional response through the stimulation of the vagal nerve (Harding and Leek, 1973). In this respect, ghrelin secretion may be induced by the vagal neural system in the same manner as the secretion of saliva and gastric acid.

Like in humans and rodents, also in our dogs the time-specific mean plasma ghrelin concentrations decreased shortly after food intake, but this decline did not reach statistical significance. However, in another study it was demonstrated that also in dogs circulating

plasma ghrelin concentrations decrease significantly after eating (Yokoyama et al., 2005). The results of the present study do show that feeding results in a significantly lower overall mean plasma ghrelin concentration compared to fasting in dogs. The mechanism by which nutrients suppress ghrelin concentrations are beginning to be elucidated. Absorbed nutrients are thought to be the most likely mediators of the postprandial decrease in plasma ghrelin concentrations in rodents (Tschop et al., 2000). Ingested nutrients suppress ghrelin release in rats and humans with an efficacy of carbohydrates > proteins > lipids (Overduin et al., 2005). Surprisingly, food-related ghrelin suppression does not require luminal nutrient exposure in the stomach or duodenum, the principal sites of ghrelin production (Williams et al., 2003a; Overduin et al., 2005). Instead, signals mediating this response originate further downstream in the intestines and from post-absorptive events. In addition to nutrients, also changes in plasma insulin concentrations, intestinal osmolarity, and enteric neural signalling probably play a role, whereas gastric distension, vagal nerve activity, and glucagon-like peptide-1 are not required (Williams et al., 2003b; Gelling et al., 2004).

There was considerable interindividual variation in time-specific mean plasma ghrelin concentrations in our dogs, as has been reported previously in humans (Cummings et al., 2001; Janssen et al., 2001). Interestingly, a strong correlation in plasma ghrelin concentrations has been found within one individual when samples are collected with an interval of 1 year (Janssen et al., 2001). This suggests that the variation in plasma ghrelin concentrations is mainly determined by the variation between individuals and less by intra-individual factors. Also, a large heterogeneity was found in the pre-food surge and post-food decline between dogs, indicating that not only basal ghrelin concentrations but also the ghrelin responses have a strong individual variation.

Time-specific mean plasma GH concentrations increased just before the time when feeding normally occurred. Also in cows a single GH surge during feeding has been demonstrated (Gaynor et al., 1995). The two principal hypothalamic regulators of GH secretion, GHRH and somatostatin, do not seem to be responsible for the rise in circulating GH concentrations around feeding (McMahon et al., 2000). Because ghrelin is also a potent GH-releasing peptide, it can be hypothesized that a preprandial rise in circulating ghrelin concentrations may be responsible for the preprandially increased GH secretion. Indeed, some studies in humans (Cummings et al., 2001), goats, and sheep (Sugino et al., 2002a,b) have demonstrated that a preprandial rise in plasma ghrelin concentration is associated with a GH surge. The results of the present study, however, do not provide evidence for such a relationship in dogs, because the significant preprandial GH surge was not associated with a

significant preprandial ghrelin surge. Moreover, in contrast to the overall mean plasma ghrelin profiles the overall mean plasma GH profiles did not differ significantly in the fed compared to the fasted state. In addition, the overall mean plasma IGF-I concentrations did not differ between the several food-fast regimens. Similar to the situation in our dogs, a link between plasma ghrelin concentrations and plasma GH concentrations could not be demonstrated in cows (Miura et al., 2004).

The overall mean plasma profiles of ghrelin on the one hand and the overall mean profiles of insulin and glucose on the other hand changed reciprocally after feeding and fasting in our dogs. While the overall mean plasma ghrelin concentrations were significantly lower after feeding than after fasting, the opposite was true for the overall mean serum insulin concentrations. These findings are in agreement with a study in humans, in which plasma ghrelin concentrations changed oppositely to circulating insulin concentrations (Cummings et al., 2001). The observation that both ghrelin and insulin are involved in the physiological response to food intake as well as in body weight regulation and that they display reciprocal 24-h profiles (Cummings et al., 2001), raises the question whether insulin negatively regulates ghrelin or *vice versa*. The former hypothesis has been investigated by many groups (Saad et al., 2002; Flanagan et al., 2003; Kamegai et al., 2004). Taken together, these studies demonstrated that while insulin can suppress ghrelin release when administered in supraphysiologic doses or at high-normal concentrations for prolonged periods of time, physiological concentrations of insulin do not appear to regulate ghrelin release (Caixas et al., 2002; Schaller, et al., 2003; Soriano-Guillen et al., 2004). It has also been suggested that ghrelin may act as a counter-regulatory hormone blocking insulin secretion and insulin action to maintain blood glucose concentrations (Broglio et al., 2001; Cummings et al., 2005). Indeed, several studies have shown that ghrelin can inhibit glucose-mediated insulin secretion, both *in vitro* and *in vivo* (Egido, et al., 2002; Reimer, et al., 2003; Colombo, et al., 2003). Similarly, exogenous ghrelin administration decreases circulating insulin concentrations in mice (Reimer, et al., 2003) and humans (Broglio et al., 2003). Recently, a novel ghrelin-producing pancreatic islet cell type has been identified, the ε-cell (Wierup et al., 2004). These cells are derived from the same progenitors as are the four classical islet cell types (producing insulin, glucagon, somatostatin, and pancreatic polypeptide), and can replace the other islet cells when the latter are eliminated by for example genetic deletion of vital transcription factors. Because ghrelin is highly expressed in the fetal pancreas (6-7 times more than in the stomach) (Chanoine and Wong, 2004), it may participate in pancreatic islet development. Preliminary evidence also suggests that ghrelin has paracrine effects on insulin

secretion in adults. If ghrelin derived from the pancreatic islets, rather than from the gastrointestinal tract, is the more critical regulator of insulin release, this raises the interesting possibility of an intra-islet ghrelin/insulin glucoregulatory axis.

In conclusion, in dogs, fasting and food intake are associated with higher and lower circulating ghrelin concentrations, respectively, suggesting that also in this species ghrelin participates in the control of feeding behaviour and energy homeostasis. The changes in plasma ghrelin concentrations are not associated with similar changes in plasma GH concentrations, whereas circulating insulin and glucose concentrations appear to change reciprocally with the ghrelin concentration.

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Chapter 8

Role of progestin-induced mammary-derived growth hormone in the pathogenesis of cystic endometrial hyperplasia in the bitch

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Abstract

Endogenous progesterone and synthetic progestins may induce hypersecretion of growth hormone (GH) of mammary origin, hyperplastic ductular changes in the mammary gland, and the development of cystic endometrial hyperplasia (CEH) in dogs. It was investigated whether progestin-induced mammary GH plays a role in the pathogenesis of CEH in the bitch. During one year, bitches with surgically excised mammary glands and healthy control bitches received 10 mg medroxyprogesterone acetate (MPA)/kg body weight at 4-week intervals. The 6-h plasma profile of GH was studied before and 3, 6, 9, and 12 months after the onset of MPA administration. Before and after MPA treatment, uterine and mammary tissues were collected for histological examination, for the immunohistochemical presence of GH, and for the expression of the genes encoding for GH, insulin-like growth factor-I (IGF-I), the GH receptor (GHR), and the progesterone receptor (PR).

In the control group, MPA administration resulted in higher basal plasma GH concentrations, lower area under the curves (AUCs) above the baseline for GH, lower GH pulse frequencies, and higher AUCs above the zero-level for GH compared with the mastectomized dogs. After MPA administration, the mammary tissue in the control dogs had differentiated into lobulo-alveolar structures and CEH was present in all uteri of both dog groups. In the MPA-exposed mammary tissue of the control dogs, GH could only be demonstrated immunohistochemically in proliferating epithelium. After treatment with MPA the dogs of both groups had immunohistochemically demonstrable GH in the cytoplasm of hyperplastic glandular uterine epithelial cells. RT-PCR analysis of the mammary gland tissue after MPA administration demonstrated a significant higher GH gene expression, and lower GHR and PR gene expression than before MPA administration. In the uterus, the expression of the gene encoding for GH was significantly increased in the mastectomized dogs, whereas in the control dogs the expression of the gene encoding for IGF-I had significantly increased with MPA administration. MPA treatment significantly down regulated PR gene expression in the uterus in both dog groups.

These results indicate that progestin-induced GH of mammary origin is not an essential component in the development of CEH in the bitch. Nevertheless, the presence of immunoreactive GH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.

Introduction

Cystic endometrial hyperplasia (CEH) is a common disorder of the canine uterus and may result in infertility (McEntee, 1990; Arthur et al., 1996; Niskanen and Thrusfield, 1998). The condition begins with endometrial glandular hyperplasia and progresses to cystic transformation of the glands. When CEH is accompanied by inflammation and usually by bacterial infection, the disorder is called CEH-endometritis or, if the cervix is closed, pyometra (Dow, 1958; Hardy and Osborne, 1974; Sevelius et al., 1990; Schaefers-Okkens, 1996; Noakes et al., 2001). This systemic disease may result in death due to toxaemia, kidney problems and peritonitis (Arthur et al., 1989). The pathogenesis of CEH remains incompletely understood, but progestins play an important role in the development of CEH in bitches (Teunissen, 1952). Consequently, CEH is frequently seen in bitches treated repeatedly with progestins for oestrus prevention (Capel-Edwards et al., 1973; Sokolowski and Zimbelman, 1973; Goyings et al., 1977). Cystic endometrial hyperplasia may also develop spontaneously during the luteal phase of the oestrous cycle of middle-aged and elderly bitches, i.e. bitches that repeatedly have been under the influence of high concentrations of endogenous progesterone (Dow, 1958).

In dogs, endogenous progesterone or synthetic progestins such as medroxyprogesterone acetate (MPA) may induce growth hormone (GH) hypersecretion, leading to acromegalic features and insulin resistance (Eigenmann and Rijnberk, 1981; Eigenmann et al., 1983; Selman et al., 1994a). This GH excess originates in the mammary gland. Progestins induce the production of GH in foci of hyperplastic ductular epithelium (Selman et al., 1994b, van Garderen et al., 1997). The gene encoding GH in the mammary gland is identical to that in the pituitary gland (Mol et al., 1995a). Moreover, the progestin-induced mammary-derived protein is identical to pituitary GH and biologically active (Selman et al., 1994b). The progestin-induced elevations of plasma GH concentrations do not have a pulsatile secretion pattern (Watson et al., 1987), characteristic of pituitary GH secretion in healthy dogs (Kooistra et al., 2000). Additionally, the progestin-induced GH overproduction can neither be stimulated with GH-releasing hormone (GHRH), nor can it be inhibited by somatostatin, indicating autonomous secretion (Watson et al., 1987; Selman et al., 1991).

Locally produced GH probably plays a paracrine role in the progestin-induced proliferation and differentiation of mammary epithelium (Feldman et al., 1993; Mol et al., 1995a). Because of the similarity of the progestin-induced epithelial changes in both the

mammary gland and the uterus, it can be hypothesized that GH is also involved in the development of progestin-induced CEH. Although immunoreactive GH (iGH) has been found in uterine epithelial cells of progestin-treated dogs, the absence of mRNA encoding GH in uterine tissue suggests that it does not originate in the uterus (Kooistra et al., 1997). This finding refutes the hypothesis that local production of GH is involved in the pathogenesis of progestin-induced CEH. However, there is still the possibility that mammary-derived GH plays a role in the pathogenesis of CEH. To address this hypothesis, the effects of monthly injections with MPA on the development of CEH were investigated in bitches with surgically excised mammary glands and in a control group of bitches with intact mammary glands.

Materials and methods

Dogs

Thirteen Beagle bitches, 3 to 9 years of age, were housed with outdoor access, fed a commercial dog food once a day, and given water *ad libitum*. The dogs were randomly assigned to two groups (mastectomized and control dogs). The mean age and body weight of the eight mastectomized dogs (5 years and 8 months and 10.5 kg, respectively) did not differ significantly from those of the five control dogs (5 years and 2 months and 9.5 kg, respectively). The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples. Inspection of the vulva (swelling, discharge) was performed and serum progesterone concentration was measured on a regular basis to determine the stage of the oestrous cycle.

Surgical procedure

Prior to surgery food was withheld for 18h. After pre-anaesthetic medication with acepromazine (Placivet 2 %®, Codifar, Wommelgem, Belgium) and methadone (Mephenon®, Federa S.A., Brussels, Belgium) (iv), anaesthesia was induced with propofol (Diprivan 1 %®, Astra Zeneca, Brussels, Belgium) (iv) and maintained with isoflurane (Isoba®Vet, Schering-Plough Animal Health, Middlesex, England) in oxygen. Epidural anaesthesia (between lumbar vertebra L7 and sacrum) was performed in dogs that underwent mastectomy and consisted of lidocain hydrochloride (Xylocaïne 2 %®, NV Astra Zeneca, Brussels, Belgium), bupivacain hydrochloride (Marcaïne 0.5 %®, NV Astra Zeneca, Brussels, Belgium), and morphine hydrochloride (Stellorphine®, Stella, Liège, Belgium). Antibiotics (amoxicillin/clavulanic acid (Synulox®, Pfizer Animal Health, Borgo San Michele, Italy))

were administered pre- and postoperatively. Post surgical analgesia consisted of carprofen (Rimadyl®, Pfizer Animal Health, Dundee, Scotland, UK) and buprenorphine (Temgesic®, Schering-Plough, Brussels, Belgium).

All surgical procedures were performed during anoestrus. The tip of the right uterine horn, the corresponding ovary and the entire mammary gland were excised in eight dogs (mastectomized group). Surgical removal of all mammary gland tissue was performed in 2 sessions with an interval of 4-6 weeks. In the five control dogs only the tip of the right uterine horn and the corresponding ovary were excised.

At the end of the series of MPA injections, the remainder of the uterus and the left ovary were removed (both dog groups), and a sample of the mammary gland was collected (control dogs). In all dogs intra-uterine fluid was collected for bacteriological examination.

After surgical removal, a part of the tissue samples was fixed in a phosphate-buffered formalin solution, processed, and embedded in paraffin; another part was frozen in liquid nitrogen and stored at -80° C until analysis.

Treatment

Treatment was started in both groups after the first surgical procedures, i.e., during anoestrus, and consisted of subcutaneous MPA injections (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) in a dose of 10 mg/kg body weight at intervals of 4 weeks, for a total of 13 administrations.

Blood sample collection

In all dogs, two blood samples, with an interval of 15 min, for determination of the plasma insulin-like growth factor-I (IGF-I) concentrations were collected by jugular venipuncture before surgery and the first MPA administration, and 1, 4, 7, and 10 months after the onset of treatment.

In all dogs, blood samples for the determination of the plasma profiles of GH were collected at 15-min intervals between 0800h and 1400h before surgery and the first MPA administration, and 3, 6, 9, and 12 months after the onset of treatment.

The blood samples were collected after an overnight fast, immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determination

Plasma progesterone concentrations were determined with a previously validated radioimmunoassay (RIA) (Henry et al., 1987). The intra-assay and interassay coefficients of variation were 7.05 % and 8.75 %, respectively. The sensitivity of the assay was 0.005 ng.

Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS, USA). The intra-assay coefficient of variation was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l.

Total plasma IGF-I concentrations were measured after acid-ethanol extraction to remove interfering IGF-binding proteins. Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 g at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7.4), 13 mM Na_2EDTA, and 0.25 % (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7 %. Plasma IGF-I concentrations were measured in a heterologous RIA validated for the dog (Favier et al., 2001). The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Programme (Harbor-UCLA Medical Center, Torrance CA).

Histology and immunohistochemistry

The formalin fixed, paraffin embedded uterine and mammary tissues were cut at 5 µm for histological and immunohistological staining. For standard histological examination the slides were stained with haematoxylin and eosin (HE). Periodic Acid Shiff (PAS) stainings were made of all uteri with CEH (i.e. after treatment with MPA) and were used to assess the secretory activity of the endometrial glands.

For immunohistochemistry, a polyclonal rabbit anti-porcine GH antibody (generous gift of S.J. Dieleman, Department of Herd Health and Reproduction, Faculty of Veterinary Medicine, Utrecht University) was used in an indirect immunoperoxidase staining procedure (PAP method) as follows. The paraffin wax of the sections was removed with xylene and the sections were cleared in 100 % alcohol. Endogenous peroxidase was blocked by incubation in 1 % H_2O_2 in methanol for 20 min at room temperature. The sections were rehydrated by passage through 96 % and 70 % alcohol to distilled water. They were then rinsed in

phosphate-buffered saline (PBS) (3 x 5 min) and preincubated with normal goat serum in PBS (1:20) for 20 min at room temperature. Incubation with the polyclonal rabbit anti-porcine GH antibody in a 1:5000 dilution in PBS took place overnight at 4° C and then for 30 min at room temperature. After incubation, the sections were rinsed for 3 x 5 min in PBS and incubated with goat anti-rabbit serum (Dakopatts Inc., Glostrup) diluted 1:20 for 60 min. Thereafter, the sections were washed in PBS for 3 x 5 min and incubated with rabbit peroxidase-antiperoxidase complex (Dakopatts Inc., dilution 1:100). Immunoreactive GH was visualized using 0.3 % (v/v) H_2O_2 and 0.5 % (w/v) 3,3,-diaminobenzidine tetrahydrochloride (Sigma, Brussels, Belgium) diluted in 0.05 mol Tris/l in HCl buffer, during a 10 min incubation step. After rinsing in distilled water for 2 min the sections were counterstained with Mayer's haematoxylin for 1 min. A canine pituitary gland served as a positive control. Approximately 70 % of the cells in the adenohypophysis stained positive for GH. The negative controls were sections of uterine and mammary tissue processed in the same way, except that normal rabbit antiserum was used instead of rabbit anti-porcine GH antibody.

Isolation of total RNA and cDNA synthesis

Surgically removed mammary and uterine specimens were snap frozen in liquid nitrogen and stored at -80° C until used for RNA isolation. Total RNA was extracted from 0.2 to 1 g tissue using TRIzol reagent (Invitrogen, Groningen, The Netherlands). Purification was performed with the Rneasy Midi Kit (Qiagen, Leusden, The Netherlands) according to the manufacturer's protocol. Purified total RNA was subjected to DNase treatment using the DNA free kit (Qiagen, Leusden, The Netherlands). cDNA synthesis was carried out from 1.5 µg total RNA in 60 µl reaction mix using the iScript^{tm} cDNA synthesis kit (Biorad, Veenendaal, The Netherlands) according to the manufacturer's protocol.

Real time polymerase chain reaction (RT-PCR)

Primers (Table 1) were designed using primer select software of DNA star and primer3 (Rozen and Skaletsky, 2000) according to the parameters outlined in the Biorad icycler manual. Specificity of each primer pair was confirmed by sequencing its product. HPRT and GAPDH genes were used as the non-regulated reference genes for normalization of target gene expressions.

RT-PCR was performed using the Biorad MyiQ detection system (Biorad Laboratories Ltd.) with SYBR green fluorophore. Reactions were performed in a total volume of 25 µl

containing 12.5 µl 2x SYBR green super mix (Biorad Laboratories Ltd.), 1 µl of each primer at 400 nM concentration, 0.8 µl of cDNA, and 9.7 µl RNase and DNase free water.

RT-PCR reactions for each primer set were optimized by performing reactions under a gradient of annealing temperature using five serial 10x dilutions of pooled cDNA from all tissue samples. The protocol used was as follows: denaturation (95° C for 5 min), amplification cycle repeated 40 times (95° C for 30 sec, a PCR specific annealing temperature (Table 1) for 30 sec, 72° C for 30 sec). A melt cure analysis was performed following every run to ensure a single amplified product for every reaction.

All reactions were performed in triplicate for every sample. The reference standard dilution series was repeated on every plate. Triplicate negative controls were run with every experimental plate to assess the specificity and to identify any potential contamination.

Table 1. Primers were designed using primer select software of DNA star and primer3 (Rozen and Skaletsky, 2000) according to the parameters outlined in the Biorad icycler manual.

Target gene	Forward primer	Reverse primer	Optimum annealing temperature (°C)
HPRT	agcttgctggtgaaaaggac	ttatagtcaagggcatatcc	56
GH	ctgctgctcatccagtcgt	caggtccttgagcttctcgt	60
GHR	gcgcatcccagagtctaca	accatgacgaaccccatct	58
IGF-I	tgtcctcctcgcatctctt	gtctccgcacacgaactg	60
PR	caatggaagggcagcataac	cagcactttctaaggcgaca	58
GAPDH	tgtccccacccccaatgtatc	ctccgatgcctgcttcactacctt	58

Statistical analysis

The 6-h plasma profiles of GH were analyzed by means of the Pulsar programme developed by Merriam and Wachter (1982). The programme identifies secretory peaks by height and duration from a smoothed baseline, using the assay standard deviation (SD) as a scale factor. The cut-off parameters G1-G5 of the Pulsar programme were set at 3.98, 2.40, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 5h. The splitting cut-off parameter was set at 0.5 and the weight assigned to peaks was 0.05. The A-, B-, and C- values of the Pulsar programme, used to calculate the variance of the assay, were set at A=0, B=7.2, and C=5. The values extracted from the Pulsar analysis included the mean of the smoothed baseline, the pulse frequency, and the area under the curve (AUC). The AUC was calculated above the zero-level (AUC_0) as well as above the baseline (AUC_{base}).

The AUC_0 for GH, the basal plasma GH concentration and the plasma IGF-I concentration were analyzed by a mixed model with dog as random effect and time, group (mastectomized and control dog group) and their interaction as categorical fixed effects. Using this model, the mastectomized and control dog group were compared. Additionally the evolution over time of these parameters was evaluated in each group separately.

Because the GH pulse frequency and the AUC_{base} for GH were not distributed normally, the two dog groups were compared at each time point (before surgery and MPA injection and 3, 6, 9, and 12 months after the onset of MPA treatment) based on the Mann-Whitney test. Furthermore, the differences within a dog group between 0 months (= before surgery and MPA treatment) and 3, 6, 9, and 12 months after the onset of MPA administration were also analyzed by the Mann-Whitney test.

All statistical tests were performed at a global 5 % significance level, applying the Bonferroni correction for multiple comparisons. All values are expressed as mean ± SEM except for the AUC_{base} for GH and the GH pulse frequency which are expressed as median values. Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

The difference in expression of the target genes by RT-PCR between dogs before and after treatment with MPA were assessed using the pair wise fixed reallocation randomization test incorporated in the software programme REST-XL (Pfaffl et al., 2002) at the 5 % significance level. Dogs which were subjected to mastectomy before treatment with MPA and dogs with intact mammary gland tissue were treated as separate groups for data analysis.

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Results

In both dog groups, the 6-h plasma profile of GH before surgery and MPA treatment was characterized by a fluctuating baseline with occasional distinct elevations, indicating pulsatile secretion of GH. During treatment with MPA, in the control dogs the basal secretion of GH increased and the pulsatile secretion of GH decreased, whereas in the mastecomized dogs the pulsatile secretion persisted, without changes in the basal GH concentration (Figure 1).

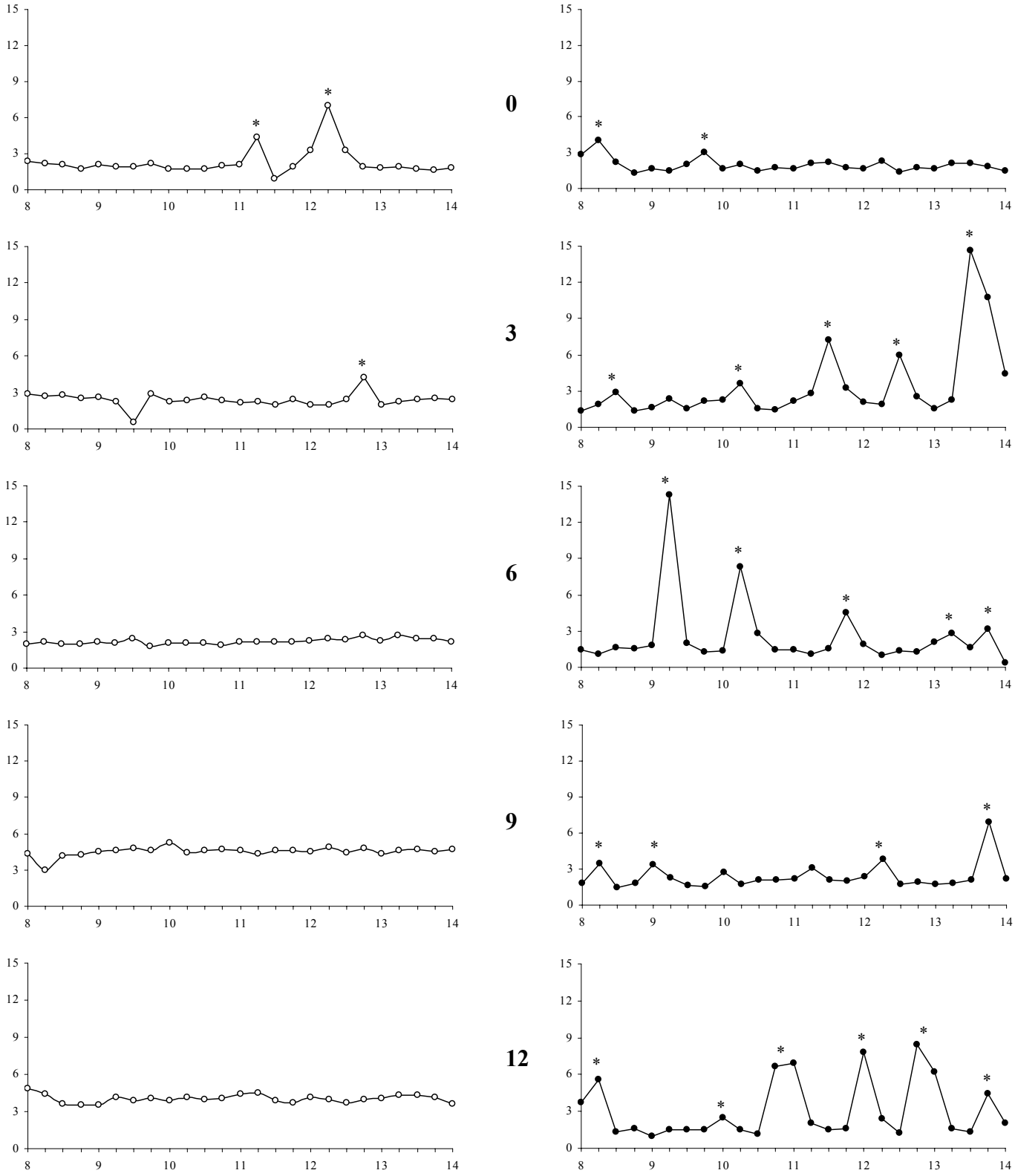

Figure 1. The secretory profiles of GH (µg/l) (=Y-axis) in a five-year-old mastectomized bitch (●) and in a five-year-old control bitch (○). Blood samples were collected at 15-min intervals for 6 h (from 0800h to 1400h = X-axis), before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment. Significant pulses, calculated by the Pulsar programme, are indicated by an asterisk.

The mean basal plasma GH concentration evolved significantly (P = 0.0002) different over time between both dog groups. In the control group the mean basal plasma GH concentration was significantly higher after 9 months (3.4 ± 0.5 µg/l, P < 0.0001) and 12 months (2.8 ± 0.6 µg/l, P = 0.001) of MPA administration than in the mastectomized group (1.4 ± 0.2 µg/l and 1.6 ± 0.2 µg/l, respectively). Within the mastectomized group the mean basal plasma GH concentration before surgery and treatment with MPA (1.6 ± 0.1 µg/l) did not differ significantly from that after 3 (1.9 ± 0.2 µg/l), 6 (1.1 ± 0.1 µg/l), 9 (1.4 ± 0.2 µg/l), and 12 (1.6 ± 0.2 µg/l) months of MPA treatment. Within the control group the mean basal plasma GH concentration before surgery and treatment with MPA (1.7 ± 0.1 µg/l) was significantly lower than that after 9 months (P < 0.0001) and 12 months (P = 0.0002) of MPA treatment (Figure 2a).

The mean AUC_0 for GH evolved significantly (P = 0.0002) different over time between both dog groups. In the control group the mean AUC_0 for GH after 9 months of MPA treatment (20.4 ± 2.9 µg/lx6h) was significantly higher (P = 0.0002) than that in the mastectomized group (9.5 ± 1.5 µg/lx6h). Within the mastectomized group the mean AUC_0 for GH before surgery and treatment with MPA (11.4 ± 0.4 µg/lx6h) did not differ significantly from that after 3 (14.2 ± 1.5 µg/lx6h), 6 (9.8 ± 1.9 µg/lx6h), 9 (9.5 ± 1.5 µg/lx6h), and 12 (11.9 ± 1.3 µg/lx6h) months of MPA treatment. Within the control group the mean AUC_0 for GH before surgery and treatment with MPA (11.3 ± 1.0 µg/lx6h) was significantly lower compared to that after 9 months (20.4 ± 2.9 µg/lx6h, P < 0.0001) and 12 months (17.0 ± 3.6 µg/lx6h, P = 0.005) of MPA treatment (Figure 2b).

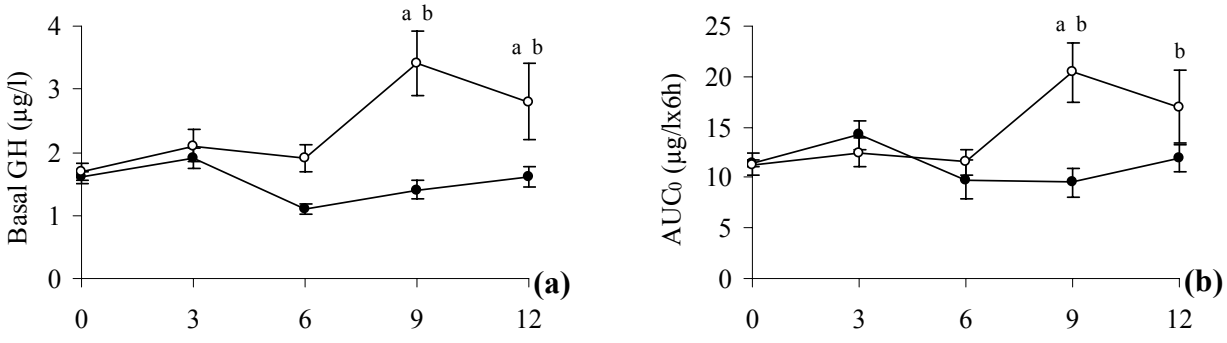

Figure 2. (a) The mean (± SEM) basal plasma GH concentration, **(b)** the mean (± SEM) area under the curve above the zero-level (AUC_0) for GH in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○). Blood samples were collected at 15-min intervals for 6 h before surgery and treatment with MPA (0 months) and 3, 6, 9, and 12 months after the onset of MPA treatment (= X-axis). 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.

The median AUC_{base} for GH in the mastectomized group was significantly higher after 3 months (2.3 µg/lx6h; range = 0 to 9.5 µg/lx6h, P = 0.01) and 9 months (0.2 µg/lx6h; range = 0 to 5.5 µg/lx6h, P = 0.003) of MPA administration than in the control group (at both time points median AUC_{base} = 0 and range = 0 to 0). In both groups, the median AUC_{base} for GH before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The median GH pulse frequency in the mastectomized group was significantly higher after 6 months (2 peaks per 6h; range = 0 to 5 peaks per 6h, P = 0.01) and 9 months (1 peak per 6h; range = 0 to 5 peaks per 6h, P = 0.01) of MPA treatment than that in the control group (at both time points median GH pulse frequency = 0 peaks and range = 0 to 0 peaks per 6h). In both groups the median GH pulse frequency before surgery and MPA treatment did not differ significantly with that after 3, 6, 9, and 12 months of MPA treatment.

The mean plasma IGF-I concentration evolved significantly (P < 0.0001) different over time between both dog groups. In the control group, the mean plasma IGF-I concentration was significantly higher after 4 (116 ± 18 µg/l, P = 0.006), 7 (143 ± 29 µg/l, P = 0.001) and 10 months (180 ± 30 µg/l, P < 0.0001) of MPA treatment than in the mastectomized group (54 ± 9 µg/l, 68 ± 12 µg/l, and 57 ± 8 µg/l, respectively). Within the mastectomized group the mean plasma IGF-I concentration before surgery and treatment with MPA (35 ± 7 µg/l) did not differ significantly from that after 1 (35 ± 7 µg/l), 4, 7, and 10 months of MPA administration. Within the control dog group the mean plasma IGF-I concentration before surgery and treatment with MPA (28 ± 6 µg/l) was significantly lower than that after 4, 7, and 10 months of MPA treatment (P < 0.0001 at all time points) (Figure 3).

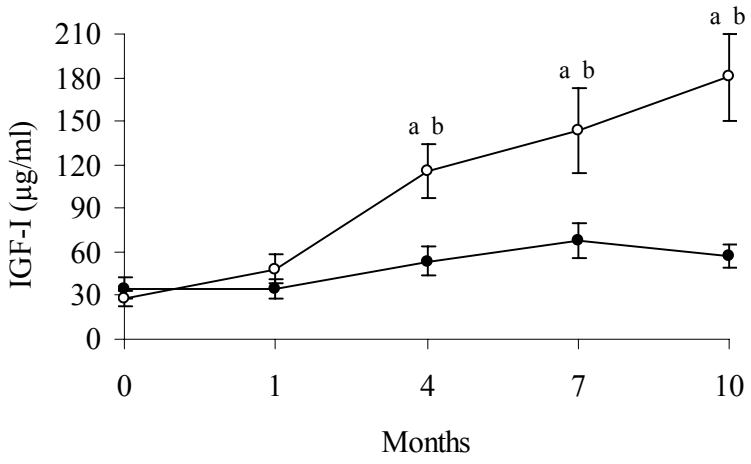

Figure 3. The mean (± SEM) plasma IGF-I concentration in eight mastectomized Beagle bitches (●) and in five control Beagle bitches (○) before surgery and treatment with MPA (0 months) and 1, 4, 7, and 10 months after the onset of MPA treatment. 'a' indicates significant difference between both dog groups and 'b' indicates significant difference within the group compared with the value before surgery and MPA treatment.

Histological examination of the mammary gland tissue before MPA treatment (Figure 4a) revealed inactive mammary tissue characterized by the presence of involuted ductular structures outlined by flattened epithelial cells, absent or small alveolar lumina, and a high stroma/parenchyma ratio. After MPA treatment (Figure 4b), the mammary tissue had differentiated into lobulo-alveolar structures in which milk protein synthesis occurred. The epithelial cells were cuboidal and surrounded by myoepithelial cells, and the stroma/parenchyma ratio was low. In one dog there was proliferating mammary epithelium, with focal hyperplastic changes of ductular epithelial cells, i.e. ductal budding structures. In the mammary tissues before treatment with MPA, immunohistochemical examination did not reveal iGH. Also after MPA treatment no iGH was found in the mammary tissues, except for the dog with focal hyperplastic changes of ductular epithelial cells (Figure 5a). In this dog, iGH was observed in hyperplastic ductular epithelial cells (Figure 5b).

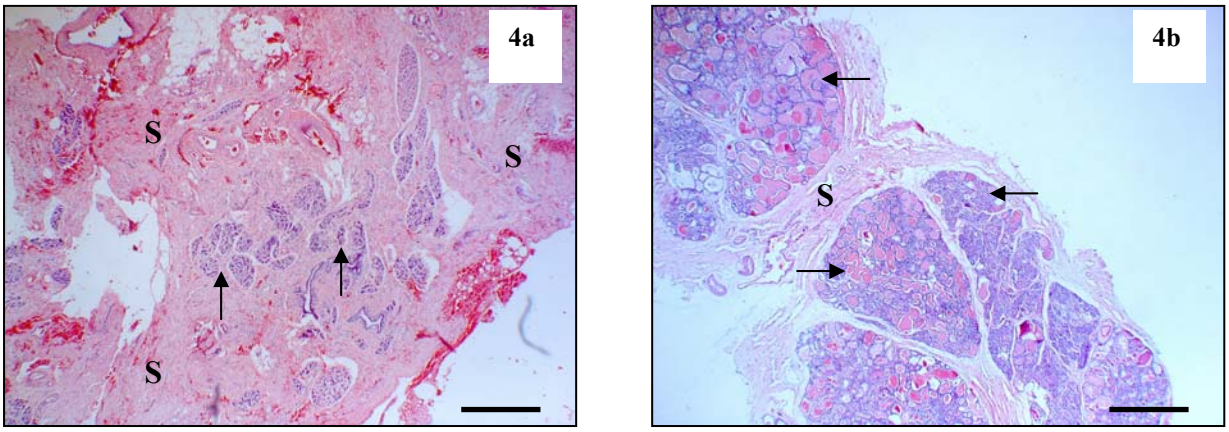

Figure 4. (a) Histology of mammary gland tissue before MPA treatment revealed inactive mammary tissue characterized by the presence of involuted ductular structures with only remnants of lobules (arrows) and an overrepresentation of stromal cells (S). **(b)** Histology after one year of MPA treatment revealed differentiated lobulo-alveolar glandular mammary tissue with milk protein synthesis (arrows). The amount of stroma is strongly reduced (S). HE staining - Bar = 200 µm

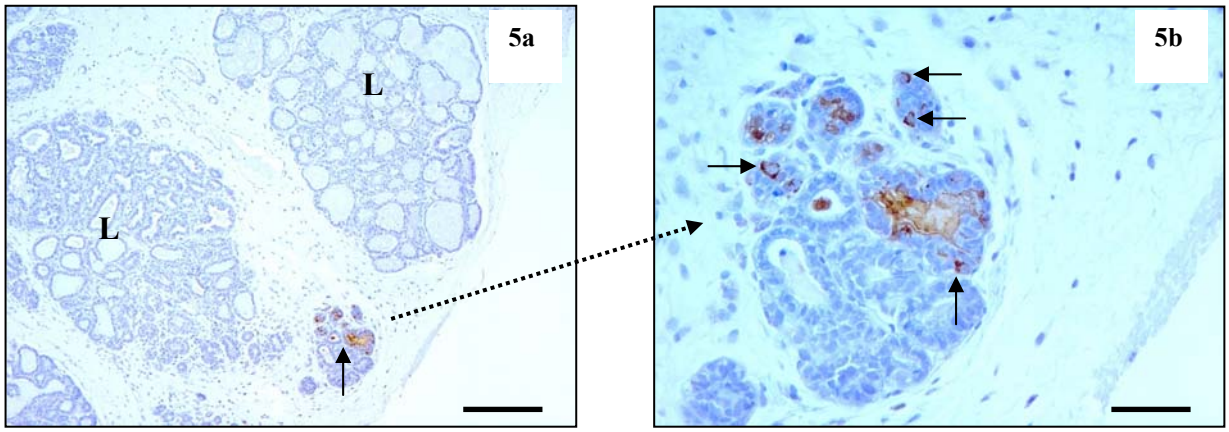

Figure 5. (a) Immunohistochemical examination of mammary gland tissue in a four-year-old Beagle dog after one year of MPA treatment. Immunoreactive GH is absent in lobulo-alveolar structures (L) and is focally present in hyperplastic ductular epithelial cells ("ductal buds") (arrow). Bar = 100 µm. **(b)** Enlargement of a part of figure 5a (dotted arrow). Immunohistochemical localization of GH in the cytoplasm of hyperplastic epithelial cells of mammary gland tissue after one year of treatment with MPA (arrows). Bar = 25 µm.

Histological examination of the uterine tissue before treatment with MPA confirmed that all dogs were in the anoestrous phase of the ovarian cycle and that none of the uteri showed histological signs of CEH (Figure 6a). The inactive endometrium contained small endometrial glands outlined by cuboidal cells. The myometrium consisted of smooth muscle cells with dense nuclei and a small amount of cytoplasm. After MPA administration, CEH was macro- and microscopically present in all dogs of both dog groups. Histological changes were severe and included multiple, large, mucus-filled cystic, PAS-positive endometrial glands, hyperplasia of the epithelium of the endometrial glands, labyrinth-like proliferations of the surface epithelium and endometrial stroma (Figure 6b). In all dogs, there was a variable degree of necrosis in the endometrium characterized by dense, small nuclei indicating pyknosis, and a small to moderate infiltration with inflammatory cells (neutrophils and foci of lymphocytes and plasma cells). Bacteriological examination of the intra-uterine fluid was negative in all dogs. Immunohistochemical examination of the uterine tissues before MPA treatment did not demonstrate the presence of iGH in any dog. Immunohistochemical examination of the uterine tissues after MPA treatment revealed iGH in all dogs of both dog groups. In general, iGH was located in the cytoplasm of hyperplastic glandular epithelial cells (Figures 7a and b).

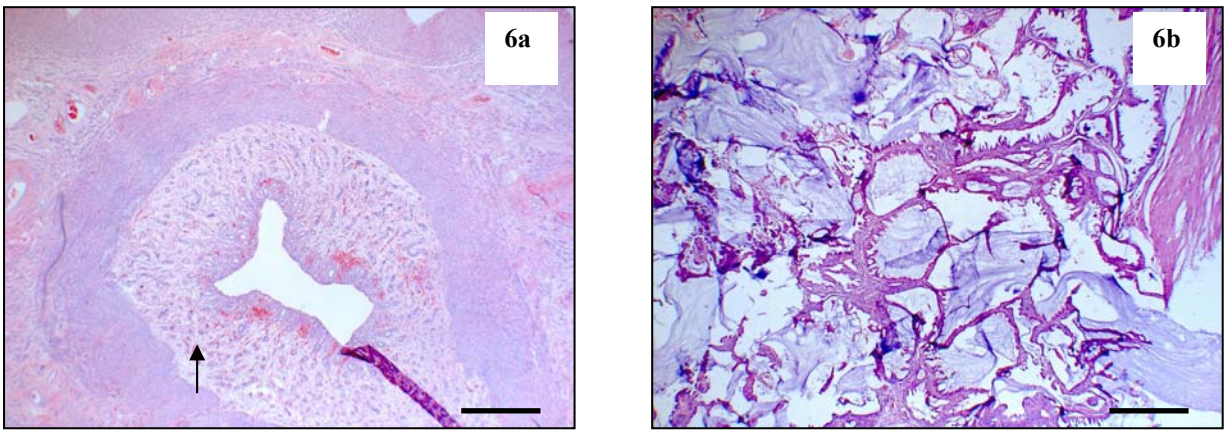

Figure 6. (a) Histology of the uterus before MPA administration with the characteristics of anoestrus, including the endometrium containing small endometrial glands (arrow). **(b)** Severe CEH after MPA administration characterized by multiple, large mucus-filled endometrial cysts and labyrinth-like proliferations of the surface epithelium. HE staining - Bar = 200 µm

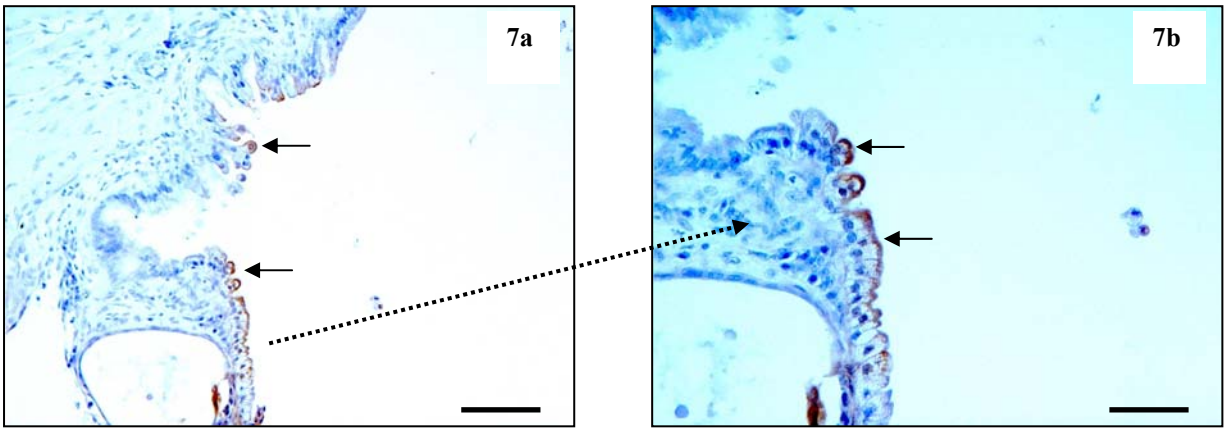

Figure 7. (a) Immunohistochemical examination of the uterus after one year of MPA administration shows the presence of GH in hyperplastic glandular epithelial cells (arrows). Bar = 100 µm. **(b)** Enlargement of a part of figure 7a (dotted arrow). Immunoreactive GH in the uterus is present in the vacuolated cytoplasm of the hyperplastic epithelium (arrows). Bar = 25 µm.

RT-PCR analysis of the mammary gland tissue of the control dogs after MPA treatment demonstrated a significantly (P = 0.002) higher mean GH gene expression than before treatment. The mean mammary GH receptor (GHR) and progesterone receptor (PR) gene expression was significantly (P = 0.01 and P = 0.03, respectively) lower after MPA administration than before treatment. No significant difference was observed in the mean mammary IGF-I mRNA content after MPA treatment compared to that before treatment (Figure 8). In the mastectomized dogs, the mean GH gene expression in the uterus was significantly (P = 0.002) higher after treatment with MPA than before treatment, whereas in the control dogs, the mean IGF-I gene expression was significantly (P = 0.001) higher after MPA administration. A trend (P = 0.052) for a lower mean GHR gene expression after treatment with MPA was found in the uterus of the control dogs compared with that before MPA administration. In both dog groups the mean mRNA content encoding the PR after treatment with MPA was significantly (P = 0.001 for both groups) down regulated compared to the PR mRNA content before treatment (Figure 9).

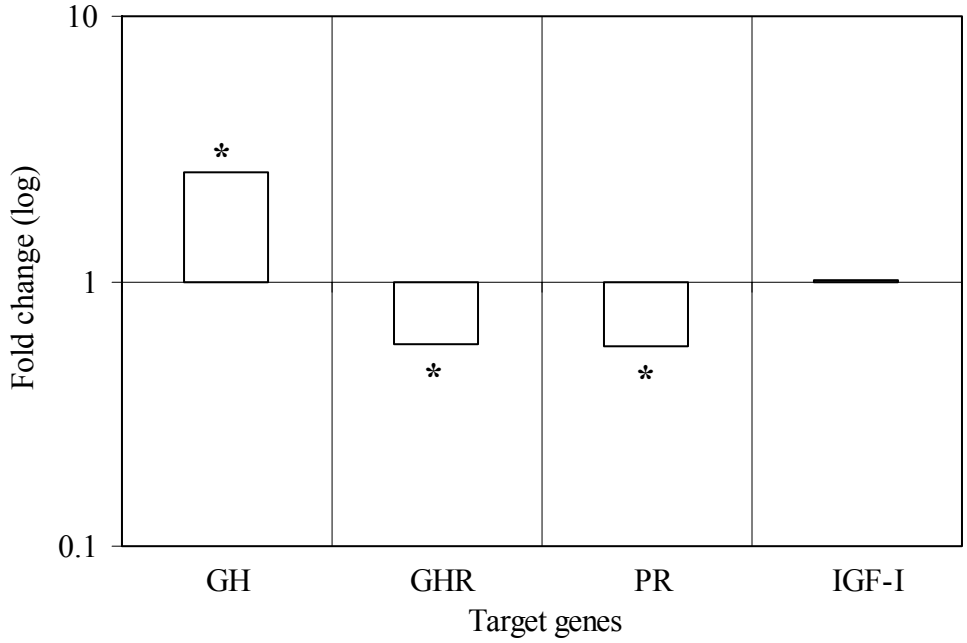

Figure 8. Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the mammary gland of Beagle dogs demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.

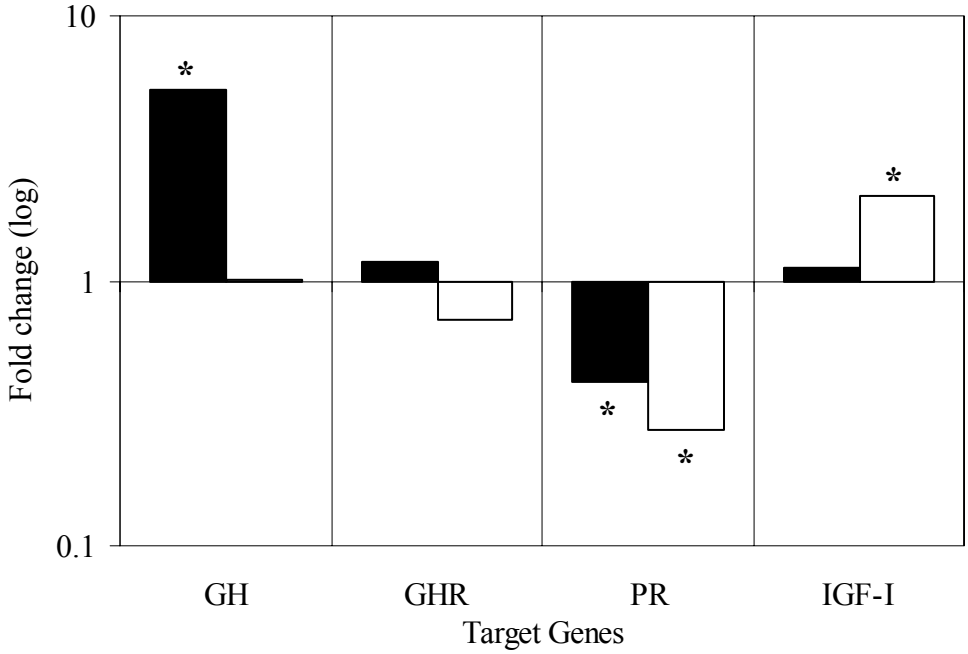

Figure 9. Difference in mean gene expression of growth hormone (GH), the GH receptor (GHR), the progesterone receptor (PR), and insulin like growth factor-I (IGF-I) between before and after treatment with MPA in the uterus of five control Beagle dogs (□) and eight mastectomized dogs (■) demonstrated by RT-PCR. Significant differences in gene expression before and after MPA treatment are indicated with an asterisk.

Discussion

In the control dogs MPA treatment resulted in a higher basal GH secretion and less GH secreted in pulses compared to the mastectomized dogs. This is consistent with (partial) suppression of pituitary GH secretion by progestin-induced GH secretion from the mammary gland. In the mastectomized dogs MPA treatment did not result in a change of the basal GH concentration, the AUC_0 for GH, the AUC_{base} for GH, and the GH pulse frequency, also demonstrating the mammary origin of progestin-induced GH excess. Dogs are not unique among other mammalian species in that the gene encoding GH is expressed in the mammary gland. RT-PCR has revealed that this gene is also expressed in the mammary gland of women and cats (Mol et al., 1995a and 1995b). However, until now the dog is the only species in which it has been demonstrated unequivocally that progestin-induced mammary-derived GH reaches the systemic circulation, and is biologically active (Selman et al., 1994b).

After several months of MPA treatment, there was a low GH pulse frequency and a corresponding low AUC_{base} for GH in the control dogs compared to the mastectomized dogs.

A similarly decreased GH pulsatility has also been reported in women during the second half of pregnancy (Eriksson et al., 1989). In these women the loss of GH pulsatility is due to the release of a placental GH variant (Eriksson et al., 1989). Thus, in both species the loss of GH pulsatility can be ascribed to the negative feedback effects of non-episodically secreted extra-pituitary GH. It has been demonstrated in humans that GH exerts its negative feedback by a stimulation of hypothalamic somatostatin secretion (Berelowitz et al., 1981). Additionally, in the control dogs the progestin-induced elevated GH concentrations had induced raised IGF-I concentrations, which will have contributed to the inhibition of pulsatile pituitary GH secretion (Hartman et al., 1993).

With regard to the physiological role of the progestin-induced mammary GH production, local autocrine and paracrine effects in the mammary gland as well as systemic endocrine effects have to be considered. It is thought that the progestin-induced GH production in the mammary gland leads to local production of IGFs, whereby the growth-promoting effect is modulated by locally synthesized IGF-binding proteins (Mol et al., 1996). Thus a proliferative environment for the glandular epithelium is created, i.e., the autocrine/paracrine background for the physiological proliferation and differentiation of mammary gland tissue. This is in agreement with the results of studies in rats, indicating that GH and GH-induced IGF-I are necessary for mammary development (Feldman et al., 1993; Walden et al., 1998). Also in the ewe mammogenesis is partly dependent upon GH (Kann, 1997). However, until now it has not been demonstrated in this species that this GH originates from the mammary gland.

In the dog, the histological features of the mammary gland vary strongly with the stage of the ovarian cycle (van Garderen et al., 1999). In the anoestrous phase, the glandular tissue is inactive, and microscopically only involuted ductular structures and remnants of lobules are encountered. These were also the characteristics of the mammary gland tissue of our dogs before treatment with MPA. In the luteal phase, after ovulation, there is nodular epithelial proliferation resulting in ductal buds that parallels high endogenous progesterone concentrations in dogs. A similar proliferation of these epithelial cells can be induced by exogenous progestins. In the bitch, ovulation is followed by a relatively long luteal phase, irrespective of pregnancy (Concannon et al., 1975). During this longstanding progesterone-dominated phase of approximately two months, epithelial cells in budding structures proliferate and finally differentiate into lobulo-alveolar structures, fully equipped for milk synthesis. In the present study, mammary gland tissue was examined histologically after a long period of progestin administration. This may be the reason that epithelial buds were

found in only one dog and that most of the glandular tissue had differentiated into lobulo-alveolar structures in which milk synthesis occurred.

In canine mammary tissue iGH and GH gene expression is found predominantly in the ductal epithelial buds in the early and midluteal phase of the ovarian cycle (van Garderen et al., 1997). In contrast, the GH gene expression is diminished in differentiated lobulo-alveolar glandular tissue and in the anoestrous phase of the canine ovarian cycle (van Garderen et al., 1997). Similar immunohistochemical features were found in the present study. Immunoreactive GH was not detected in the mammary gland tissue of the anoestrous dogs before treatment with MPA. Additionally, iGH was absent in the mammary gland tissue of the control bitches treated for twelve months with MPA, except for one dog. In this dog, iGH appeared to be present only in hyperplastic ductular epithelium that consisted of more than 2 cell layers, i.e. epithelial cells in budding structures.

RT-PCR analysis, which is more sensitive than immunohistochemical examination demonstrated that MPA administration increased GH gene expression in mammary gland tissue of the control dogs. Also in another study, increased GH mRNA levels were found in mammary gland tissue of dogs after prolonged treatment with progestins (Mol et al., 1995a). Whereas in primates, GH can bind to the GHR and the prolactin receptor, in nonprimate mammals, such as the dog, GH can only bind to its specific receptor, the GHR (Rutteman et al., 1986). van Garderen et al. (1999) demonstrated that immunohistochemical expression of the GHR is down regulated in completely differentiated alveolar epithelial cells at the end of the luteal phase. Similarly, RT-PCR analysis of the mammary tissue in our control dogs after prolonged MPA treatment confirmed that GHR gene expression was significantly lower compared to this expression before MPA administration.

A possible systemic endocrine effect of mammary GH is the effect on uterine epithelium. After MPA treatment the uterine tissues of both the intact and the mastectomized dogs had iGH. This indicates that progestin-induced mammary-derived GH does not play a key role in the development of CEH in the bitch. Nevertheless, the widespread presence of iGH in uterine epithelial cells of dogs with CEH suggests an association between GH and this uterine pathology. Next to GH, a previous study (De Cock et al., 2000) also demonstrated the presence of iIGF-I in the uterus of dogs with CEH. These growth factors have an intrinsic growth promoting effect on most cell types in the body (Schoenle et al., 1982; Simmen, 1991; Jones and Clemmons, 1995; McCusker, 1998). The growth promoting effect of IGF-I on the uterus is further illustrated by the fact that IGF-I knock out mice have severe hypoplastic uteri (Baker et al., 1996). In addition, IGF-I is involved in the postnatal growth of the uterus in the

rat (Gu et al., 1999) and the pig (Simmen et al., 1992). IGF-I treatment of ovariectomized rats induces uterine endometrial hyperplasia (Sahlin et al., 1994).

RT-PCR revealed that, after MPA treatment, the GH mRNA content of uterine tissue was only increased in the mastectomized dogs and not in the control dogs. Comparable with the progestin-induced GH gene expression in canine mammary tissue during development of ductal epithelial buds (van Garderen et al., 1997), MPA treatment also resulted in more GH gene expression in the uterine epithelial tissue. Apparently, in the control dogs the elevated circulating concentrations of GH of mammary origin and the subsequently increased plasma IGF-I concentrations suppressed uterine GH gene expression, similar to what has been reported for the pituitary (Hartman et al., 1993). MPA treatment also resulted in increased expression of the IGF-I gene in the uterine tissue, but this was significant only in the control dogs. This may be explained by the stimulating effect of the elevated circulating concentrations of GH, originating from the mammary gland, on uterine IGF-I gene expression in these dogs. As the presence of mRNA encoding for the GHR has already been demonstrated in the human uterus (Mercado et al., 1994; Sharara and Nieman, 1995), circulating GH may indeed influence intracellular effects in uterine cells.

In this study MPA did not promote the expression of GHRs in uterine epithelium. This makes it unlikely that increased numbers of GHRs can explain the presence of iGH in uterine cells, as proposed earlier (Kooistra et al., 1997).

Uterine steroid hormone receptor status is a crucial element in pathological conditions such as CEH and pyometra (De Cock et al., 1997; Noakes et al., 2001). Progesterone exposure is considered the initiating step in the development of CEH (Teunissen, 1952; Capel-Edwards et al., 1973; Sokolowski and Zimbelman, 1973; Goyings et al., 1977; Evans and Sutton, 1989; Allen, 1992; Von Berky and Townsend, 1993). In the present study one year of MPA treatment decreased the expression of the gene encoding for PR. In line with this observation, immunohistochemical studies (De Bosschere et al., 2002) found a severe reduction in the canine uterine PR after 12 weeks of MPA administration. The uterine concentration and distribution of progesterone receptors are influenced by hormonal changes during the oestrous cycle and by administration of MPA in the bitch (Dhaliwal, 1997). An increase in plasma progesterone concentration results in a decline of PR content in the uterus (Fernandes et al., 1989; Clarke and Sutherland, 1990; Vesanen et al., 1991; Graham and Clarke, 1997; Vermeirsch et al., 2000) and explains the decrease of PR mRNA with MPA treatment in the present study.

In conclusion, MPA administration caused hypersecretion of GH and IGF-I in the control dogs, but failed to do so in the mastectomized dogs. All dogs of both groups developed CEH, indicating that mammary GH is not a requirement for the development of progestin-induced CEH. Nevertheless, the presence of iGH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.

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Chapter 9

Adenohypophyseal function in bitches treated with medroxyprogesterone acetate

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Abstract

The aim of this study was to investigate the effects of treatment with medroxyprogesterone acetate (MPA) on canine adenohypophyseal function. Five Beagle bitches were treated with MPA (10 mg/kg, every 4 weeks) and their adenohypophyseal function was assessed in a combined adenohypophyseal function test. Four hypophysiotrophic hormones (corticotrophin-releasing hormone, growth hormone (GH)-releasing hormone, gonadotrophin-releasing hormone (GnRH), and thyroid-releasing hormone (TRH)) were administered before and 2, 5, 8, and 11 months after the start of MPA treatment, and blood samples for determination of the plasma concentrations of adrenocorticotrophic hormone (ACTH), cortisol, GH, insulin-like growth factor-I (IGF-I), luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), α-melanocyte-stimulating hormone (α-MSH), and thyroid-stimulating hormone (TSH) were collected at -15, 0, 5, 10, 20, 30, and 45 min after supra-pituitary stimulation.

Medroxyprogesterone acetate successfully prevented the occurrence of oestrus, ovulation, and a subsequent luteal phase. Treatment with MPA did not affect basal and GnRH-induced plasma LH concentrations. The basal plasma FSH concentration was significantly higher at 2 months after the start of MPA treatment than before or at 5, 8, and 11 months after the start of treatment. The maximal FSH increment and the area under the curve (AUC) for FSH after supra-pituitary stimulation were significantly higher before treatment than at 5, 8, and 11 months of MPA treatment. Differences in mean basal plasma GH concentrations before and during treatment were not significant, but MPA treatment resulted in significantly elevated basal plasma IGF-I concentrations at 8 and 11 months. Treatment with MPA did not affect basal and stimulated plasma ACTH concentrations, with the exception of a decreased AUC for ACTH at 11 months. In contrast, the maximal cortisol increment and the AUC for cortisol after supra-pituitary stimulation were significantly lower during MPA treatment than prior to treatment. Treatment with MPA did not affect basal plasma concentrations of prolactin, TSH, and α-MSH, with the exception of slightly increased basal plasma TSH concentrations at 8 months of treatment. Treatment with MPA did not affect TRH-induced plasma concentrations of prolactin and TSH.

In conclusion, the effects of chronic MPA treatment on adenohypophyseal function included increased FSH secretion, unaffected LH secretion, activation of the mammary GH-induced IGF-I secretion, slightly activated TSH secretion, suppression of the hypothalamic-pituitary-adrenocortical axis, and unaffected secretion of PRL and α-MSH.

Introduction

Progestins, such as medroxyprogesterone acetate (MPA), are commonly used to prevent oestrus in the bitch (Schaefers-Okkens, 1996; Romagnoli and Concannon, 2003). Whether the oestrus-preventing properties of progestins in the bitch are due to effects on the hypothalamus, on the pituitary gland, or at the ovarian level is not clear. McCann et al. (1987) and Colon et al. (1993) reported that basal plasma levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) do not change during progestin treatment. Information about the effect of gonadotrophin-releasing hormone (GnRH) on the secretion of LH during progestin treatment is conflicting. GnRH-induced increases in plasma LH concentrations in progestin-treated dogs did not differ from those in control dogs in one study (Colon et al., 1993), while in another study the GnRH-induced LH levels were reduced (McCann et al., 1987). In dogs there is no information on the effect of progestins on GnRH-stimulated FSH concentrations.

In women, progestins are known to prevent ovulation by inhibiting the mid-cycle surges of FSH and LH, whereas the tonic release of these gonadotrophins continues at luteal phase levels (Mishell, 1996; Jain et al., 2004). Long-term use of depot MPA in women does not affect the pituitary responsiveness of LH and FSH to GnRH administration, suggesting that the pituitary is not the primary site for ovulation inhibition in women (Ismael et al., 1987).

Prolonged treatment with progestins in bitches is associated with alterations in the release of pituitary hormones other than gonadotrophins. Progestin administration leads to a decrease in the pituitary responsiveness of growth hormone (GH) to growth hormone releasing hormone (GHRH) (Watson et al., 1987; Selman et al., 1991, 1994a). This change is due to GH release from foci of hyperplastic ductular mammary epithelium (Selman et al., 1994b; van Garderen et al., 1997), leading to elevated plasma GH levels that do not have a pulsatile plasma profile (Watson et al., 1987). The hypothalamic-pituitary-adrenocortical (HPA) axis is suppressed by progestins (McCann et al., 1987; Rutteman et al., 1987; Selman et al., 1997), due to the intrinsic glucocorticoid properties of progestins (Guthrie and John, 1980; Selman et al., 1996; Selman et al., 1997). While basal plasma concentrations of adrenocorticotrophic hormone (ACTH) are only moderately affected (Selman et al., 1997), the basal plasma concentrations of cortisol are markedly decreased (Concannon et al, 1980; McCann et al., 1987; Rutteman et al., 1989; Selman et al., 1997). In addition, the response of ACTH and cortisol to stimulation with corticotrophin-releasing hormone (CRH) may be

reduced (McCann et al., 1987; Selman et al., 1997). Also, in women the administration of MPA causes suppression of the HPA axis (Jones et al., 1974).

With regard to other anterior pituitary hormones, such as prolactin (PRL) and thyroid-stimulating hormone (TSH), there is little information about the effect of progestin treatment on their release. In the bitch, progestin treatment does not seem to affect mean PRL (Concannon et al., 1980) and TSH concentrations (Frank et al., 1979). Information with regard to pituitary responsiveness of PRL to supra-pituitary stimulation is limited to one study, in which MPA administration did not change PRL response to thyroid-releasing hormone (TRH) in ovariohysterectomized, oestradiol-primed bitches (Rutteman et al., 1987). On the other hand, there is evidence that treatment with MPA increases the pituitary PRL responsiveness to TRH in women (Mishell et al., 1977). Finally, there are no reports on the effect of progestins on the pituitary release of α-melanocyte-stimulating hormone (α-MSH).

The aim of the present study was to obtain an integral picture of the effect of progestins on the function of the adenohypophysis in the bitch. For this purpose, the effects of supra-pituitary stimulation on the release of seven adenohypophyseal hormones were studied before and several times during MPA treatment in Beagle bitches by means of a combined anterior pituitary function test (Meij et al., 1996a),

Materials and methods

Dogs

Studies were carried out in five healthy intact Beagle bitches, aged 3 to 9 years and weighing 9.0 to 10.3 kg, that never had been treated with progestins. They were housed with outdoor access, fed a commercial diet once daily, and given water *ad libitum*. They were accustomed to the laboratory environment and procedures such as collection of blood samples. Throughout the study the general condition of the dogs was monitored by physical examination and routine clinical chemistry.

Study design and blood sample collection

In the dogs used in this study, the tip of the right uterine horn and the corresponding ovary had been excised to serve as control tissues in another study. This surgical procedure had been performed 245 ± 42 days (mean ± standard deviation (SD)) before the start of the treatment with MPA. After the surgery, all of the dogs had had one complete oestrous cycle.

Treatment with the synthetic progestin depot preparation MPA (Depo-Promone®, Pharmacia Animal Health, Puurs, Belgium) was begun during anoestrus in a dose of 10 mg/kg body weight subcutaneously at intervals of 4 weeks, for a total of 13 injections. Three days before the start of the treatment with MPA, the mean plasma progesterone concentration was 0.9 ± 0.3 nmol/l (mean ± SD).

Before and at 2, 5, 8, and 11 months after the start of the treatment with MPA, a combined anterior pituitary function test was performed using four releasing hormones (4RH test) according to methods described previously (Meij et al., 1996a; Meij et al., 1996b). Briefly, an intravenous catheter was placed in the cephalic vein of each dog to facilitate rapid sequential injection. Immediately after the collection of the zero blood sample from the jugular vein, four releasing hormones were injected intravenously within 30 sec, in the following order and doses per kg body weight: 1 µg oCRH (Peninsula Laboratories Inc., Belmont, CA, USA), 1 µg hGHRH (hGHRF; Peninsula Laboratories Inc., Belmont, CA, USA), 10 µg GnRH (Fertagyl®; Intervet, Boxmeer, The Netherlands), and 10 µg TRH (Hoffman-La Roche, Basel, Switzerland). During progestin treatment, the 4RH tests were always performed immediately before the next 4-weekly administration of MPA. The clock for blood sampling was started immediately after the administration of the last releasing hormone. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min from the jugular vein and transferred to ice-chilled EDTA-coated and heparinized (for TSH) tubes. Samples were centrifuged at 4º C for 10 min. Plasma was stored at -25º C until assayed for ACTH, cortisol, GH, FSH, LH, PRL, and TSH. Plasma concentrations of α-MSH and insulin-like growth factor-I (IGF-I) were determined in the -15 and 0 min samples only.

Hormone determination

Plasma progesterone concentrations were measured in a previously validated radioimmunoassay (RIA) (Henry et al., 1987). The sensitivity of the assay was 0.0005 ng. The intra-assay and interassay coefficients of variation were 7.05 % and 8.75 %, respectively.

Plasma ACTH concentrations were measured by use of a two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, The Netherlands). The antiserum is highly specific for $ACTH_{1\text{-}39}$. The intra-assay and interassay coefficients of variation were 3.2 % and 7.8 %, respectively, and the sensitivity of the assay was 0.22 pmol/l. The antiserum cross-reacts with neither α-MSH nor ACTH precursors (Hodgkinson et al., 1984; Findling et al., 1990).

Plasma cortisol concentrations were measured by a RIA validated for the dog (Coat-A-Count® Cortisol, Diagnostic Product Corporation, Los Angeles, CA, USA). Intra-assay and

interassay coefficients of variation ranged from 3.0 to 5.1 % and from 4.0 to 6.4 %, respectively. The sensitivity of the assay was 1 nmol/l.

Plasma FSH concentrations were measured by a homologous canine IRMA (AHC004, Biocode SA, Liège, Belgium). The intra-assay and interassay coefficients of variation for values above 1.6 µg/l were 3.2 % and 15 %, respectively. The sensitivity of the assay was 1.5 µg/l.

Plasma GH concentrations were measured by a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS). The intra-assay coefficient of variation was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l

Total plasma IGF-I concentrations were measured in a heterologous RIA, validated for the dog, after acid-ethanol extraction to remove interfering IGF binding proteins (IGFBPs). Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 g at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7·4), 13 mM Na_2EDTA, and 0·25 % (w/v) BSA. The extraction efficiency was 92.5 ± 5.7 %. Plasma IGF-I concentrations were measured in a heterologous RIA validated for the dog (Favier et al., 2001). The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Programme (Harbor-UCLA Medical Center, Torrance CA).

Plasma LH concentrations were measured in a heterologous RIA described previously by Nett et al. (1975), with a few modifications. A rabbit antiserum raised against ovine LH (CSU-204, kindly supplied by G D Niswender, Colorado State University, CO, USA), radio-iodinated bovine LH-7981 as prepared for our bovine LH assay (Dieleman and Bevers, 1987), and canine pituitary standard LER 1685-1 (a gift of Dr L E Reichert, Albany Medical College, NY, USA) were used in this assay. The intra-assay and interassay coefficients of variation for values above 0.5 µg/l were 2.3 % and 10.5 % respectively. The sensitivity of the assay was 0.3 µg/l.

Plasma concentrations of α-MSH were measured by RIA without extraction according to methods described previously (Mol et al., 1987). The intra-assay and interassay coefficients of variation were less than 8 % and 23 %, respectively. The sensitivity of the assay was 5 pmol/l.

Plasma PRL concentrations were measured by a previously validated heterologous RIA (Okkens et al., 1985). The intra-assay and interassay coefficients of variation were 3.5 % and 11.5 %, respectively. The sensitivity of the assay was 0.8 µg/l.

Plasma TSH concentrations were measured in a homologous solid-phase, two-site chemoluminescent enzyme immunometric assay (Immulite canine TSH, Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions. The intra-assay coefficients of variation were 5.0 %, 4.0 % and 3.8 % at TSH levels of 0.20, 0.50, and 2.6 µg/l, respectively. The interassay coefficients of variation were 6.3 % and 8.2 % at TSH levels of 0.16 and 2.8 µg/l, respectively. The sensitivity of the assay was 0.03 µg/l.

Statistical analysis

Differences in body weight before and after the study were assessed in a paired Student's t-test. Plasma α-MSH concentrations below the limit of quantitation were assigned a value of 5 pmol/l. The following response variables were considered: basal hormone value, maximal increment from basal level, and area under the curve above the basal hormone level (AUC). The basal concentration was defined as the mean of the hormone concentrations at -15 and 0 min. The AUC for hormone concentration following stimulation was calculated by the trapezoidal method. Basal hormone concentration, maximal increment, and AUC before and after MPA treatment were compared for the different hormone concentrations using a mixed model with dog as random effect and period (0, 2, 5, 8, and 11 months after MPA treatment) as categorical fixed effects factor. The periods were compared in pairs, applying Tukey's multiple comparisons technique at a global significance level of 5% to obtain adjusted P values.

Additionally, a mixed model was used with dog as random effect and period, time (min after stimulation) and the period by time interaction as categorical fixed effects to investigate whether the time evolution after stimulation differed from period to period.

Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

Ethics of the study

The study protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium.

Results

No signs of oestrus were detected during the 12 months of MPA treatment. In addition, plasma progesterone concentrations were low at 5 months (0.6 ± 0.6 nmol/l; mean ± SD) and 12 months (0.6 ± 0.3 nmol/l) after the start of the treatment. The mean body weight of the dogs on the day of the last MPA injection (12.4 ± 1.6 kg, mean ± SD) was significantly higher (P = 0.02) than that on the day of the first injection of MPA (9.5 ± 0.7 kg).

Basal plasma LH concentrations did not change significantly. In each sampling period, supra-pituitary stimulation resulted in a significant rise (P < 0.001) in plasma LH concentration. The maximal increment and the AUC for LH did not vary significantly with time (Table 1, Figure 1).

Basal plasma FSH concentrations were significantly higher at 2 months after the start of treatment with MPA than before treatment (P = 0.004) or at 5 (P = 0.004), 8 (P = 0.002), or 11 months (P < 0.001) after the start of the treatment. In each sampling period, supra-pituitary stimulation resulted in a significant rise (P < 0.001) in the plasma FSH concentration. The maximal increment was significantly higher before treatment than after 5 (P = 0.01) or 11 months (P = 0.01) of treatment with MPA, while the difference was not significant (P = 0.07) at 8 months. The AUC for FSH was significantly higher before MPA treatment than after 5 (P < 0.001), 8 (P = 0.03), or 11 (P = 0.02) months of treatment with MPA (Table 1, Figure 1).

Table 1. Characteristics of LH and FSH secretion in 5 Beagle dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. The values are expressed as mean ± SEM or median and range. Tmax indicates the time at which maximal supra-pituitary stimulation was observed.

		Months after treatment with MPA			
	Before MPA	2	5	8	11
LH basal (µg/l)	3.0 ± 1.2	4.6 ± 1.9	2.9 ± 0.6	3.9 ± 2.1	2.5 ± 1.1
LH increment (µg/l)	57.4 ± 9.3	58.3 ± 8.9	62.3 ± 10.6	82.9 ± 21.3	82.0 ± 19.3
LH Tmax (min)	10 (5-20)	10 (10-20)	10 (10-20)	10 (10-20)	10 (10-20)
LH AUC (µg/lx45min)	1633 ± 262	1801 ± 363	1797 ± 302	2019 ± 558	2024 ± 386
FSH basal (µg/l)	7.4 ± 0.9	14.2 ± 2.1 **a**	7.5 ± 1.2	6.9 ± 1.2	5.6 ± 0.9
FSH increment (µg/l)	27.2 ± 4.3	22.2 ± 3.8	17.6 ± 3.2 **b**	20.2 ± 4.1	17.5 ± 3.6 **b**
FSH Tmax (min)	20 (10-20)	20 (20-30)	20 (20-45)	20 (10-20)	20 (10-20)
FSH AUC (µg/lx45min)	932 ± 137	756 ± 121	599 ± 116 **b**	645 ± 135 **b**	608 ± 118 **b**

a: significantly different from before treatment and at 5, 8, and 11 months after starting treatment with MPA; **b**: significantly different from before MPA treatment.

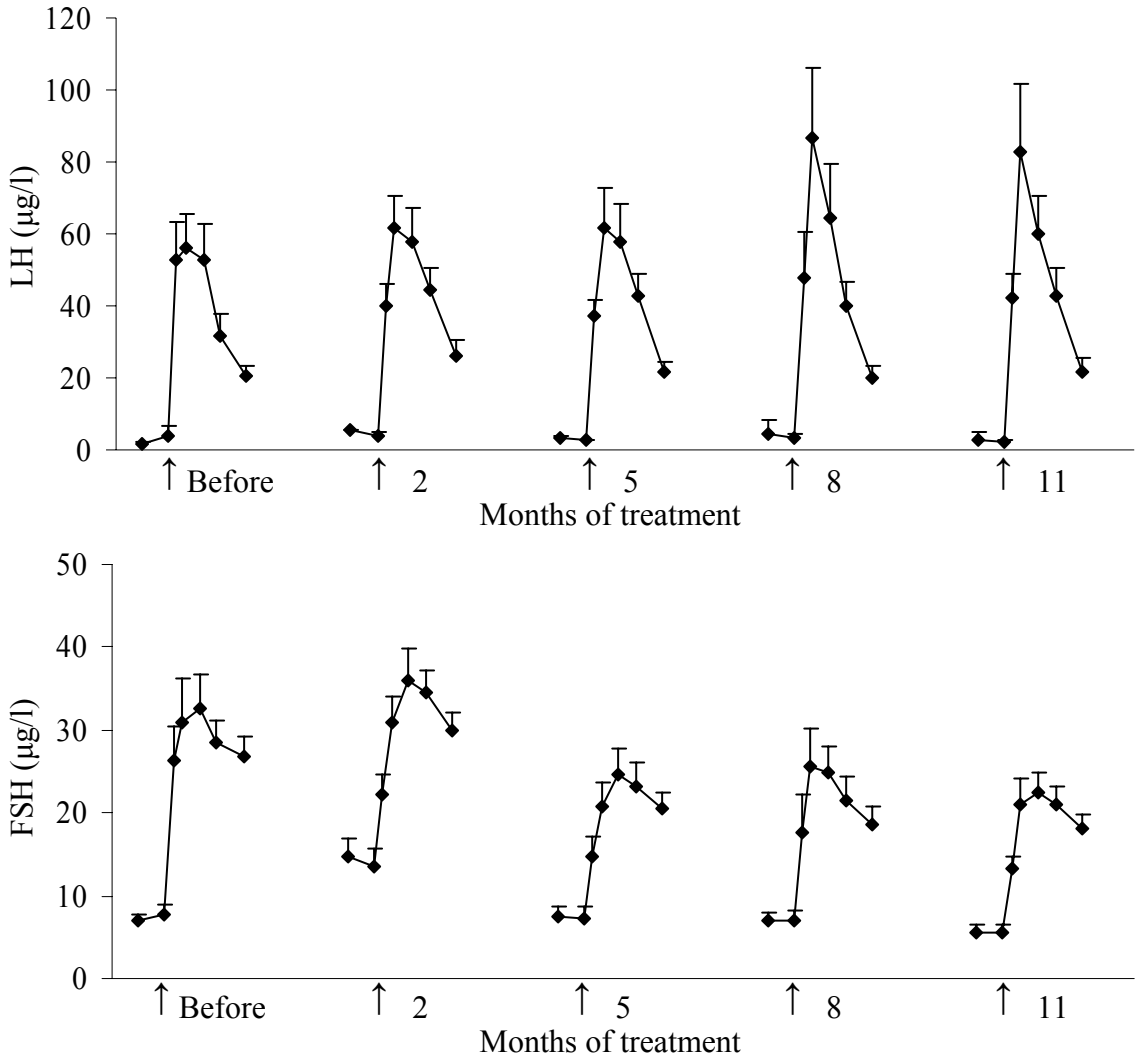

Figure 1. Plasma LH and FSH responses (mean ± SEM) in 5 dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min following the injection of the releasing hormones at 0 min (arrow).

Differences in basal plasma GH concentrations before and during treatment with MPA were not significant. At 8 months after the start of treatment with MPA supra-pituitary stimulation resulted in a significant rise ($P < 0.001$) in plasma GH concentration, while no significant effect was noted during the other periods. Differences in the maximal increment and the AUC for GH before and during the treatment with MPA were not significant (Table 2, Figure 2). Basal plasma IGF-I concentrations were significantly higher at 8 months ($P = 0.02$) and 11 months ($P < 0.001$) of treatment with MPA than before treatment. In addition, basal

plasma IGF-I concentratios were significantly higher at 11 months of treatment than at 2 months (P = 0.02) (Table 2).

Table 2. Characteristics of GH and IGF-I secretion in 5 Beagle dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. The values are expressed as mean ± SEM or median and range. Tmax indicates the time at which maximal supra-pituitary stimulation was observed.

		Months after treatment with MPA			
	Before MPA	2	5	8	11
GH basal (µg/l)	2.7 ± 0.9	2.3 ± 0.3	2.5 ± 0.3	2.7 ± 0.4	3.0 ± 0.5
GH increment (µg/l)	1.8 ± 1.5	0.6 ± 0.3	0.9 ± 0.4	1.1 ± 0.2	0.6 ± 0.4
GH Tmax (min)	5 (-15-45)	20 (10-30)	10 (-15-20)	20 (5-45)	10 (0-20)
GH AUC (µg/lx45min)	5.1 ± 44.4	13.1 ± 9.5	23.3 ± 17.6	34.5 ± 7.4	12.4 ± 13.7
IGF-I basal (µg/l)	45 ± 6	108 ± 25	135 ± 35	159 ± 30 **a**	224 ± 53 **a,b**

a: significantly different from before MPA treatment; **b**: significantly different from 2 months after starting treatment with MPA.

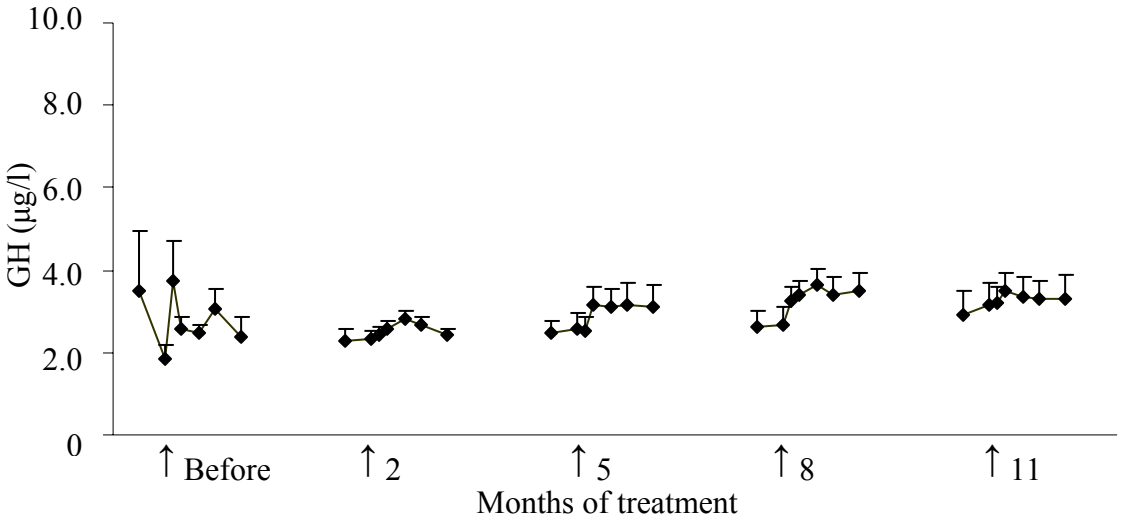

Figure 2. Plasma GH responses (mean ± SEM) in 5 dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min following the injection of the releasing hormones at 0 min (arrow).

Differences in basal plasma ACTH concentrations before and during treatment with MPA were not significant. In each sampling period, supra-pituitary stimulation resulted in a significant rise ($P < 0.001$) in plasma ACTH concentration. Differences in the maximal increment before and during the treatment with MPA were not significant. The AUC for ACTH after 11 months of MPA treatment was significantly lower ($P = 0.05$) than at 2 and 8 months after the start of the MPA treatment (Table 3, Figure 3). Differences in basal plasma cortisol concentrations before and during treatment with MPA were not significant. In each sampling period, supra-pituitary stimulation resulted in a significant rise ($P < 0.001$) in plasma cortisol concentration. The maximal increments decreased significantly during treatment ($P = 0.003$, $P = 0.01$, $P = 0.002$, and $P = 0.002$, respectively). The AUC for cortisol in the four periods of treatment with MPA was significantly lower than that before MPA treatment ($P = 0.002$, $P = 0.003$, $P = 0.002$, and $P = 0.002$, respectively) (Table 3, Figure 3).

Table 3. Characteristics of ACTH and cortisol secretion in 5 Beagle dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. The values are expressed as mean ± SEM or median and range. Tmax indicates the time at which maximal supra-pituitary stimulation was observed.

		Months after treatment with MPA			
	Before MPA	2	5	8	11
ACTH basal (pmol/l)	4.0 ± 0.9	6.0 ± 0.7	4.0 ± 0.6	5.0 ± 0.4	5.0 ± 0.4
ACTH increment (pmol/l)	68 ± 14	59 ± 7	47 ± 5	64 ± 8	42 ± 7
ACTH Tmax (min)	5 (5-10)	10 (5-30)	10 (5-30)	10 (5-10)	10 (5-45)
ACTH AUC (pmol/lx45min)	1840 ± 286	2005 ± 212	1498 ± 94	2027 ± 180	1276 ± 231 **a**
cortisol basal (nmol/l)	48 ± 5	60 ± 11	58 ± 18	53 ± 10	58 ± 14
cortisol increment (nmol/l)	380 ± 39	238 ± 12 **b**	258 ± 19 **b**	231 ± 24 **b**	226 ± 16 **b**
cortisol Tmax (min)	30 (30-45)	30 (30-45)	45 (30-45)	30 (20-45)	30 (20-30)
cortisol AUC (nmol/lx45min)	12199 ± 1121	7825 ± 331 **b**	8042 ± 572 **b**	7785 ± 707 **b**	7585 ± 433 **b**

a: significantly different from 2 and 8 months after starting treatment with MPA; **b:** significantly different from before MPA treatment.

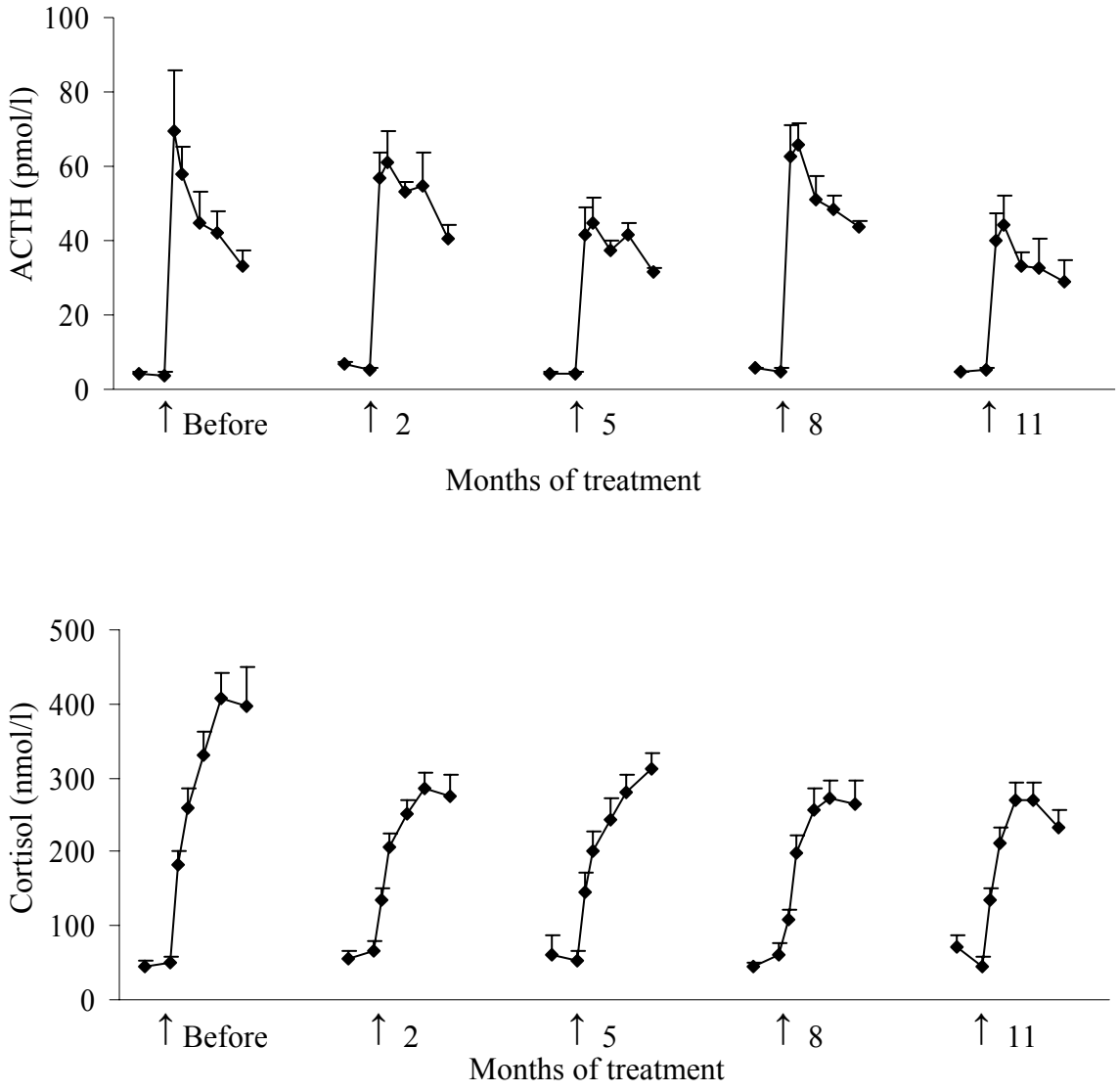

Figure 3. Plasma ACTH and cortisol responses (mean ± SEM) in 5 dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min following the injection of the releasing hormones at 0 min (arrow).

Differences in basal plasma PRL concentrations before and during treatment with MPA were not significant. In each sampling period, supra-pituitary stimulation resulted in a significant rise ($P < 0.001$) in plasma PRL concentration. Differences in the maximal increment and the AUC for PRL before and during treatment with MPA were not significant (Table 4, Figure 4).

Basal plasma TSH concentrations at 8 months of MPA treatment were significantly higher than before treatment ($P = 0.03$) and at 5 months of treatment ($P = 0.05$). In each sampling period, supra-pituitary stimulation resulted in a significant rise ($P < 0.001$) in plasma TSH concentration. Differences in the maximal increment and the AUC for TSH before and during MPA treatment were not significant (Table 4, Figure 4).

Basal plasma α-MSH concentrations were 20.7 ± 4.9 pmol/l before treatment and 20.3 ± 5.3, 21.3 ± 5.3, 25.6 ± 8.4, and 32.1 ± 4.4 pmol/l at the four sampling times during MPA treatment. Differences in the mean plasma α-MSH concentrations were not significant.

Table 4. Characteristics of TSH and PRL secretion in 5 Beagle dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. The values are expressed as mean ± SEM or median and range. Tmax indicates the time at which maximal supra-pituitary stimulation was observed.

		Months after treatment with MPA			
	Before MPA	2	5	8	11
TSH basal (µg/l)	0.09 ± 0.03	0.21 ± 0.06	0.14 ± 0.04	0.22 ± 0.08 **a**	0.17 ± 0.07
TSH increment (µg/l)	0.76 ± 0.25	1.01 ± 0.21	0.88 ± 0.17	1.13 ± 0.27	0.91 ± 0.25
TSH Tmax (min)	10 (5-20)	20 (10-30)	30 (10-30)	10 (10-20)	10 (10-30)
TSH AUC (µg/lx45min)	23.4 ± 6.1	34.1 ± 8.0	30.0 ± 5.6	36.2 ± 7.6	28.7 ± 6.1
PRL basal (µg/l)	8.5 ± 2.5	9.1 ± 3.2	11.4 ± 6.7	4.7 ± 0.8	8.0 ± 2.5
PRL increment (µg/l)	53.2 ± 18.0	42.4 ± 14.3	28.5 ± 7.2	66.2 ± 20.1	33.2 ± 6.6
PRL Tmax (min)	10 (5-20)	10 (5-10)	10 (5-20)	10 (5-10)	10 (5-20)
PRL AUC (µg/lx45min)	1086 ± 231	1038 ± 272	698 ± 211	1418 ± 260	806 ± 148

a: significantly different from before MPA treatment and at 5 months of MPA treatment.

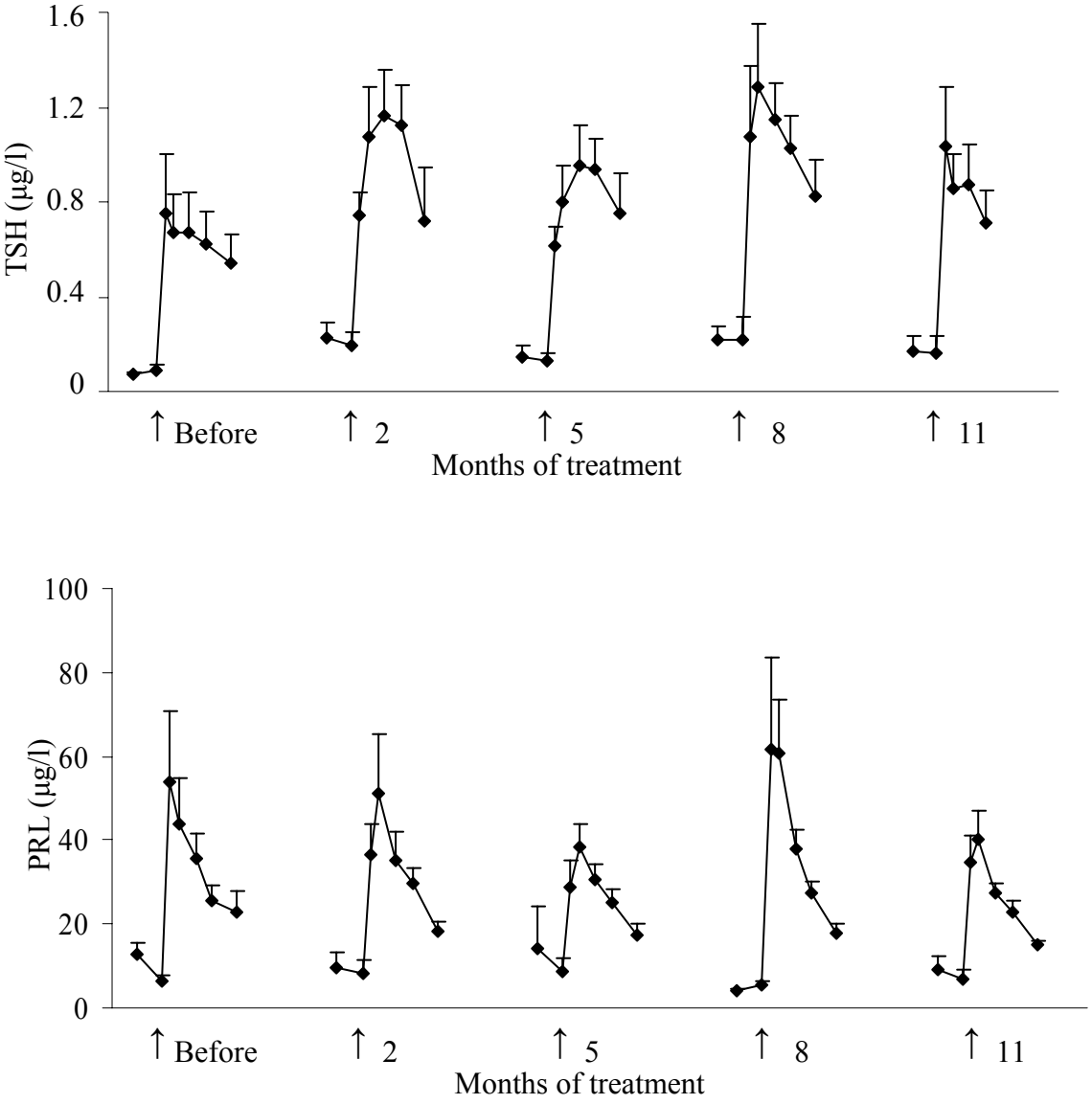

Fig 4. Plasma TSH and PRL responses (mean ± SEM) in 5 dogs in a combined anterior pituitary function test according to Meij et al. (1996a) before and 2, 5, 8, and 11 months after starting treatment with MPA. Blood samples were collected at -15, 0, 5, 10, 20, 30, and 45 min following the injection of the releasing hormones at 0 min (arrow).

Discussion

The tip of one uterine horn and the corresponding ovary were used as control tissues in another study. Nevertheless, all bitches had an oestrous cycle between the surgical procedure and the start of the MPA treatment. Chaffaux and co-workers (1981) demonstrated in dogs

that basal plasma gonadotrophin levels and the response of these hormones to an intramuscular injection of a GnRH analogue were unaffected by unilateral ovariectomy. Furthermore, the difference in plasma FSH concentration between intact and unilateral oophorectomized women was not significant (Cooper and Thorp, 1999). Thus the dogs used in this study can be regarded as having an intact hypothalamus-pituitary-ovarian axis.

The results of this study demonstrate that treatment with MPA affects the hypothalamic-pituitary-ovarian axis. Oestrus, ovulation, and a subsequent luteal phase did not occur in any of the bitches during treatment with MPA, as judged by the lack of external signs of oestrus and low plasma levels of progesterone. The prevention of oestrus by MPA in the present study cannot be ascribed to a significant reduction in circulating levels of either FSH or LH. On the contrary, during the first months of MPA treatment there was an increase in basal plasma FSH without a concomitant change in basal plasma LH. The progestin-induced change in FSH concentration was not observed by Colon et al. (1993) and its recognition may be explained by the repeated sampling employed in the present study.

The results of this study thus indicate that the progestin-induced changes in gonadotrophin release are confined to FSH secretion. One mechanism for the differential control of LH and FSH secretion is gonadal feedback. Both oestradiol and inhibin can specifically suppress FSH synthesis and secretion (Mann et al, 1992; Shupnik, 1996). Reduced secretion of these ovarian hormones can explain the elevated circulating FSH concentration during the first months of MPA treatment. On the other hand, the gonadal peptide activin specifically stimulates FSH secretion. Consequently, a temporary progestin-induced change in the secretion of activin might also explain the initial divergent basal levels of LH and FSH. Other important factors in gonadotrophin control are the frequency and amplitude of GnRH pulses, which have been shown to differentially alter LH and FSH gene expression and secretion (Haisenleder et al., 1991; Shupnik, 1996; Vizcarra et al., 1997).

The elevated basal plasma FSH level during the first months of MPA treatment may thus be due to a direct inhibitory effect of MPA at the ovarian level, resulting in suppression of the ovarian secretion of oestradiol or inhibin, or stimulation of activin release. In this context, increased plasma gonadotrophin levels and low ovarian oestradiol production have been reported in women treated with progestins (Poindexter et al., 1993; Heikinheimo et al., 1996). Observations in monkeys indicate that the inhibitory effects of progesterone on follicular development persist even in the presence of elevated plasma FSH levels, providing additional evidence that progestins may have a direct effect at the ovarian level (Goodman et al., 1982). In women, there are also indications for a hypothalamic site of progestin action

(Couzinet and Schaison, 1993). An initial progestin-induced change in the pattern of hypothalamic secretion of GnRH may therefore be an alternative explanation for the rise in the basal plasma FSH level during the first months of MPA treatment observed in this study.

With continuing MPA treatment, basal plasma FSH returned to pretreatment levels and the pituitary FSH response to supra-pituitary stimulation decreased. These observations may be explained by down-regulation of the pituitary GnRH receptors due to continuous GnRH stimulation (Belchetz et al., 1978). The high GnRH secretion associated with MPA treatment postulated in the previous paragraph may therefore have resulted in desensitization of the response of the gonadotrophs to GnRH. The decline to pretreatment FSH levels and the decrease in responsiveness of pituitary FSH secretion to GnRH are probably part of the oestrus-preventing effects of MPA, because increased FSH secretion is a critical event in the initiation of ovarian folliculogenesis (Kooistra et al., 1999a,b). In other words, MPA treatment for oestrus prevention may prohibit the normal rise in plasma FSH concentration during late anoestrus.

The present results confirm previous findings that progestins alter the GH-IGF-I axis in the bitch (Eigenmann and Rijnberk, 1981). Basal plasma GH concentration tended to increase gradually during the course of the MPA treatment, but the change was not significant. In another study (Concannon et al., 1980), plasma GH concentration did not rise in 27 out of 36 MPA-treated bitches. However, the significant increase in circulating IGF-I concentration during MPA treatment in the present study is consistent with excessive exposure to GH (Selman et al., 1994a). Plasma IGF-I concentration may thus be a more sensitive indicator than plasma GH concentration for the effect of the progestin treatment on the GH-IGF-I axis.

In contrast to previous observations (Meij et al., 1996a), the GH response to supra-pituitary stimulation prior to MPA treatment was not significant. This may be due to ageing (Bhatti et al., 2002), since the dogs in our study were considerably older than those in the study of Meij et al. (1996a). In addition, a relatively high plasma GH level before supra-pituitary stimulation in one of the dogs, probably explained by sampling during a GH pulse, had a substantial influence on basal plasma GH concentration. In agreement with previous observations (Selman et al., 1991), there was no pituitary GH response to supra-pituitary stimulation during MPA treatment in three of the four tests. This may be ascribed to the negative feedback effect of the nonepisodically secreted mammary GH. It has been demonstrated in humans that GH exerts its negative feedback effect by stimulating

hypothalamic somatostatin secretion (Berelowitz et al., 1981). Additionally, the GH-induced elevated circulating IGF-I levels also inhibit pituitary GH secretion (Hartman et al., 1993).

In addition to interacting with the progesterone receptor, MPA also has a relatively high affinity for the glucocorticoid receptor (Selman et al., 1996). Suppression of the HPA axis was thus expected during MPA treatment, as was reported in both humans (Willemse et al., 1990) and dogs (Selman et al., 1994c; Selman et al., 1996). Indeed, we found the cortisol response to stimulation to be decreased with MPA treatment , although ACTH secretion was not demonstrably changed, possible because MPA affects the HPA axis for only 2-3 weeks (Selman et al, 1994c). Although this leads to adrenocortical atrophy (Selman et al, 1997), by 3 weeks (Selman et al., 1994c) or 4 weeks (present study) after administration of MPA, the initially suppressed ACTH (and cortisol) concentrations can have returned to normal.

Treatment with MPA causes significant increases in body weight. The intrinsic glucocorticoid properties of MPA (Selman et al., 1996), leading to increased appetite, may have contributed to this increase in body weight. However, it is difficult to attribute this effect to the glucocorticoid action of MPA alone, since treatment with MPA also affected the GH-IGF-I axis.

As the luteal phase in the bitch progresses, circulating progesterone concentration decreases and PRL secretion increases (Kooistra and Okkens, 2002). This association has also been demonstrated in pregnant and pseudopregnant bitches (Steinetz et al., 1990, Okkens et al. 1997). Moreover, administration of a progesterone receptor antagonist to pregnant bitches causes plasma PRL levels to rise sharply (Galac et al., 2000). Nevertheless, in agreement with previous studies (Concannon et al., 1980; Rutteman et al., 1987), progestin treatment did not affect PRL secretion in the present study. This may be explained by persistently high progestin status.

Mean basal plasma TSH concentration was higher at 8 months after the start of MPA treatment than it was before treatment and at 5 months after the start of treatment. There were no significant alterations in the TSH response to supra-pituitary stimulation. Although basal plasma TSH concentrations were elevated at 8 months after the start of the treatment, they were still within the reference range for TSH in our laboratory. Others found that MPA treatment had no effect on mean circulating TSH levels (Frank et al., 1979), but several authors have reported that total T4 and/or T3 values increase during the progesterone-dominated luteal phase and pregnancy in bitches (Reimers et al., 1984; Dixon, 2004; Feldman and Nelson, 2004). It remains to be seen whether these changes have clinical significance, but

the results of the present study emphasize the importance of considering progestin use when studying the hypothalamic-pituitary-thyroid axis.

In conclusion, MPA successfully prevented the occurrence of oestrus, ovulation, and a subsequent luteal phase. The effects of chronic MPA treatment on adenohypophyseal function included increased FSH secretion, unaffected LH secretion, activation of the mammary GH-induced IGF-I secretion, slightly activated TSH secretion, suppression of the HPA axis, and unaffected secretion of PRL and α-MSH

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Chapter 10

Treatment of growth hormone excess in dogs with the progesterone receptor antagonist aglépristone

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Abstract

Acromegaly or hypersomatotropism in dogs is almost always due to progestin-induced hypersecretion of growth hormone (GH) originating from the mammary gland. The aim of this study was to investigate whether aglépristone, a progesterone receptor antagonist, can be used to treat this form of canine acromegaly. In five Beagle bitches hypersomatotropism was induced by administration of medroxyprogesterone acetate (MPA) for over 1 year. Subsequently, aglépristone was administered. Blood samples were collected before MPA administration, and immediately before, during, and 3.5 and 5.5 weeks after the last administration of aglépristone for determination of the plasma concentrations of GH and insulin-like growth factor-I (IGF-I). In addition, blood samples for the determination of the 6-h plasma profile of GH were collected before MPA administration, before aglépristone administration and 1 week after the last aglépristone treatment.

Administration of MPA resulted in a significant increase of the mean plasma IGF-I concentration, whereas analysis of the pulsatile plasma profile demonstrated a trend ($P = 0.06$) for a higher mean basal plasma GH concentration and a higher mean area under the curve above the zero-level (AUC_0) for GH. Treatment with aglépristone resulted in a significant decrease of the mean plasma GH and IGF-I concentrations. Analysis of the pulsatile plasma profile showed a trend ($P = 0.06$) for a lower mean basal plasma GH concentration and a lower mean AUC_0 for GH 1 week after the last aglépristone treatment compared with these values before aglépristone administration. Three and a half weeks and 5.5 weeks after the last aglépristone administration the mean plasma IGF-I concentration increased again.

In conclusion, aglépristone can be used successfully to treat dogs with progestin-induced hypersomatotropism.

Introduction

Acromegaly is characterized by bony and soft tissue overgrowth due to excessive growth hormone (GH) secretion. The syndrome is known to occur in humans, dogs, and cats. However, the pathogenesis differs among these species. Acromegaly in humans and cats is commonly caused by a somatotroph adenoma of the pituitary gland (Rijnberk et al., 2003) whereas in dogs the GH excess usually originates from an extra-pituitary site (Selman et al., 1994). In dogs, endogenous progesterone secreted during the luteal phase of the oestrous cycle or exogenous progestins such as medroxyprogesterone acetate (MPA) used for oestrus prevention may promote hypersecretion of GH from foci of hyperplastic ductular epithelium of the mammary gland (Eigenmann et al., 1983; Selman et al., 1994; van Garderen et al., 1997). In contrast to the pulsatile secretion pattern of GH in healthy dogs (Takahashi et al., 1981; French et al., 1987, Kooistra et al., 2000), the plasma GH profile in bitches with progestin-induced acromegaly is not pulsatile (Watson et al., 1987). In addition, progestin-induced GH hypersecretion cannot be stimulated with GH-releasing hormone (GHRH) and α-adrenergic agonists, nor can it be inhibited by somatostatin (Watson et al., 1987; Selman et al., 1991). The progestin-induced increase in plasma GH concentrations are associated with increased plasma concentrations of insulin-like growth factor-I (IGF-I) (Selman et al., 1994).

The physical changes of progestin-related hypersomatotropism in dogs tend to develop gradually and consist of prominent skin folds, abdominal distension, and widening of the interdental spaces (Rijnberk, 1996). Due to the insulin-antagonistic action of GH, hyperglycaemia and eventually diabetes mellitus may occur (Eigenmann, 1983a). Ovariectomy is the treatment of choice in female dogs with spontaneous progesterone-induced acromegaly. Plasma GH concentrations rapidly return to normal after ovariohysterectomy (Eigenmann and Venker-van Haagen, 1981). However, in dogs with acromegaly due to progestin administration the detrimental effects of the depot-progestins may continue for a long time after cessation of administration (Eigenmann, 1983b, Rijnberk, 1996).

Progesterone receptor blockers such as aglépristone (RU 46534) and mifepristone (RU 38486) are competitive antagonists of the progesterone receptor (Cadepond et al., 1997; Van Look and Bygdeman, 1989). Aglépristone is the first progesterone receptor blocker licensed for veterinary use and has been used efficiently to terminate pregnancy (Galac et al., 2000; Fieni et al., 2001) and to induce parturition (Baan et al., 2005). Furthermore, it is successfully used for the treatment of fibroadenomatous mammary hyperplasia in cats (Wehrend et al.,

2001; Görlinger et al., 2002; Meisl et al., 2003) and may be a useful adjunct in the medical treatment of endometritis and pyometra in the dog (Trash et al., 2003).

The presence of progesterone receptors in mammary gland tissue of dogs (Lantinga-van Leeuwen et al., 2000) allows for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced hypersomatotropism. The aim of this study was therefore to investigate whether the progesterone receptor antagonist aglépristone can be used to treat canine acromegaly.

Materials and methods

Dogs

Five intact Beagle bitches were housed with outdoor access, fed on a commercial dog food once a day, and given water *ad libitum*. The ages and body weights of the dogs ranged from 3 to 9 years (mean 5 years) and 9.0 to 10.3 kg (mean 9.5 kg), respectively. The dogs were accustomed to the laboratory environment and procedures such as collection of blood samples.

Treatments

The five Beagle bitches were treated with the synthetic progestin depot preparation Depo-Promone® (medroxyprogesterone acetate (MPA), Pharmacia Animal Health, Puurs, Belgium). MPA treatment was started during anoestrus and consisted of subcutaneous injections in a dosage of 10 mg/kg body weight at 4-week intervals for a total of 14 (3 dogs) or 15 (2 dogs) injections.

Five days (= day 0) and six days (= day 1) after the last MPA injection (= day -5), aglépristone (Alizin®, Virbac Animal Health, Barneveld, The Netherlands) was administered subcutaneously in a dosage of 10 mg/kg body weight. One (= day 8), two (= day 15), and three (= day 22) weeks later a single aglépristone injection was given in the same dose. Three randomly chosen dogs received the first aglépristone injection after the 14th MPA injection, and the other two dogs after the 15th MPA injection so that these two dogs could serve as control dogs for the three dogs that received the aglépristone injection first.

Blood sample collection

Blood samples for determination of the plasma progesterone concentrations were collected 5 and 12 months after the start of the MPA treatment.

Blood samples for determination of the plasma concentrations of GH and IGF-I were collected before MPA treatment, at days -9, -8, -7, -5, -3, -2, -1, 0 (= immediately before aglépristone treatment and after MPA treatment for over 1 year), at days 1, 3, 5, 7, 8, 11, 13, 15, 18, 20, 22, 25 (= during aglépristone treatment), and at days 46 and 60 (= 3.5 and 5.5 weeks after the last aglépristone injection). On days of treatment (MPA or aglépristone), blood samples were collected prior to the drug administration.

Blood samples for determination of the pulsatile plasma profiles of GH were collected at 15-min intervals between 0800h and 1400h before MPA administration, before aglépristone administration, and 1 week after the last administration of aglépristone (at day 28).

All blood samples were collected by jugular venipuncture after an overnight fast, immediately transferred to ice-chilled EDTA-coated tubes and centrifuged at 4^{o} C for 10 min. Plasma was stored at -25^{o} C until assayed.

Hormone determination

Plasma progesterone concentrations were determined with a previously validated radioimmunoassay (RIA) (Henry et al., 1987). The intra-assay and interassay coefficients of variation were 8.8 % and 7.1 %, respectively. The sensitivity of the assay was 0.005 ng.

Plasma GH concentrations were measured using a commercially available RIA for porcine and canine GH (PGH-46HK; Linco Research, St. Charles MS). The intra-assay coefficient of variation was 7.6 % at a plasma concentration of 4.4 µg/l. The sensitivity of the assay was 1 µg/l.

Total plasma IGF-I concentrations were measured after acid-ethanol extraction to remove interfering IGF binding proteins. Plasma IGF was extracted using a mixture of 87.5 % (v/v) ethanol and 12.5 % 2 M formic acid. Tubes containing 100 µl plasma and 400 µl of the ethanol-formic acid mixture were mixed thoroughly and incubated for 30 min at room temperature. After centrifugation for 30 min at 5500 g at 4° C, a 50 µl aliquot of the supernatant was diluted 1:50 with assay buffer containing 63 mM Na_2HPO_4 (pH 7.4), 13 mM Na_2EDTA, and 0.25 % (w/v) BSA. The extraction efficiency amounted to 92.5 ± 5.7 %. Plasma IGF-I concentrations were measured in a heterologous RIA validated for the dog (Favier et al., 2001). The intra-assay coefficient of variation was 8.6 % at a plasma concentration of 100 µg/l. The sensitivity of the assay was 10 µg/l. IGF-I antiserum AFP4892898 and human IGF-I for iodination were obtained from the National Hormone and Peptide Programme (Harbor-UCLA Medical Center, Torrance CA).

Statistical analysis

To study the effect of MPA administration, the plasma GH and IGF-I concentrations before and after MPA treatment were compared using a mixed model with dog as random effect and period (2 levels: before and after MPA treatment) as categorical fixed effect.

In order to assess the overall effect of aglépristone on the plasma GH and IGF-I concentrations, a mixed model was fitted with dog as random effect and period (3 levels: immediately before aglépristone, during aglépristone, and 3.5 and 5.5 weeks after the last aglépristone treatment) as categorical fixed effect. The three periods were compared pairwise using Tukey's multiple comparisons technique.

To study the evolution of the GH and IGF-I concentrations during the aglépristone period, a mixed model was fitted with dog as random effect and time since start of aglépristone treatment as continuous fixed effect at a global significance level of 5 %.

The plasma GH and IGF-I concentrations before MPA treatment were compared with the concentrations 3 days after the last aglépristone injection (i.e. at day 25) using a mixed model with dog as random effect and period (2 levels: before MPA treatment and 3 days after the last aglépristone injection) as categorical fixed effect.

To evaluate the effect of withdrawal of aglépristone treatment, the two last measurements during aglépristone treatment (days 22 and 25) were compared with the two measurements after aglépristone treatment (days 46 and 60) using a mixed model with dog as random effect and period (2 levels: days 22 and 25 and days 46 and 60) as categorical fixed effect.

The 6-h plasma profiles of GH were analyzed by means of the Pulsar programme developed by Merriam and Wachter (1982). The programme identifies secretory peaks by height and duration from a smoothed baseline, using the assay standard deviation (SD) as a scale factor. The cut-off parameters G1-G5 of the Pulsar programme were set at 3.98, 2.40, 1.68, 1.24, and 0.93 times the assay SD as criteria for accepting peaks 1, 2, 3, 4, and 5 points wide, respectively. The smoothing time, a window used to calculate a running mean value omitting peaks, was set at 5h. The splitting cut-off parameter was set at 0.5 and the weight assigned to peaks was 0.05. The A-, B-, and C- values of the Pulsar programme, used to calculate the variance of the assay, were set at A=0, B=7.2, and C=5. The values extracted from the Pulsar analysis included the mean of the smoothed baseline, the pulse frequency, and the area under the curve (AUC). The AUC was calculated above the zero-level (AUC_0) as well as above the baseline (AUC_{base}). The difference in variables before MPA treatment,

before aglépristone administration, and 1 week after the last aglépristone injection (i.e. at day 28), were analyzed by the signed rank test with dog as block.

All values are expressed as mean ± SEM or median. Statistical significance was defined at $P \leq 0.05$. Analyses were performed with SAS version 9.1 for Windows (Insightful Corp., Seattle, US).

Ethics of the study

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University.

Results

During MPA administration none of the dogs showed signs of oestrus and the mean plasma progesterone concentration was low 5 months (0.2 ± 0.2 ng/l) and 12 months (0.2 ± 0.1 ng/l) after the start of the MPA treatment. The mean body weight of the dogs on the day of the last injection of MPA (12.4 ± 0.7 kg) was significantly higher ($P < 0.02$) than that on the day of the first injection of MPA (9.5 ± 0.3 kg) (paired Student's t-test). Signs of acromegaly became apparent in three of the five dogs after 6 months of MPA treatment and consisted of prominent skin folds especially on the head, an increase in the interdental spaces, inspiratory stridor, and snoring.

MPA administration for over 1 year resulted in a higher mean plasma GH concentration (2.3 ± 0.5 µg/l) compared to that before MPA treatment (1.9 ± 0.3 µg/l), although this difference did not reach statistical significance (Figure 1a). However, the mean plasma IGF-I concentration after 1 year of MPA administration (146 ± 25 µg/l) was significantly ($P = 0.003$) higher compared to that before MPA treatment (36 ± 6 µg/l) (Figure 1b).

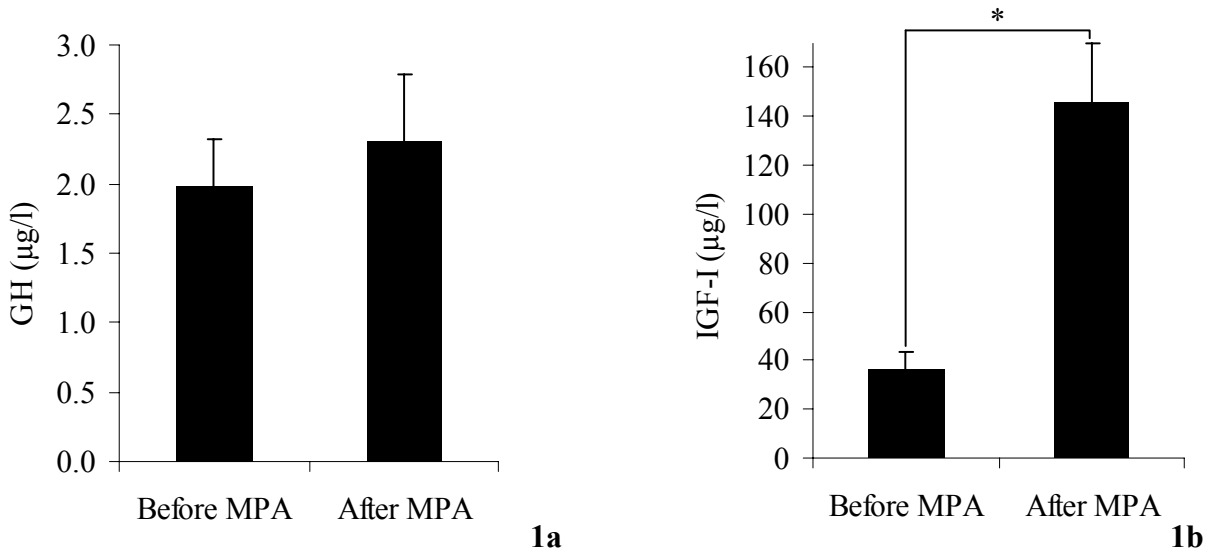

Figure 1. Mean (+ SEM) plasma concentrations of GH **(1a)** and IGF-I **(1b)** in 5 Beagle dogs before administration of medroxyprogesterone acetate (before MPA) and after 1 year treatment with MPA (after MPA). Significant differences between periods are indicated with an asterisk.

Analysis of the pulsatile plasma GH profiles after 1 year of MPA administration revealed a trend (P = 0.06) for a higher mean basal plasma GH concentration and a higher mean AUC_0 for GH compared to these values before MPA treatment (Table 1 and Figure 2).

Table 1. Area under the curve above the baseline (AUC_{base}) for GH (µg/lx6h), AUC above the zero-level (AUC_0) for GH (µg/lx6h), basal plasma GH concentration (µg/l), and GH pulse frequency (peaks per 6h) in 5 Beagle dogs before MPA administration (before MPA), after 1 year of MPA administration (= before aglépristone administration) (before A), and 1 week after the last aglépristone administration (after A).

	AUC_{base} (mean ± SEM)	AUC_0 (mean ± SEM)	GH pulse frequency (median)	Basal GH (mean ± SEM)
Before MPA	1 ± 0.4	11.3 ± 1.1	1	1.7 ± 0.1
Before A	0 ± 0.0	17.0 ± 3.6	0	2.8 ± 0.6
After A	0.3 ± 0.2	8.8 ± 0.7	0	1.4 ± 0.1

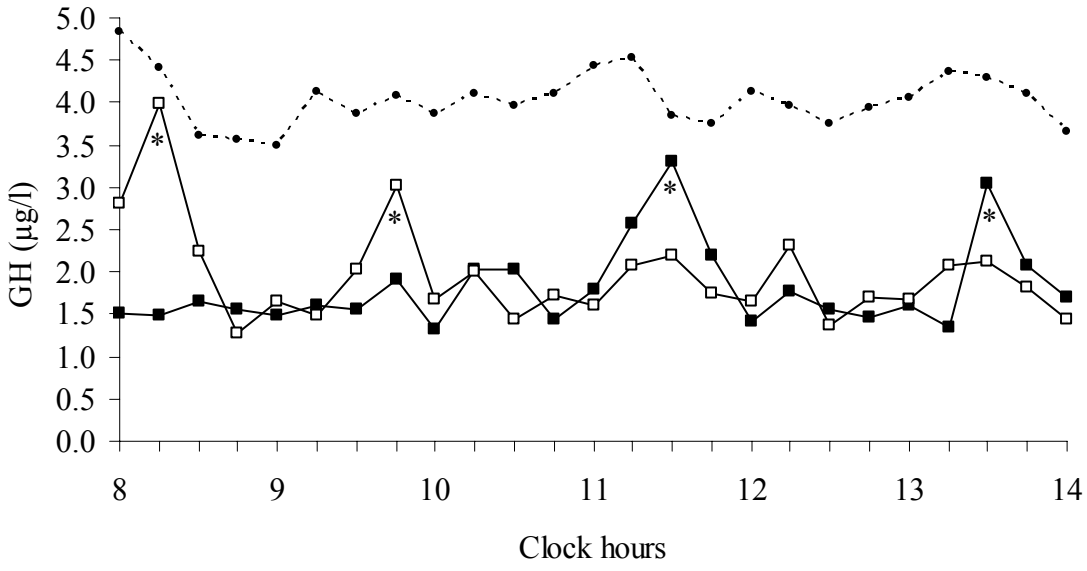

Figure 2. The plasma profiles of GH in a 4-year-old Beagle bitch. Blood samples were collected at 15-min intervals between 0800h and 1400h, before MPA administration (□), after 1 year of MPA administration (= before aglépristone administration) (dotted line), and 1 week after the last aglépristone treatment (■). Significant pulses, calculated by the Pulsar programme, are indicated by an asterisk.

The administration of aglépristone caused no side effects except a short-term skin irritation at the site of the injection in one dog. The mean plasma GH concentration immediately before aglépristone administration (2.3 ± 0.5 µg/l) was significantly higher than that during (1.7 ± 0.3 µg/l; $P < 0.0001$) and 3.5 and 5.5 weeks after (1.8 ± 0.3 µg/l; $P = 0.018$) the last administration of aglépristone (Figure 3a). Also the mean plasma IGF-I concentration immediately before aglépristone administration (146 ± 25 µg/l) was significantly higher than that during (108 ± 27 µg/l; $P < 0.0001$) administration of aglépristone (Figure 3b). In the weeks that aglépristone was administered, analysis of the course of the circulating hormone concentrations indicated a significant decrease in plasma GH ($P = 0.005$) and IGF-I ($P < 0.0001$) concentrations (Figures 4a and b, respectively).

The plasma GH and IGF-I concentrations before MPA treatment did not differ significantly from these concentrations 3 days after the last aglépristone injection (i.e. day 25).

Analysis of the pulsatile plasma GH profiles revealed a trend ($P = 0.06$) for a lower mean basal plasma GH concentration and a lower mean AUC_0 for GH 1 week after the last aglépristone administration (i.e. day 28) compared with these concentrations before aglépristone treatment. The AUC_{base} for GH increased again 1 week after the last aglépristone

injection compared with this concentration before aglépristone administration, although this difference did not reveal statistical significance (Table 1 and Figure 2).

The mean plasma GH concentration at the end of the period of aglépristone administration (i.e. days 22 and 25) (1.5 ± 0.1 µg/l) did not differ significantly from that at 3.5 and 5.5 weeks (i.e. days 46 and 60) after withdrawal of aglépristone (1.8 ± 0.3 µg/l). However, the mean plasma IGF-I concentration at the end of the period of aglépristone administration (88 ± 22 µg/l) was significantly ($P < 0.0001$) lower compared with that 3.5 and 5.5 weeks after withdrawal of aglépristone (124 ± 29 µg/l).

In the two control dogs that did not receive aglépristone after the 14th MPA injection, the mean plasma concentrations of GH and IGF-I before the 14th administration of MPA (1.6 ± 0.1 µg/l and 105 ± 28 µg/l, respectively) were not different from those before the 15th administration of MPA (1.7 ± 0.1 µg/l and 116 ± 26 µg/l, respectively).

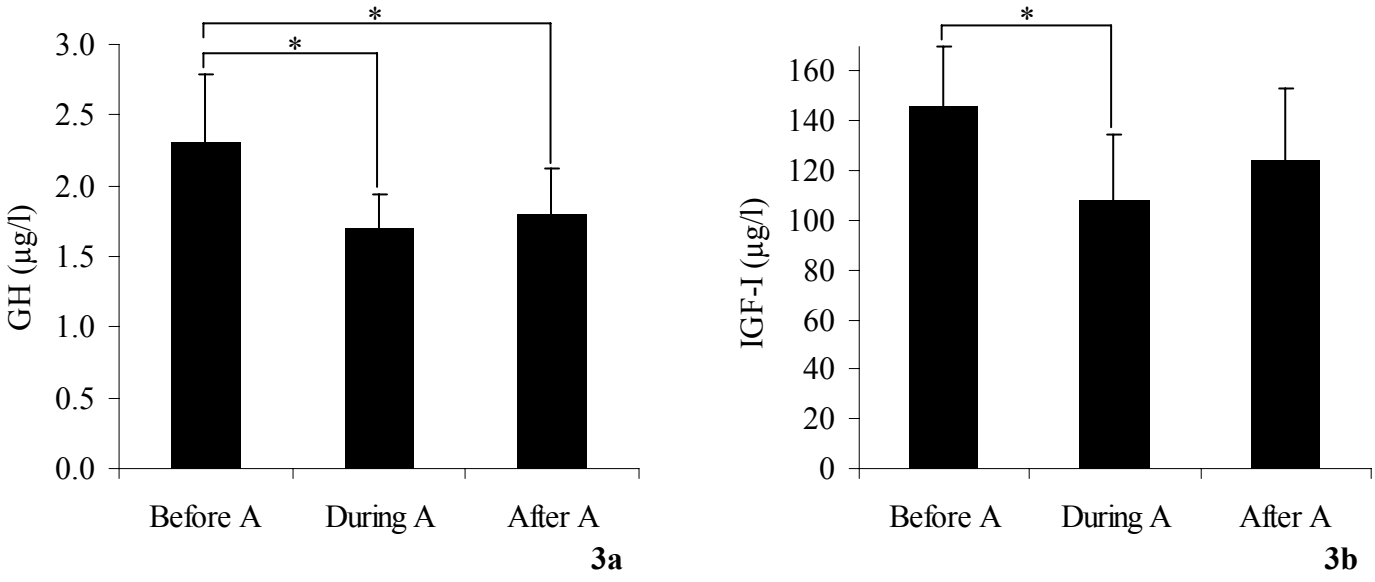

Figure 3. Mean (+ SEM) plasma concentrations of GH **(3a)** and IGF-I **(3b)** in 5 Beagle dogs immediately before aglépristone (before A), during aglépristone (during A), and 3.5 and 5.5 weeks after the last aglépristone administration (after A). Significant differences between periods are indicated with an asterisk.

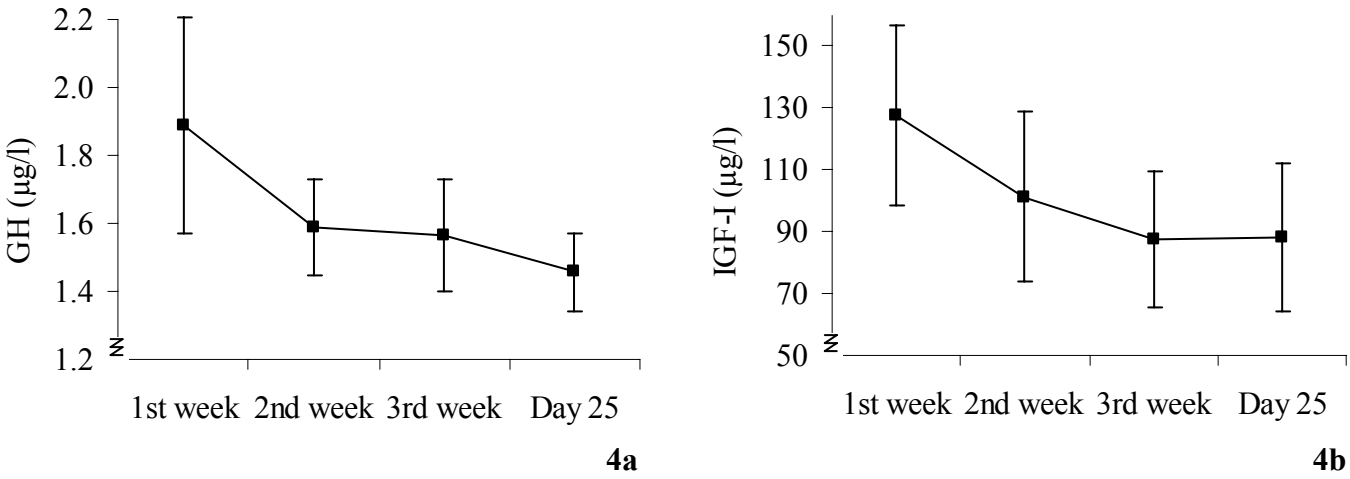

Figure 4. Mean (± SEM) plasma concentrations of GH **(4a)** and IGF-I **(4b)** in 5 Beagle dogs at 1 week, 2 weeks, 3 weeks, and at day 25 during aglépristone administration.

Discussion

In three of the five Beagle dogs, signs of acromegaly became apparent after 6 months of MPA administration. In line with these changes, the mean plasma IGF-I concentrations were raised. Moreover, analysis of the pulsatile plasma profile showed a trend for a higher mean basal GH concentration and a higher mean AUC_0 for GH in the five Beagle dogs. These findings are consistent with progestin-induced hypersecretion of GH (Concannon et al., 1980; Rijnberk et al., 1980; Eigenmann and Rijnberk, 1981; Eigenmann et al., 1983).

Previous studies identified foci of hyperplastic ductular epithelium of the mammary gland as the site of origin of GH excess induced by progestins (Selman et al., 1994; van Garderen et al., 1997). The expression of the gene encoding GH has been demonstrated in canine mammary gland tissue, and sequence analysis has revealed that this gene is identical to the pituitary GH gene (Mol et al., 1995; Mol et al., 1996). Immunohistochemical studies have demonstrated the intracellular co-localization of both the progesterone receptor and GH in progestin-exposed, hyperplastic canine mammary epithelial tissue, whereas immunoreactive GH could not be demonstrated in progesterone receptor-negative epithelial cells (Lantinga-van Leeuwen et al., 2000). These observations are consistent with the central role of progestins in GH gene expression in the canine mammary gland and allow for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH hypersecretion.

To the authors' knowledge, treatment of acromegalic dogs with the progesterone receptor antagonist aglépristone (RU 46534) has not been reported before. The results of the present study demonstrate that progestin-induced elevations in circulating GH and IGF-I concentrations decrease significantly during treatment with aglépristone. At the end of the aglépristone treatment period the plasma GH and IGF-I concentrations did not differ significantly from those before MPA administration. Our findings are in agreement with those of Watson et al. (1987) who found that administration of the antiprogestin mifepristone (RU 38486) resulted in a decrease of plasma GH concentrations and normalization of plasma IGF-I concentrations in bitches with progestin-induced acromegaly.

The mean basal plasma concentrations of GH and IGF-I in the two control dogs that did not receive aglépristone after the 14th MPA injection remained high and did not decrease. This indicates that indeed aglépristone is responsible for the lowering of the plasma GH and IGF-I concentrations in the dogs treated with the progesterone receptor blocker, and that this lowering is not due to, for example, a waning effect of MPA on GH and IGF-I secretion.

The 6-h pulsatile plasma profile of GH represents a more sensitive way of analyzing the effects of different treatments on the secretion of GH than the plasma GH concentration itself. Analysis of the plasma GH profiles revealed that the mean basal plasma GH concentration and AUC_0 for GH tended to decrease at the end of the treatment with the progesterone receptor blocker compared with these values before aglépristone administration. In addition, the AUC_{base} for GH, i.e., the amount of GH secreted in pulses, increased again during aglépristone treatment, although this difference did not reveal statistical significance. Thus, treatment with aglépristone resulted in partial restoration of the normal pulsatile GH secretion. Higher dosages of aglépristone may result in complete normalization of the secretion pattern of GH.

Plasma IGF-I concentrations are generally regarded as more sensitive indicators of the GH status than plasma GH concentrations (Clemmons and Strasburger, 2004). Consequently, the significantly higher plasma IGF-I concentrations at days 46 and 60 compared with those at days 22 and 25 therefore suggest increased GH exposure, despite the fact that analysis of the plasma GH concentrations did not reveal a significant increase. The recurrence of IGF-I hypersecretion after withdrawal of aglépristone treatment is not surprising as all dogs received injections of a depot progestin preparation for a period of 1 year, and the progestin effect of this depot preparation is much longer than the duration of aglépristone treatment in the present study. Similarly, in a cat with fibroadenomatous mammary hyperplasia due to treatment with a depot progestin preparation hyperplasia recurred 8 days after discontinuation

of aglépristone administration (Wehrend et al., 2001). This indicates that treatment with an antiprogestin is required as long as the action of the synthetic progestin is present. Also in our 3 dogs with acromegalic signs, no physical changes were visible during or after treatment with aglépristone.

Due to the insulin-antagonistic action of GH, progestin-induced hypersecretion of GH may also result in hyperglycaemia and eventually manifest diabetes mellitus may ensue (Eigenmann and Rijnberk, 1981; Eigenmann, 1983a). Disappearance of these catabolic abnormalities depends on the functional status of the pancreatic β-cells. If an adequate population of functional β-cells is present at the time the progestin effect is blocked, hyperinsulinemia, carbohydrate intolerance, and hyperglycaemia may be reversible after correction of the hypersomatotropism (Eigenmann, 1983b). If the population of functional β-cells is severely decreased, permanent diabetes mellitus can be anticipated. Because the effects of depot progestins may persist for several months, prevention or reversal of the catabolic effects of progestin-induced GH excess is especially important when, in case of hyperglycaemia, the depot progestin has been administered only recently. The results of the present study illustrate that in these cases aglépristone offers an effective treatment option.

In conclusion, administration of aglépristone significantly decreases plasma GH and IGF-I concentrations in dogs with progestin-induced hypersomatotropism.

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Chapter 11

Summarizing discussion and conclusions

The pulsatile secretion of growth hormone (GH) is regulated predominantly by the opposing actions of the hypothalamic peptides GH-releasing hormone (GHRH) and somatostatin (SS), stimulating and inhibiting pituitary GH secretion, respectively (Tannenbaum and Ling, 1984). In addition, the pituitary secretion of GH is regulated by the negative feedback of both insulin-like growth factor-I (IGF-I) (Voss et al., 2000) and GH (Conway et al., 1985; Lanzi and Tannenbaum, 1992). The amplitude and frequency of GH secretory pulses are influenced by a variety of factors such as age, nutrition, body composition, exercise, and several hormones (Hartman, 2000).

In 1999, ghrelin, a 28-amino-acid peptide with strong GH-releasing activity was discovered. It is predominantly produced by the stomach. The action of this peptide is mediated by the activation of the GH secretagogue (GHS) receptor type 1a (Kojima et al., 1999). Before the discovery of ghrelin, this orphan receptor had been shown to be specific for a family of synthetic, peptidyl and nonpeptidyl GHSs such as GH-releasing peptide-6 (GHRP-6), hexarelin, and MK-0677 (Momany et al., 1981; Momany et al., 1984; Bowers, 1993). Apart from a potent GH releasing action, ghrelin has other activities including stimulation of appetite, control of energy balance, control of gastric motility, and influence on glucose metabolism. For a review on pituitary GH secretion and its regulation, and the diverse endocrine and nonendocrine effects of synthetic GHSs and ghrelin, the reader is referred to **Chapter 2** of this thesis.

Several pathological (e.g. obesity and chronic hypercortisolism) and non-pathological (e.g. ageing) states in humans are characterized by a reduction in pituitary GH secretion (Casanueva, 1992; Leal-Cerro et al., 1994). With regard to the effects of chronic glucocorticoid excess on the plasma GH profile, the results of the study described in **Chapter 3** demonstrate that GH is secreted in a pulsatile fashion in dogs with pituitary-dependent hypercortisolism. However, the pulsatile plasma GH profile in these dogs is characterized by less GH secreted per pulse, while the basal plasma GH concentration is similar to that of healthy control dogs of comparable age.

Chronic hypercortisolism in humans is not only associated with reduced pituitary GH release but also with an impaired GH response to various stimuli (Casanueva, 1992; Leal-Cerro et al., 1994). Even a combination of GHRH and GHRP-6, which is a very powerful GH-releasing stimulus, is unable to induce significant GH release in humans with Cushing's syndrome (Leal-Cerro et al., 1994). Also in dogs with pituitary-dependent hyperadrenocorticism (PDH) administration of GHRP-6 results in a blunted GH response compared to healthy dogs of similar age (**Chapter 4**). The GH response after administration

of ghrelin to dogs with PDH was also low but not significantly different from that in healthy dogs.

The impaired pulsatile GH secretion in dogs with PDH is not yet fully understood but may be ascribed to changes in supra-pituitary stimulation. Enhancement of hypothalamic SS release (Wehrenberg et al., 1990; Lima et al., 1993; Wajchenberg et al., 1996; Terzolo et al., 2000), a decrease in hypothalamic GHRH synthesis and secretion (Miell et al., 1991; Senaris et al., 1996; Ohyma et al., 1997), or a combination of both (Leal-Cerro et al., 1998) may be involved. In addition to their effect at the hypothalamic level, glucocorticoids may also influence GH secretion by acting directly at the pituitary level (Leal-Cerro et al., 1994). As mentioned above, chronic glucocorticoid excess results in a decreased response of GH to GH-releasing stimuli (Peterson and Altszuler, 1981; Wehrenberg et al., 1983; Hotta et al., 1988; Burguera et al., 1990; Voltz et al., 1995; Meij et al., 1997; Ohyama et al., 1997; Watson et al., 2000). It has been postulated that post-GHRH receptor signalling is impaired in somatotrophs exposed to high doses of dexamethasone for long periods (Ohyama et al., 1997). The decrease in hypothalamic GHRH secretion may also result in a lack of priming of the somatotrophs and, subsequently, in reduced GH synthesis and secretion and decreased responsiveness to exogenous GHRH (Thakore and Dinan, 1994). Finally, it has been demonstrated that administration of glucocorticoids to young rats decreases the number of somatotrophs in the pituitary gland (Niimi et al., 1993).

A cardinal physical feature of PDH in dogs is centripetal obesity with abdominal enlargement (Rijnberk, 1996). As not only chronic hypercortisolism but also obesity is associated with an impaired GH response to GH-releasing stimuli (Bowers, 1993), it can be hypothesized that the suppressed GH release in Cushing's syndrome is related to obesity as well. However, in contrast to the situation of chronic hypercortisolism (Leal-Cerro et al., 1994), intravenous administration of the combination of GHRH and GHRP-6 results in an elevated GH response in obese humans (Bowers, 1993). This indicates that the impaired GH response in individuals with Cushing's syndrome cannot be explained solely by obesity.

Both basal and stimulated GH secretion as well as circulating IGF-I concentrations decline with age in several mammalian species (Finkelstein et al., 1972; Rudman, 1985; Zadik et al., 1985; Corpas et al., 1992; Wilshire et al., 1995; Muller et al., 2002; Lee et al., 2004). Little is known about how age affects the GH response to GH-releasing stimuli in dogs. The results of the study described in **Chapter 5** demonstrate the existence of age-related differences with regard to the GH-releasing activity of intravenously administered GHSs in dogs. In young and old healthy dogs, ghrelin caused a significant rise in plasma GH

concentrations when compared with the administration of 0.9 % NaCl. In young dogs, ghrelin was a more potent stimulator of GH release than GHRH and GHRP-6. In old dogs, however, GHRH administration caused higher elevations in plasma GH concentrations than GHRP-6 or ghrelin. These results also illustrate remarkable species-related differences, as studies in rats demonstrate that the GH-releasing potency of ghrelin is similar to that of GHRH (Kojima et al., 1999), whereas in humans ghrelin is a more potent stimulus of GH secretion than GHRH or the synthetic GHS hexarelin (Takaya et al., 2000; Arvat et al., 2001).

The mean ghrelin-induced plasma GH response was significantly lower in the old dogs than in the young dogs. The mean plasma GH concentration after GHRH and GHRP-6 administration was also lower in the old dogs compared with the young dogs, but this difference did not reach statistical significance. These observations are compatible with findings in humans, indicating that not only the GH-releasing effect of ghrelin (Broglio et al., 2003) but also that of GHRH and peptidyl or nonpeptidyl synthetic GHSs undergoes an age-related decrease (Bowers et al., 1992; Aloi et al., 1994; Chapman et al., 1996; Muccioli et al., 2002; Broglio et al., 2003). In old rats, the GH response to synthetic GHSs is impaired as well (Ceda et al., 1986; Walker et al., 1990). Also in old dogs, the GH responsiveness to the synthetic GHS hexarelin has been reported to be low (Cella et al., 1995). In humans, it has been demonstrated that the age-related reduction of both spontaneous and stimulated GH secretion reflects age-related changes in the neural control of somatotroph function (Giustina and Veldhuis; 1998; Ghigo et al., 1999). These changes include a concomitant reduction in the secretion of GHRH and enhancement in SS release (Kelijman, 1991; Giustina and Veldhuis; 1998; Ghigo et al., 1999; Muller et al., 1999). A recent study of the hypothalamic release of GHRH and SS in monkeys has demonstrated that the GHRH pulse frequency and amplitude and baseline GHRH levels are much lower in aged animals than in young adult animals. In contrast, the amplitude of SS pulses and baseline SS levels are significantly higher in aged monkeys than in young adult monkeys (Nakamura et al., 2003). It seems that an impairment of pituitary function does not play a major role (Muller et al., 1999). Repeated GHRH injections in elderly subjects, combined administration of GHRH and clonidine in old dogs, or GHRH + GHRP-6 injection in aged rats (Walker et al., 1991) significantly increases circulating GH concentrations (Cella et al., 1993; Nicolas et al., 1994). These observations support the idea that the pituitary somatotrophs retain their capacity to synthesize and secrete adequate concentrations of GH during ageing (Corpas et al., 1992; Cella et al., 1993; Muller et al., 1999; Muccioli et al., 2002). This despite the fact that the number and size of GH-producing cells in the human pituitary decrease with increasing age (Sun et al., 1984) and that

the decreased GH secretion in elderly rats is associated with reduced pituitary GH content (Sonntag et al., 1980), reduced pituitary GH mRNA (Takahashi et al, 1990), and low pituitary GHRH-receptor mRNA concentration (Kamegai et al., 1999). The diminished GH response to GHRH in aged humans, rats, and dogs indicates that pituitary somatotrophs also become less sensitive to GHRH in older individuals (Pavlov et al., 1986; Cella et al., 1989; Arce et al., 1990).

In the studies on the effects of GHSs on the release of adenohypophyseal hormones other than GH, interesting species-related differences were observed (**Chapter 5**). The action of ghrelin and GHRP-6 is GH-specific in old and young dogs, i.e., both stimulants did neither stimulate the pituitary-adrenocortical axis nor the release of thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and prolactin (PRL). These results are in line with observations in anaesthetized rats reported by Kojima et al. (1999), who found that intravenous administration of ghrelin specifically stimulated GH release but did not affect other adenohypophyseal hormones. However, Thomas et al. (1997) have shown that GHRP-6 mediates the release of ACTH and cortisol in conscious rats. Studies in healthy humans demonstrated that intravenous administration of ghrelin and synthetic GHSs apart from stimulating GH release, also increase circulating concentrations of PRL, adrenocorticotrophic hormone (ACTH), and cortisol (Arvat et al., 2001; Muccioli et al., 2002; Takaya et al., 2002).

The diagnosis of GH deficiency should be based upon the results of a stimulation test because basal plasma concentrations of GH and IGF-I may overlap between pituitary dwarfs and healthy individuals (Gill et al., 1998; Kooistra et al., 2000). In young dogs, ghrelin is a more potent stimulator of GH release than GHRH. Therefore ghrelin might be used to diagnose GH deficiency. We investigated the effects of intravenous administration of ghrelin on the plasma concentration of GH in German shepherd dogs with congenital combined pituitary hormone deficiency and in healthy dogs of a similar age (**Chapter 6**).

In none of the dwarf dogs ghrelin administration resulted in a rise of the plasma GH concentration above 5 µg/l. This finding corresponds with observations in humans with isolated childhood-onset GH deficiency, in whom the GH response to ghrelin is also markedly reduced (Aimaretti et al., 2002). However, in some of the healthy dogs the plasma GH concentration also remained low after ghrelin administration. Thus, while a ghrelin-induced plasma GH concentration higher than 5 µg/l seems to exclude GH deficiency, false-negative results may occur.

Through activation of pathways distinct from those needed for GH secretion, ghrelin causes weight gain by increasing food intake and reducing fat utilization. In several

mammalian species this gastric peptide seems to play a role in meal initiation (Kamegai et al., 2000; Tschop et al., 2000; Wren et al., 2000; Wren et al., 2001). **Chapter 7** is a report on investigations on the effects of food intake and fasting in healthy Beagle dogs. Therefore, the plasma concentrations of ghrelin, GH, IGF-I, glucose, and insulin were measured when food was administered at the usual time, after a 1-day fast, after a 3-day fast, and after re-feeding at the usual time the next day. In agreement with observations in rodents (Tschop et al., 2000; Asakawa et al., 2001), administration of a meal lowered plasma ghrelin concentrations and fasting increased plasma ghrelin concentrations in our dogs. The high plasma ghrelin concentrations during fasting fits in with a physiological role for this hormone in increasing appetite and initiation of food intake. Similar to the situation in rodents, circulating ghrelin concentrations in humans are rapidly suppressed by food intake, and 24-hour plasma ghrelin profiles reveal marked preprandial increases and postprandial decreases associated with every meal (Cummings et al., 2001).

In our dogs, the highest plasma ghrelin concentrations were observed immediately before the administration of food on the first day. Possibly this preprandial rise occurs as an anticipatory response to feeding as the dogs received their food for several years at the same time of the day. Sugino et al. (2002) demonstrated in sheep that expectation of food may stimulate ghrelin secretion . The transient increase in ghrelin secretion just before feeding is most likely elicited by a conditioned emotional response. It is well known that secretion of saliva and gastric acid preceding food intake is induced by a conditioned emotional response through the stimulation of the vagal nerve (Harding and Leek, 1973). Ghrelin secretion may be induced by the vagal system in the same manner as the secretion of saliva and gastric acid. Like in humans and rodents, also in our dogs the plasma ghrelin concentrations decreased shortly after food intake, but this decline did not reach statistical significance. In a very recent study in dogs this post-prandial decrease was found to be statistically significant. (Yokoyama et al., 2005). The mechanism by which nutrients suppress ghrelin concentrations are beginning to be elucidated; changes in plasma insulin concentrations, intestinal osmolarity, and enteric neural signalling probably play a role, whereas gastric distension, vagal nerve activity, and glucagon-like peptide-I are not required (Williams et al., 2003; Gelling et al., 2004).

In contrast with studies in humans and sheep (Cummings et al., 2001; Sugino et al., 2002), the results of the present study, did not provide evidence for an association between a preprandial rise in plasma ghrelin concentrations and a GH surge in dogs. Similar to the

situation in our dogs, a link between plasma ghrelin and plasma GH concentrations has not been demonstrated in cows (Miura et al., 2004).

In our dogs the plasma profiles of ghrelin on the one hand and the profiles of insulin and glucose on the other hand were reciprocal after food intake and fasting. These findings are in agreement with a study in humans, in which plasma ghrelin concentrations evolved oppositely to plasma insulin concentrations (Cummings et al., 2001). This raises the question whether insulin negatively regulates ghrelin or *vice versa*. The former hypothesis has been investigated by several groups (Saad et al., 2002; Flanagan et al., 2003; Kamegai et al., 2004). Taken together, these studies demonstrated that while insulin can suppress ghrelin release when administered in supraphysiologic doses or at high-normal concentrations for prolonged periods of time, physiological concentrations of insulin do not appear to regulate ghrelin release (Caixas et al., 2002; Schaller, et al., 2003; Soriano-Guillen et al., 2004). It has also been suggested that ghrelin may act as a counter-regulatory hormone blocking insulin secretion and insulin action to maintain blood glucose concentrations (Broglio et al., 2001; Cummings et al., 2005). Indeed, several studies have shown that ghrelin can inhibit glucose-mediated insulin secretion, both *in vitro* and *in vivo* (Egido, et al., 2002; Colombo, et al., 2003; Reimer, et al., 2003). Similarly, exogenous ghrelin administration decreases circulating insulin concentrations in mice (Reimer, et al., 2003) and humans (Broglio et al., 2003).

The production and release of GH has been demonstrated in a variety of human extra-pituitary tissues such as the central nervous system (Render et al., 1995) and the immune system (Clark, 1997; Van Buul-Offers and Kooijman, 1998). Expression of GH mRNA has also been found in bone marrow (Kooijman et al., 1997) and testis (Untergasser et al., 1997).

In dogs, a pre-eminent example of extra-pituitary GH production is the progestin-induced synthesis in the mammary gland (Selman et al., 1994a; Mol et al., 1995 a,b; Mol et al., 1996; van Garderen et al., 1997). In this species, mammary GH reaches the systemic circulation and may give rise to a syndrome of GH excess (Selman et al., 1994b). The progestin-induced elevations of plasma GH concentrations do not have a pulsatile pattern (Watson et al., 1987). Additionally, the progestin-induced GH overproduction can neither be stimulated with GHRH, nor can it be inhibited by SS (Watson et al., 1987; Selman et al., 1991). Endogenous progesterone and synthetic progestins, such as medroxyprogesterone acetate (MPA), primarily induce the expression of GH in areas of hyperplastic mammary epithelium, suggesting that locally produced GH promotes epithelial proliferation and differentiation in an autocrine and/or paracrine fashion (van Garderen et al., 1997).

Locally produced GH may also play a role in tumourigenesis in the mammary gland. GH expression has been found in benign and malignant mammary tumours of dogs, and in fibroadenomatous hyperplasia of the mammary gland of cats that have been treated with progestins (Mol et al., 1995a). In cats mammary GH does not seem to reach the systemic circulation. In woman GH is expressed in unaffected mammary tissue and in mammary neoplasms (Mol et al., 1995a). The GH genes expressed in mammary tissues of dogs and women are identical to the genes encoding GH in the pituitary gland (Mol et al., 1995 a,b). For an overview on the effects of progesterone and synthetic progestins in the bitch, the reader is referred to **Chapter 2** of this thesis.

In agreement with previous publications (Takahashi et al., 1981; French et al., 1987; Kooistra et al., 2000), the results of the study reported in **Chapter 8** demonstrate that GH is secreted in a pulsatile fashion in the bitch. Administration of MPA during one year resulted in higher basal plasma GH and IGF-I concentrations, higher area under the curves (AUCs) above the zero-level for GH, and lower AUCs above the baseline for GH (i.e., less GH secreted in pulses) in the healthy control dogs compared to dogs with a complete excision of the mammary gland. The findings in the control dogs are consistent with partial suppression of pituitary GH release by progestin-induced mammary-derived GH secretion and by elevated plasma IGF-I concentrations. Before treatment with MPA, in the anoestrous phase of the ovarian cycle, the mammary tissue of our dogs was inactive on histological examination. After one year of MPA administration, most of the glandular tissue had differentiated into lobulo-alveolar structures in which milk synthesis occurred, except in one dog where nodular epithelial proliferation resulting in ductal buds was present. These findings are in agreement with the the observations of van Garderen et al. (1997).

In canine mammary tissue immunoreactive GH (iGH) and GH gene expression is found predominantly in ductal epithelial buds during the early and midluteal phase of the ovarian cycle. The GH gene expression is diminished in differentiated lobulo-alveolar glandular tissue, and in the inactive tissue during the anoestrous phase of the canine ovarian cycle (van Garderen et al., 1997). Similarly, iGH was not detected in the mammary gland tissue of the anoestrous dogs before treatment with MPA. Additionally, iGH was absent in the mammary gland tissue of all control bitches treated for one year with MPA, except for one dog. In this dog, iGH appeared to be present only in hyperplastic ductular epithelium that consisted of more than 2 cell layers, i.e. epithelial cells in budding structures.

RT-PCR analysis demonstrated that MPA administration increased the GH gene expression and decreased the GH receptor (GHR) gene expression in mammary tissue of the

control dogs. Increased GH mRNA concentrations in mammary gland tissue of dogs after prolonged treatment with progestins have been reported earlier (Mol et al., 1995a). Immunohistochemical expression of the GHR may be down regulated in completely differentiated alveolar epithelial cells at the end of the luteal phase (van Garderen et al., 1999).

In this study it was hypothesized that progesterone-induced mammary GH production may have endocrine effects on other tissues such as the uterine epithelium. Cystic endometrial hyperplasia (CEH) is frequently seen in bitches treated repeatedly with progestins for prevention of oestrus (Capel-Edwards et al., 1973; Sokolowski and Zimbelman, 1973; Goyings et al., 1977). Cystic endometrial hyperplasia may also develop spontaneously during the luteal phase of the oestrous cycle of middle-aged and elderly bitches (Dow, 1958). Because of the similarity of the progestin-induced epithelial changes in both the mammary gland and the uterus, it was hypothesized that GH is also involved in the development of progestin-induced CEH. Although iGH has been found in uterine epithelial cells of progestin-treated dogs, the absence of GH mRNA in uterine tissue suggests that it does not originate in the uterus (Kooistra et al., 1997). Both the control dogs and the mastectomized dogs developed CEH, macroscopically and histologically, with treatment of MPA. After MPA administration, iGH was present in uterine epithelial cells of both dog groups, whereas no uterine GH immunoreactivity was observed in these groups before MPA administration. These findings indicate that progestin-induced mammary GH does not play an essential role in the development of CEH in the bitch. Nevertheless, the presence of iGH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may be involved in the pathogenesis of CEH.

RT-PCR analysis revealed that the GH mRNA content was only increased in uterine tissue of the mastectomized dogs and not in the control dogs after MPA treatment. Comparable with the progestin-induced GH gene expression in canine mammary tissue during development of ductal epithelial buds (van Garderen et al., 1997), MPA treatment also resulted in more GH gene expression in the uterine epithelial tissue. Probably in the control dogs the elevated circulating concentrations of GH of mammary origin and the consequently elevated plasma IGF-I concentrations suppressed uterine GH gene expression, as has been reported for the pituitary (Hartman et al., 1993). MPA treatment also resulted in increased expression of the IGF-I gene in uterine tissue, but this increase was significant only in the control dogs. This may be explained by the stimulating effect of the elevated circulating concentrations of GH, originating from the mammary gland, on uterine IGF-I gene expression

in these dogs. MPA treatment did not promote the expression of GHRs in uterine epithelium. This makes it unlikely that increased numbers of GHRs can explain the presence of iGH in uterine cells, as proposed earlier (Kooistra et al., 1997).

In **Chapter 9**, an integral picture of the effects of progestins on the function of the adenohypophysis in the bitch is reported. The effects of supra-pituitary stimulation, using a combined anterior pituitary function test (Meij et al., 1996), on the release of seven adenohypophyseal hormones was studied in Beagle bitches before and several times during one year of MPA treatment. The prevention of oestrus by MPA in our bitches cannot be ascribed to a significant reduction in circulating concentrations of neither follicle-stimulating hormone (FSH) nor LH. On the contrary, during the first months of MPA treatment basal plasma FSH concentrations increased, without a concomitant change in the basal plasma LH concentrations. This elevated basal plasma FSH concentration may be due to a direct inhibitory effect of MPA at the ovarian level, resulting in suppression of the ovarian secretion of oestradiol and/or inhibin or stimulation of activin release (Couzinet and Schaison, 1993; Poindexter et al., 1993; Heikinheimo et al., 1996; Shupnik, 1996). With progression of the MPA treatment, basal plasma FSH concentrations declined to pre-treatment concentrations, while the pituitary FSH response to supra-pituitary stimulation decreased. These observations may be explained by down-regulation of the pituitary GnRH receptors due to continuous GnRH stimulation (Belchetz et al., 1978).

The results of the study in **Chapter 9** confirmed previous findings that progestins alter the GH-IGF-I axis in the bitch (Eigenmann et al., 1983; Selman et al., 1994b). Basal plasma GH concentrations tended to increase gradually during the course of the MPA treatment, although this rise was not statistically significant. This is in agreement with results of an earlier study, in which in 27 out of 36 MPA treated bitches plasma GH concentrations did not rise significantly (Concannon et al., 1980). However, the significant increase in circulating IGF-I concentrations during MPA treatment in our study indicates indirectly excessive exposure to GH (Selman et al., 1994b). Plasma IGF-I concentrations may thus be a more sensitive indicator than plasma GH concentrations for the effect of progestin treatment on the GH-IGF-I axis.

Besides an interaction with the progesterone receptor, MPA also has a relatively high affinity for the glucocorticoid receptor (Selman et al., 1996). Consequently, suppression of the hypothalamic-pituitary-adrenocortical (HPA) axis was expected during MPA treatment, as has been reported before in both humans (Willemse et al., 1990) and dogs (Selman et al., 1994c, Selman et al., 1996). However, the results of the study reported in **Chapter 9** indicate

that the effects on ACTH secretion characteristics were limited. Because the supra-pituitary stimulation test was carried out four weeks after the injection of MPA, ACTH release most likely had returned to pre-treatment values within this timeframe. The suppression of the adrenocortical component of the HPA axis was more pronounced and comparable to previous observations (Selman et al., 1996). Apparently the suppression of the ACTH secretion was severe enough to cause atrophy of the adrenocortical zona fasciculata.

The basal plasma TSH concentrations were elevated at 8 months after the start of the MPA treatment, although they were still within the reference range for TSH in our laboratory. Our results conflict with those of others, who found no effect of MPA treatment on mean circulating TSH concentrations (Frank et al., 1979). One may speculate that MPA had a direct effect on the thyroid gland as a result of its inherent glucocorticoid properties, leading to a (slight) rise of the plasma TSH concentrations (Kemppainen et al., 1983).

No changes in PRL or α-melanocyte-stimulating hormone secretion were observed. The absence of an effect of MPA treatment on plasma PRL concentrations is in agreement with previous studies (Concannon et al., 1980; Rutteman et al., 1987) and may be explained by the absence of a clearcut decrease in progestational activity, that is known to be a trigger of PRL release (Galac et al., 2000).

The presence of progesterone receptors in mammary gland tissue allows for a targeted endocrine therapy with progesterone receptor blockers in dogs with progestin-induced mammary-derived GH excess. The results of the study reported in **Chapter 10** indicate that administration of the progesterone receptor blocker aglépristone (RU 46534) results in a significant decrease of plasma GH and IGF-I concentrations in dogs with progestin-induced hypersomatotropism. Our findings are in agreement with those of Watson et al. (1987) who found that administration of the antiprogestin mifepristone (RU 38486) decreases plasma GH concentrations and normalizes plasma IGF-I concentrations in bitches with progestin-induced acromegaly.

Analysis of the plasma GH profiles revealed that the mean basal plasma GH concentration and AUC above the zero-level for GH tended to decrease at the end of the treatment period with the progesterone receptor blocker compared with these values before aglépristone administration. In addition, the AUC above the baseline for GH, i.e., the amount of GH secreted in pulses, increased again during aglépristone treatment, although this difference did not reach statistical significance. Thus, treatment with aglépristone resulted in partial restoration of the normal pulsatile GH secretion. Higher dosages of aglépristone may result in complete normalization of the secretion pattern of GH.

Three and a half and 5.5 weeks after the last administration of aglépristone the plasma IGF-I concentrations had increased again, suggesting recurring high GH exposure. The recurrence of IGF-I hypersecretion after withdrawal of aglépristone treatment is not surprising as all dogs received injections of a depot progestin preparation for a period of one year, and the progestin effect of this depot preparation is much longer than the duration of aglépristone treatment in the present study. This indicates that treatment with an antiprogestin is required as long as the action of the synthetic progestin is present.

The following conclusions can be drawn for dogs:

- Pituitary-dependent hyperadrenocorticism is not only associated with less GH secreted per pulse but also with an impaired response to synthetic GHSs.
- In young dogs, ghrelin is a more potent stimulator of GH release than GHRH or GHRP-6. In old dogs, GHRH administration causes higher elevations of plasma GH concentrations than ghrelin or GHRP-6 administration.
- The GH-releasing capacity of ghrelin decreases with age whereas this decline is considerably lower for stimulation with GHRP-6 or GHRH.
- Ghrelin and GHRP-6 are specific releasers of GH. They do not stimulate the pituitary-adrenocortical axis nor the release of TSH, LH, or PRL.
- A ghrelin-stimulation test may be used in the diagnosis of canine pituitary dwarfism.
- Fasting and food intake lead to higher and lower circulating ghrelin concentrations, respectively.
- During food intake and fasting, the changes in plasma ghrelin concentrations are not associated with similar changes in plasma GH concentrations.
- During food intake and fasting, circulating insulin and glucose concentrations change reciprocally with the ghrelin concentrations.
- In healthy dogs, treatment with medroxyprogesterone acetate (MPA) results in a higher basal plasma GH secretion and less GH secreted in pulses compared to dogs with surgically excised mammary gland tissue. In mastectomized dogs however, MPA treatment does not change basal plasma GH concentrations, the AUC above the zero-level for GH, the AUC above the baseline for GH, and the GH pulse frequency.
- In both healthy dogs and mastectomized dogs, cystic endometrial hyperplasia (CEH) develops after one year treatment with MPA. Thus, progestin-induced mammary-derived GH is not a requirement for the development of CEH.

- The presence of immunoreactive GH in the cytoplasm of hyperplastic glandular uterine epithelial cells of dogs with CEH suggests that GH may play a role in the pathogenesis of CEH.
- The effect of MPA on gonadotrophin secretion is confined to FSH secretion. MPA treatment increases basal plasma FSH concentration during the first months of treatment, while the pituitary FSH response to supra-pituitary stimulation decreases during MPA administration.
- The progesterone receptor blocker aglépristone allows for treatment of progestin-induced hypersomatotropism.

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Chapter 12

Samenvatting en conclusies

De pulsatiele vrijstelling van groeihormoon (GH) door de voorkwab van de hypofyse wordt vooral gestuurd door de wisselwerking van de hypothalame hormonen GH-releasing hormoon (GHRH) en somatostatine (SS). Het GHRH stimuleert en SS remt de afgifte van GH (Tannenbaum en Ling, 1984). Daarnaast wordt de hypofysaire GH-secretie beïnvloed door de negatieve terugkoppeling van de insuline-achtige groeifactor-I (IGF-I) (Voss et al., 2000) en van circulerend GH (Conway et al., 1985; Lanzi en Tannenbaum, 1992). De amplitude en de frequentie van GH pulsen zijn afhankelijk van veel factoren, waaronder leeftijd, voeding, lichaamssamenstelling, lichamelijke activiteit, en verscheidene andere hormonen (Hartman, 2000).

In 1999 werd ghreline ontdekt, een peptide dat vooral in de maag wordt geproduceerd. Het bestaat uit 28 aminozuren en bevordert de afgifte van GH via de GH-secretagoog receptor type 1a (Kojima et al., 1999). Eerder was al vastgesteld dat deze receptor specifiek de werking medieert van een groep synthetische GH-afgifte bevorderende peptiden en niet-peptiden, zoals GH-releasing peptide-6, hexareline, en MK-0677 (Momany et al., 1981; Momany et al., 1984; Bowers, 1993). Ghreline heeft naast de sterke GH-vrijstellende werking ook andere eigenschappen zoals bevordering van de eetlust, controle van de energiebalans, controle van de maagmotiliteit, en beïnvloeding van het glucosemetabolisme. Voor een overzicht van de regulatie van de secretie van het hypofysaire GH, en van de verscheidene endocriene en niet-endocriene functies van synthetische GH-secretagogen en ghreline wordt de lezer verwezen naar **hoofdstuk 2** van dit proefschrift.

Verscheidene pathologische (bv. vetzucht en hypercortisolisme) en niet-pathologische (bv. veroudering) veranderingen bij de mens gaan gepaard met een verminderde hypofysaire GH vrijstelling (Casanueva, 1992; Leal-Cerro et al., 1994). In **hoofdstuk 3** worden de effecten van chronische glucocorticoïd-overmaat op het plasma GH-profiel beschreven bij de hond. Alhoewel ook bij honden met hypofyse-afhankelijk hyperadrenocorticisme (HAH) GH in pulsen werd afgegeven, was er sprake van een afname van de afgifte van GH per puls. De basale plasma GH-concentratie veranderde niet.

Chronische overproductie van cortisol gaat bij de mens niet alleen gepaard met een afname van de hypofysaire GH-secretie maar ook met een verminderde GH-respons op diverse stimuli (Casanueva, 1992; Leal-Cerro et al., 1994). Zelfs de gecombineerde toediening van GHRH en GHRP-6, dat een sterke stimulus is voor GH-secretie, veroorzaakt geen significante stijging van de plasma GH-concentratie bij mensen met het syndroom van Cushing (Leal-Cerro et al., 1994). De resultaten van het onderzoek beschreven in **hoofdstuk 4** laten ook zien dat, vergeleken met gezonde honden van vergelijkbare leeftijd, toediening van

GHRP-6 leidt tot een verminderde GH-respons bij honden met HAH. De ghreline-geïnduceerde GH-vrijstelling bij honden met HAH was ook laag maar niet significant verschillend van de GH-vrijstelling bij gezonde honden.

De afgenomen pulsatiele secretie van GH bij honden met HAH zou een gevolg kunnen zijn van veranderingen in de aansturing van de hypofyse. Een toename van de hypothalame afgifte van SS (Wehrenberg et al., 1990; Lima et al., 1993; Wajchenberg et al., 1996; Terzolo et al., 2000), een afname van de hypothalame aanmaak en afgifte van GHRH (Miell et al., 1991; Senaris et al., 1996; Ohyma et al., 1997), of een combinatie van beide (Leal-Cerro et al., 1998) zouden de verminderde pulsatiele hypofysaire GH-afgifte kunnen verklaren. Naast hun effect op de hypothalamus, zouden glucocorticoïden ook een rechtstreekse invloed op de groeihormoonproducerende cellen van de hypofyse kunnen uitoefenen (Leal-Cerro et al., 1994). Zoals reeds eerder vermeld, leidt chronische cortisol-overmaat tot verminderde responsiviteit van GH op stimulantia zoals GHRH (Peterson en Altszuler, 1981; Wehrenberg et al., 1983; Hotta et al., 1988; Burguera et al., 1990; Voltz et al., 1995; Meij et al., 1997; Ohyama et al., 1997; Watson et al., 2000). Daarnaast is ook gepostuleerd dat het 'post-receptor'-systeem van de GHRH-receptor minder goed functioneert indien de groeihormoonproducerende cellen gedurende lange tijd worden blootgesteld aan hoge doses dexamethason (Ohyama et al., 1997). De hierboven beschreven afname van de hypothalame afgifte van GHRH zou kunnen leiden tot minder gevoelige groeihormoonproducerende cellen, en daarmee tot een afname van de aanmaak en secretie van GH, alsook een afgenomen responsiviteit op toegediend GHRH (Thakore en Dinan, 1994). Tenslotte, bij jonge ratten is aangetoond dat toediening van glucocorticoïden het aantal groeihormoonproducerende cellen in de hypofyse vermindert (Niimi et al., 1993).

Typische lichamelijke kenmerken van HAH bij de hond zijn vetzucht en een toegenomen buikomvang (Rijnberk, 1996). Aangezien niet alleen chronisch hypercortisolisme maar ook vetzucht gepaard gaat met een verminderde GH-respons op GH-stimulantia (Bowers, 1993) kan de gedachte opkomen dat de onderdrukte GH-vrijstelling bij het syndroom van Cushing ook veroorzaakt wordt door vetzucht. In tegenstelling tot de situatie bij chronisch hypercortisolisme (Leal-Cerro et al., 1994), leidt intraveneuze toediening van GHRH en GHRP-6 bij zwaarlijvige personen echter wel tot een toegenomen GH-respons (Bowers, 1993). Dit impliceert dat de verminderde GH-respons bij het syndroom van Cushing niet alleen kan worden verklaard door vetzucht.

Bij een groot aantal zoogdieren nemen de basale en gestimuleerde GH-vrijstelling, alsook de circulerende IGF-I-concentraties af met de leeftijd (Finkelstein et al., 1972;

Rudman, 1985; Zadik et al., 1985; Corpas et al., 1992; Wilshire et al., 1995; Muller et al., 2002; Lee et al., 2004). Gegevens over leeftijdsgebonden verschillen in de gestimuleerde GH-vrijstelling bij de hond zijn echter nauwelijks voorhanden. De resultaten van het onderzoek beschreven in **hoofdstuk 5** laten zien dat er ook bij honden leeftijdsgebonden verschillen zijn met betrekking tot de GH-respons op intraveneus toegediende GH-secretagogen. Bij zowel jonge als oude honden veroorzaakt toediening van ghreline, in vergelijking met 0.9 % NaCl, een significante toename in de plasma GH-concentraties. Bij jonge honden is ghreline een sterkere stimulus voor GH-secretie dan GHRH en GHRP-6. Bij oude honden echter, is GHRH-toediening een sterkere stimulus voor GH-vrijstelling dan de toediening van ghreline of GHRP-6. Er zijn ook interessante diersoortgebonden verschillen. Bij de rat is de vrijstelling van ghreline-geïnduceerd GH vergelijkbaar met die na GHRH-toediening (Kojima et al., 1999), terwijl bij de mens toediening van ghreline leidt tot hogere GH-concentraties dan toediening van GHRH of hexareline (Takaya et al., 2000; Arvat et al., 2001).

De gemiddelde ghreline-geïnduceerde plasma GH-respons was significant lager bij oude honden dan bij jonge honden. Deze plasma GH-respons was ook lager bij de oude honden dan bij de jonge honden na GHRH en GHRP-6 toediening, maar dit verschil was niet significant. Deze observaties zijn vergelijkbaar met die bij de mens waar niet alleen het GH-vrijstellend effect van ghreline (Broglio et al., 2003), maar ook dat van GHRH en van synthetische GH-secretagogen een leeftijdsgebonden afname vertoont (Bowers et al., 1992; Aloi et al., 1994; Chapman et al., 1996; Muccioli et al., 2002; Broglio et al., 2003). Ook bij oude ratten (Ceda et al., 1986; Walker et al., 1990) en oude honden (Cella et al., 1995) is de GH-respons op synthetische GH-secretagogen afgenomen. De leeftijdsgebonden afname van zowel basaal als gestimuleerd GH wordt bij de mens toegeschreven aan veranderingen in de neurale controle van de groeihormoonproducerende cellen in de hypofysevoorkwab (Giustina en Veldhuis; 1998; Ghigo et al., 1999). Deze veranderingen kunnen het gevolg zijn van wijzigingen in de hypothalame aansturing van de hypofyse, waarbij vooral gedacht moet worden aan het gelijktijdig optreden van verminderde GHRH-secretie en toegenomen SS-afgifte (Kelijman, 1991; Giustina en Veldhuis; 1998; Ghigo et al., 1999; Muller et al., 1999). Bevindingen van een recente studie over de hypothalame afgifte van GHRH en SS bij apen laten zien dat de frequentie en de amplitude van de GHRH-pulsen en de basale GHRH-concentratie bij oude apen veel lager zijn dan bij jongvolwassen apen. Daarentegen zijn de amplitude van de SS-pulsen en de basale SS-concentratie significant hoger bij oude apen dan bij jongvolwassen apen (Nakamura et al., 2003). Veranderingen op het niveau van de groeihormoonproducerende cellen spelen waarschijnlijk geen essentiële rol (Muller et al.,

1999). Inderdaad, herhaalde GHRH-injecties bij oude mensen, gecombineerde toediening van GHRH en clonidine bij oude honden, of GHRH + GHRP-6 injectie bij oude ratten (Walker et al., 1991) veroorzaken een significante toename van circulerend GH (Cella et al., 1993; Nicolas et al., 1994). Deze bevindingen tonen aan dat de groeihormoonproducerende cellen van de hypofysevoorkwab in staat blijven om voldoende GH te synthetiseren en vrij te stellen tijdens veroudering (Corpas et al., 1992; Cella et al., 1993; Muller et al., 1999; Muccioli et al., 2002), ondanks het feit dat het aantal en de grootte van de groeihormoonproducerende cellen afneemt met voortschrijdende leeftijd (Sun et al., 1984) en dat de afgenomen GH-afgifte bij oude ratten gepaard gaat met een afname van de hoeveelheid hypofysair GH (Sonntag et al., 1980), een afname van mRNA coderend voor GH (Takahashi et al, 1990) en een lage concentratie mRNA coderend voor de GHRH-receptor in de hypofyse (Kamegai et al., 1999). De verminderde GH-respons op GHRH-toediening bij oude mensen, ratten en honden geeft aan dat de groeihormoonproducerende cellen in de hypofyse minder gevoelig zijn voor GHRH (Pavlov et al., 1986; Cella et al., 1989; Arce et al., 1990).

De resultaten van het onderzoek beschreven in **hoofdstuk 5** tonen aan dat ghreline en GHRP-6 GH-specifiek zijn bij jonge en oude honden, dit wil zeggen dat beide stimulantia noch de hypofyse-bijnierschors-as, noch de vrijstelling van thyroïd-stimulerend hormoon (TSH), luteïniserend hormoon (LH) of prolactine (PRL) beïnvloeden. Deze bevindingen zijn vergelijkbaar met die in studies bij ratten onder narcose waarbij intraveneuze toediening van ghreline enkel GH-vrijstelling stimuleerde zonder verandering in de plasmaconcentraties van de andere hypofysevoorkwabhormonen (Kojima et al., 1999). Toediening van GHRP-6 induceert echter bij ratten bij bewustzijn vrijstelling van adrenocorticotroop hormoon (ACTH) en cortisol (Thomas et al.,1997). Bij de mens is aangetoond dat intraveneuze toediening van ghreline en synthetische GH-secretagogen, niet alleen de GH-afgifte bevordert, maar ook leidt tot verhoogde circulerende concentraties van PRL, ACTH, en cortisol (Arvat et al., 2001; Muccioli et al., 2002; Takaya et al., 2002).

De diagnose GH-deficiëntie dient gebaseerd te zijn op resultaten van een stimulatietest, daar basale plasma GH- en IGF-I-concentraties kunnen overlappen bij hypofysaire dwergen en gezonde individuen (Gill et al., 1998; Kooistra et al., 2000). Aangezien toediening van ghreline bij jonge honden een sterkere stimulus voor GH-secretie bleek te zijn dan GHRH, zou dit peptide mogelijk ook gebruikt kunnen worden om GH-deficiëntie vast te stellen. In **hoofdstuk 6** worden de effecten van intraveneuze toediening van ghreline op de plasma GH-concentratie beschreven bij Duitse herders met een aangeboren tekort van meerdere hypofysevoorkwabhormonen en bij gezonde honden van vergelijkbare

leeftijd. Ghreline-toediening leidde bij geen enkele hypofysaire dwerg tot een toename van de plasma GH-concentratie boven de 5 µg/l. Dit is in overeenstemming met waarnemingen bij de mens waar geïsoleerde GH-deficiëntie bij kinderen ook gekenmerkt wordt door een sterk verminderde GH-respons op ghreline (Aimaretti et al., 2002). Echter, bij enkele gezonde honden bleef de plasma GH-concentratie ook laag na toediening van ghreline. Dus, terwijl een ghreline-geïnduceerde plasma GH-waarde hoger dan 5 µg/l GH deficiëntie blijkt uit te sluiten kunnen fout-negatieve resultaten voorkomen.

Ghreline veroorzaakt, via andere wegen dan die betrokken bij GH-secretie, ook gewichtstoename door stimulatie van de eetlust en vermindering van vetafbraak. Bij verscheidene zoogdieren blijkt dit peptide ook een rol te spelen bij de initiatie van voedselopname (Kamegai et al., 2000; Tschop et al., 2000; Wren et al., 2000; Wren et al., 2001). In **hoofdstuk 7** is het onderzoek bij gezonde honden beschreven naar de effecten van voedselopname en vasten. Hiertoe werden de plasmaconcentraties van ghreline, GH, IGF-I, glucose en insuline bepaald na voedselopname op het gebruikelijke voedertijdstip, na 1 dag vasten, na 3 dagen vasten, en na hervatting van de voedselverstrekking op het gebruikelijke voedertijdstip de volgende dag. Onze bevindingen waren vergelijkbaar met onderzoeksresultaten bij knaagdieren (Tschop et al., 2000; Asakawa et al., 2001) en mensen (Cummings et al., 2001). Toediening van een maaltijd resulteerde in lagere plasma ghrelineconcentraties en vasten leidde tot hogere plasma ghrelineconcentraties. De hogere plasma ghrelineconcentratie tijdens vasten zou de fysiologische rol van dit peptide bij de toename van eetlust en initiatie van voedselopname kunnen weerspiegelen. De hoogste plasma ghrelinewaarden werden bij onze honden waargenomen net voor de toediening van voedsel op de eerste dag. Het is mogelijk dat deze preprandiale toename van de plasma ghrelineconcentratie een anticiperende respons is op voedselopname, aangezien de honden gedurende jaren het voeder kregen toegediend op hetzelfde moment van de dag. Sugino et al. (2002) toonden aan dat emotionele factoren, en vooral de verwachting dat voedsel zal worden verstrekt, bij schapen ghrelinesecretie stimuleert. Deze tijdelijke toename van de ghrelinesecretie zou het gevolg kunnen zijn van een geconditioneerde emotionele respons. Het is bekend dat secretie van speeksel en maagzuur vlak voor voederopname op gang komt doordat een dergelijke geconditioneerde emotionele respons leidt tot stimulatie van de *nervus vagus* (Harding en Leek, 1973).

Overeenkomstig de bevindingen bij knaagdieren en mensen, namen bij onze honden de plasma ghrelineconcentraties ook af vlak na inname van voedsel, maar deze afname was niet significant. Zeer recent hebben Yokoyama et al. (2005) bij honden een significante daling

van de circulerende ghrelineconcentraties vlak na voederopname gerapporteerd. Het mechanisme dat hiervoor verantwoordelijk is begint stilaan duidelijk te worden: veranderingen in de plasma insulineconcentratie, intestinale osmolariteit, en intestinale neurale signalen lijken een rol te spelen, terwijl uitzetting van de maag, vagale activiteit en glucagon-achtig peptide geen rol lijken te spelen (Williams et al., 2003; Gelling et al., 2004).

In overeenstemming met de resultaten van een onderzoek bij koeien (Miura et al., 2004) werd bij onze honden geen GH-stijging gezien parallel aan de preprandiale toename van de plasma ghrelineconcentratie. Dit is in tegenstelling met bevindingen bij de mens en bij schapen (Cummings et al., 2001; Sugino et al., 2002), waarbij de anticipatie van voedselverstrekking wel leidt tot een stijging van de plasma ghrelineconcentratie.

Evenals bij de mens (Cummings et al., 2001), waren de veranderingen in het plasmaprofiel van ghreline tijdens voedselopname en vasten tegengesteld aan de veranderingen in het circulerend profiel van glucose en insuline. De vraag is dan of insuline de vrijstelling van ghreline negatief beïnvloedt of omgekeerd. De eerste hypothese werd onderzocht door verscheidene onderzoeksgroepen (Saad et al., 2002; Flanagan et al., 2003; Kamegai et al., 2004). Deze onderzoeken toonden aan dat insuline de plasmaconcentraties van ghreline kan onderdrukken als het wordt toegediend in supra-fysiologische doses of hoog-normale doses worden gegeven gedurende een lange periode. Fysiologische insulineconcentraties blijken de vrijstelling van ghreline niet te reguleren (Caixas et al., 2002; Schaller, et al., 2003; Soriano-Guillen et al., 2004). Er is ook gesuggereerd dat ghreline de secretie en de werking van insuline zou kunnen blokkeren om zo het bloedglucosegehalte te handhaven (Broglio et al., 2001; Cummings et al., 2005). Verscheidene studies hebben aangetoond dat ghreline de glucose-gemedieerde insulinesecretie kan remmen, zowel *in vivo* als *in vitro* (Egido, et al., 2002; Colombo, et al., 2003; Reimer, et al., 2003). Ook exogeen toegediend ghreline verlaagt circulerende insulineconcentraties bij muizen (Reimer, et al., 2003) en mensen (Broglio et al., 2003).

De aanmaak en vrijstelling van GH is aangetoond in diverse humane extra-hypofysaire weefsels zoals het centrale zenuwstelsel (Render et al., 1995) en het immuunsysteem (Clark, 1997; Van Buul-Offers en Kooijman, 1998). Expressie van mRNA coderend voor GH is ook gevonden in het beenmerg (Kooijman et al., 1997) en de testis (Untergasser et al., 1997).

Een prachtig voorbeeld van extra-hypofysaire GH-synthese bij de hond is de progesteron-geïnduceerde aanmaak in melkklierweefsel. De productie van GH in de melkklier kan worden geïnduceerd door endogeen progesteron en door de toediening van progestativa. Activatie van de progesteronreceptor door progesteron of progestativa wordt dan ook

beschouwd als de eerste stap in dit proces (Selman et al., 1994a; Mol et al., 1995 a,b; Mol et al., 1996; van Garderen et al., 1997). Bij de hond bereikt het mammaire GH de systemische circulatie, waardoor het beeld van hypersomatotropisme of acromegalie kan ontstaan (Selman et al., 1994b). Het plasma GH-profiel bij teven met progesteron-geïnduceerde acromegalie heeft geen pulsatiel karakter (Watson et al., 1987). Bovendien kunnen de plasma GH-concentraties bij deze honden niet gestimuleerd worden met GHRH noch geremd worden met SS (Watson et al., 1987; Selman et al., 1991). Endogeen progesteron en synthetische progestativa zoals medroxyprogesteron acetaat (MPA) induceren de expressie van GH voornamelijk in het epitheel van de ductale, knopvormig uitgroeiende structuren van de melkklier tijdens de proliferatiefase. Dit suggereert dat lokaal geproduceerd GH de proliferatie van epitheel bevordert op een autocriene en/of paracriene wijze (van Garderen et al., 1997). Het lokaal gevormde GH speelt dus waarschijnlijk een rol bij de proliferatie en differentiatie van ductale structuren, waaruit uiteindelijk alveolair, secernerend klierepitheel ontstaat.

Expressie van GH is ook aangetoond in goed- en kwaadaardige melkkliertumoren bij de hond (Mol et al., 1995a). GH-genexpressie is ook gevonden in fibroadenomateuze melkklierhyperplasie bij katten die behandeld waren met progestativa (Mol et al., 1995a). Bij deze diersoort lijkt het mammaire GH de algemene circulatie echter niet te bereiken. Ook in humaan onaangetast melkklierweefsel en in melkkliertumoren werd GH-expressie aangetoond (Mol et al., 1995a). Het GH-gen in melkklierweefsel en melkkliertumoren van mens en hond is identiek aan het gen coderend voor GH in de hypofysevoorkwab (Mol et al., 1995 a,b). Voor een overzicht van de effecten van progesteron en synthetische progestativa bij de teef, wordt de lezer verwezen naar **hoofdstuk 2** van dit proefschrift.

In overeenstemming met eerdere publicaties (Takahashi et al., 1981; French et al., 1987; Kooistra et al., 2000) laten de in **hoofdstuk 8** beschreven bevindingen zien dat bij de gezonde teef GH pulsatiel wordt afgegeven. Toediening van MPA gedurende 1 jaar resulteerde bij gezonde controlehonden in hogere basale plasma GH- en IGF-I-concentraties, hogere oppervlaktes onder de curve (AUCs) boven het nulniveau voor GH, en lagere AUCs boven de basaallijn voor GH (= minder GH afgegeven in pulsen) dan bij honden die een volledige mastectomie hadden ondergaan. De bevindingen bij de controlehonden zijn te verklaren door een negatieve terugkoppeling van progesteron-geïnduceerd mammair GH en de toegenomen plasma IGF-I-concentraties op de pulsatiele afgifte van hypofysair GH.

In de anoestrusfase van de ovariële cyclus en vóór MPA-behandeling was het melkklierweefsel bij histologisch onderzoek inactief. Na 1 jaar MPA-behandeling was het

grootste deel van het mammaweefsel gedifferentieerd tot alveolair melk-secernerend klierepitheel, behalve bij één hond waarbij melkklierweefsel in de proliferatiefase werd gevonden. Het epitheel bij deze hond bestond uit ductale, knopvormig uitgroeiende structuren. Deze bevindingen zijn vergelijkbaar met de bevindingen in een eerder onderzoek bij honden (van Garderen et al.,1997).

In de hondenmelkklier worden immunoreactief GH (iGH) en GH-genexpressie voornamelijk gevonden in ductale, knopvormig uitgroeiende structuren in de vroege en midluteale fase van de ovariële cyclus. GH-genexpressie neemt sterk af in gedifferentieerd alveolair secernerend klierepitheel en in de anoestrusfase van de ovariële cyclus (van Garderen et al., 1997). Ook in onze studie was geen iGH aantoonbaar in de melkklier van de honden in anoestrus vóór behandeling met MPA. Bij de controlehonden die gedurende 1 jaar zijn behandeld met MPA werd bij één hond iGH gevonden in het epitheel van ductale, knopvormig uitgroeiende structuren.

RT-PCR-analyse, een veel gevoeliger techniek dan immuunhistochemie, liet zien dat onder invloed van de MPA-behandeling in het melkklierweefsel van de controlehonden de GH-genexpressie toenam en de GH-receptor (GHR)-genexpressie afnam. Ook in een andere studie werd een toegenomen concentratie GH-mRNA gevonden in de hondenmelkklier na langdurige toediening van progestativa (Mol et al., 1995a). Passend bij onze bevindingen is ook met immunohistochemisch onderzoek aangetoond dat de expressie van de GHR vermindert in gedifferentieerd alveolair secernerend klierepitheel op het einde van de luteale fase (van Garderen et al., 1999).

Naast de effecten in de melkklieren, heeft het door progesteron-geïnduceerde mammaire GH mogelijk ook een endocrien effect op het baarmoederslijmvlies. Zo is door ons gepostuleerd dat het mammaire GH een rol speelt bij de ontwikkeling van cysteuze endometriumhyperplasie (CEH). Cysteuze endometriumhyperplasie wordt frequent waargenomen bij teven die herhaaldelijk zijn behandeld met progestativa ter preventie van de loopsheid (Capel-Edwards et al., 1973; Sokolowski en Zimbelman, 1973; Goyings et al., 1977). CEH kan zich echter ook spontaan ontwikkelen gedurende de luteale fase van de ovariële cyclus bij teven van middelbare en hoge leeftijd. (Dow, 1958). Gezien de gelijkenis tussen de progesteron-geïnduceerde epitheliale veranderingen in de melkklier en de baarmoeder, kan de vraag gesteld worden of mammair GH ook betrokken is bij de ontwikkeling van progesteron-geïnduceerde CEH. Met immunohistochemie werd reeds GH aangetoond in uterusepitheel van honden die behandeld werden met progestativa. Echter de

afwezigheid van mRNA coderend voor GH suggereerde dat dit niet wordt aangemaakt in de uterus zelf (Kooistra et al., 1997).

De bevindingen van het onderzoek beschreven in **hoofdstuk 8** tonen aan dat bij beide groepen honden (controlehonden en gemastectomeerde honden) MPA-behandeling leidt tot uteriene veranderingen met macroscopische als microscopische kenmerken van CEH. In het uterusepitheel van beide groepen honden was ná MPA-behandeling immunoreactiviteit voor GH aanwezig, terwijl er vóór behandeling geen iGH was. Deze bevindingen tonen aan dat progesteron-geïnduceerd mammair GH geen essentiële rol speelt bij de ontwikkeling van CEH. Echter, de aanwezigheid van iGH in het cytoplasma van hyperplastisch uterusepitheel bij honden met CEH suggereert dat GH mogelijk wel betrokken is bij het ontstaan van CEH.

Bij onderzoek met RT-PCR bleek dat na MPA-behandeling GH-mRNA alleen was toegenomen in het uterusepitheel van gemastectomeerde honden en niet bij de gezonde controlehonden. Vergelijkbaar met de progesteron-geïnduceerde GH genexpressie in de hondenmelkklier (van Garderen et al., 1997), leidde MPA-behandeling ook tot meer GH genexpressie in het uterusepitheel. Waarschijnlijk leidde bij de controlehonden de toename van de plasmaconcentraties van mammair GH en de daaruit voortkomende toename van plasma IGF-I-concentraties tot een onderdrukking van de GH-genexpressie, op eenzelfde manier als reeds aangetoond voor de hypofyse (Hartman et al., 1993). MPA-behandeling resulteerde ook in een toegenomen expressie van het IGF-I-gen in de baarmoeder, maar dit was alleen significant bij de controlehonden. Een verklaring hiervoor zou het stimulerend effect van de toegenomen plasmaconcentraties van mammair GH op de IGF-I-genexpressie kunnen zijn. MPA-behandeling stimuleerde de expressie van GHRn in uterusepitheel bij onze honden niet, waardoor het niet waarschijnlijk lijkt dat toename van de GHRn de aanwezigheid van iGH in uterusepitheel, zoals eerder gesuggereerd door Kooistra et al. (1997), zou kunnen verklaren

In **hoofdstuk 9** worden de effecten van MPA op de functie van de hypofysevoorkwab bij de teef beschreven. De effecten van supra-hypofysaire stimulatie, waarbij gebruik gemaakt werd van een gecombineerde functietest voor de hypofysevoorkwab (Meij et al., 1996), op de vrijstelling van zeven hypofysevoorkwabhormonen werd bestudeerd vóór MPA-toediening en op diverse tijdstippen tijdens een 1 jaar durende MPA-behandeling. Oestruspreventie door middel van MPA bij onze honden kon niet worden toegeschreven aan een significante afname van de plasmaconcentraties van follikelstimulerend hormoon (FSH) of LH. Sterker nog, MPA-behandeling werd zelfs gekenmerkt door een toename van de basale plasma FSH-concentratie, zonder verandering van de basale plasma LH-concentratie gedurende de eerste

maanden van MPA-behandeling. Deze toegenomen basale FSH-concentratie zou het gevolg kunnen zijn van een direct remmend effect van MPA op het niveau van het ovarium, resulterend in een vermindering van de ovariële oestradiol- en/of inhibinesecretie of stimulatie van de activinevrijstelling (Couzinet en Schaison, 1993; Poindexter et al., 1993; Shupnik, 1996; Heikinheimo et al., 1996). Bij voortgaande MPA-behandeling namen de basale FSH-concentraties weer af tot hun niveau vóór MPA-behandeling, terwijl de hypofysaire FSH-respons op supra-hypofysaire stimulatie daalde. Onderdrukking van de hypofysaire GnRH-receptoren door continue GnRH-stimulatie zou hiervoor een verklaring kunnen zijn (Belchetz et al., 1978).

Eerdere bevindingen toonden aan dat progestativa de GH-IGF-I-as (Eigenmann et al., 1983; Selman et al., 1994b) bij de teef beïnvloeden, en dit werd bevestigd bij het onderzoek vermeld in **hoofdstuk 9**. Basale plasma GH-concentraties stegen geleidelijk gedurende de 1 jaar durende MPA-behandeling, echter de stijging was niet statistisch significant. Concannon et al. (1980) vonden eerder dat plasma GH-concentraties niet significant stegen bij 27 van de 36 MPA-behandelde honden. Echter, de significante stijging van circulerend IGF-I gedurende MPA-behandeling in ons onderzoek impliceert indirect de overmatige blootstelling aan GH (Selman et al., 1994b). Plasma IGF-I-concentraties lijken dus een meer gevoelige indicator te zijn dan plasma GH-concentraties voor het documenteren van de effecten van progestativa op de GH-IGF-I-as.

Naast een interactie met de progesteronreceptor heeft MPA ook een relatief hoge affiniteit voor de glucocorticoïdreceptor (Selman et al., 1996). Onderdrukking van de hypothalamus-hypofyse-bijnierschors-as gedurende MPA-behandeling werd verwacht, aangezien dit eerder al aangetoond was bij de mens (Willemse et al., 1990) en de hond (Selman et al., 1994c, Selman et al., 1996). Echter, de resultaten van de studie beschreven in **hoofdstuk 9** laten zien dat de effecten op de ACTH-secretie gedurende MPA-behandeling beperkt bleven. Aangezien de supra-hypofysaire stimulatie uitgevoerd werd ongeveer 4 weken na injectie van MPA is het mogelijk dat de ACTH-synthese zich intussen had hersteld. De onderdrukking van de cortisolsecretie was evenwel meer uitgesproken en vergelijkbaar met resultaten in vorige studies (Selman et al., 1996). Blijkbaar was de onderdrukking van de ACTH-secretie sterk genoeg om aanleiding te geven tot atrofie van de zona fasciculata van de bijnierschors.

De basale plasma TSH-concentraties waren 8 maanden na MPA-behandeling gestegen, maar ze bleven toch binnen de referentiegrenzen van ons laboratorium. Frank et al. (1979) vonden geen effect van MPA-behandeling op de circulerende TSH-concentratie. Men

zou kunnen veronderstellen dat MPA door de inherente glucocorticoïde werking een direct effect heeft op de schildklier (Kemppainen et al., 1983). Via terugkoppeling op de hypofyse zou dit een (lichte) stijging van de plasma TSH-concentraties tot gevolg kunnen hebben.

MPA-behandeling veroorzaakte geen veranderingen in de secretie van PRL of α-melanocyt-stimulerend hormoon. Een verklaring voor het uitblijven van een effect op de PRL-secretie gedurende MPA-behandeling zou de afwezigheid van een duidelijke afname van progestagene activiteit kunnen zijn. Van dergelijke abrupte dalingen is bekend dat ze leiden tot verhoogde afgifte van PRL (Galac et al., 2000).

De aanwezigheid van progesteronreceptoren in de hondenmelkklier maakt het mogelijk om honden met progesteron-geïnduceerde mammaire GH-hypersecretie doelgericht te behandelen met progesteronreceptor-blokkers. De resultaten van het onderzoek beschreven in **hoofdstuk 10** tonen aan dat toediening van de progesteronreceptor-blokker aglépristone aan honden met progesteron-geïnduceerd hypersomatotropisme leidt tot een significante daling van de plasma GH- en IGF-I-concentraties. Dit stemt overeen met de bevindingen van Watson et al. (1987) die bij honden met progesteron-geïnduceerde acromegalie vonden dat met de toediening van de progesteronreceptor-antagonist mifepristone de plasma GH-concentratie significant daalde en de plasma IGF-I-concentratie normaliseerde. Bij analyse van het plasma GH-profiel bleek dat de gemiddelde basale GH-concentratie en de AUC boven het nulniveau voor GH bij onze honden met GH-overmaat op het einde van de behandeling met aglépristone gering waren afgenomen, vergeleken met deze waarden vóór aglépristonebehandeling. De AUC boven de basaallijn voor GH, dat is de hoeveelheid GH geproduceerd in pulsen, was aan het einde van de aglépristonebehandeling gestegen, hoewel niet significant. Dus, behandeling met aglépristone resulteerde in gedeeltelijk herstel van de normale pulsatiele GH-secretie. Een hogere dosis aglépristone zou mogelijk kunnen leiden tot complete normalisatie van het pulsatiele GH patroon.

Drie en een half en 5,5 week na de laatste toediening van aglépristone namen de plasma IGF-I-concentraties weer toe; een teken van toegenomen blootstelling aan GH. De terugkeer van IGF-I hypersecretie na het stoppen van aglépristone-toediening was niet verbazingwekkend aangezien alle honden een depot-progesteronpreparaat toegediend hadden gekregen gedurende een periode van 1 jaar, en aangezien het progesteroneffect van dit preparaat veel langer is dan de duur van de aglépristone-behandeling in deze studie. Dit impliceert dat behandeling met een progesteronreceptor-antagonist zo lang moet duren als het te verwachten effect van het progestageen.

De volgende conclusies kunnen worden getrokken voor de hond:

- Hypofyse-afhankelijk hyperadrenocorticisme gaat niet alleen gepaard met een afname van de afgifte van GH in pulsen, maar ook met een verminderde respons op GH-stimulantia.
- Bij jonge honden is ghreline een potentere stimulus voor GH-afgifte dan GHRH en GHRP-6. Bij oude honden leidt GHRH-toediening tot hogere plasma GH-concentraties dan de toediening van ghreline of GHRP-6.
- De ghreline-geïnduceerde GH-secretie neemt af met toenemende leeftijd; deze leeftijdsafhankelijke afname is veel lager bij toediening van GHRP-6 of GHRH.
- Ghreline en GHRP-6 stimuleren specifiek de vrijstelling van GH en veroorzaken geen toename van de secretie van ACTH, cortisol, TSH, LH, of PRL.
- Een ghreline-stimulatietest is een bruikbaar alternatief voor stimulatie met GHRH bij de diagnostiek van hypofysaire dwerggroei.
- Vasten en voedselopname gaan gepaard met, respectievelijk, hogere en lagere circulerende ghrelineconcentraties.
- De veranderingen in plasma ghrelineconcentraties gedurende voedselopname en vasten gaan niet gepaard met vergelijkbare veranderingen in de plasma GH-concentraties.
- Gedurende vasten en voedselopname zijn de veranderingen in de circulerende insuline- en glucoseconcentraties omgekeerd aan de veranderingen in de plasma ghrelineconcentraties.
- Bij gezonde controlehonden leidt toediening van medroxyprogesteron acetaat (MPA) tot een hogere basale plasma GH-concentratie en minder GH afgegeven in pulsen dan bij honden zonder melkklieren. Bij gemastectomeerde honden leidt behandeling met MPA niet tot een verandering van de basale plasma GH-concentratie, de AUC boven het nulniveau en boven de basaallijn voor GH, en de frequentie van de GH pulsen.
- Zowel bij gezonde honden als bij gemastectomeerde honden ontwikkelt zich na behandeling met MPA cysteuze endometriumhyperplasie (CEH). Dus, progesteron-geïnduceerd mammair GH speelt geen essentiële rol bij de ontwikkeling van CEH.
- De aanwezigheid van immunoreactief GH in het cytoplasma van hyperplastisch baarmoederepitheel suggereert echter een rol van GH in de pathogenese van CEH.
- Het effect van MPA op de secretie van gonadotrope hormonen blijft beperkt tot de FSH-secretie. Gedurende de eerste maanden leidt MPA-behandeling tot een toename van de basale plasma FSH-concentratie, terwijl de FSH respons op supra-hypofysaire stimulatie afneemt.
- Progesteron-geïnduceerd hypersomatotropisme kan met de progesteronreceptor-antagonist aglépristone worden behandeld.

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Sofie

Curriculum vitae

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List of publications and manuscripts

Curriculum vitae

Sofie Bhatti werd op 24 november 1973 geboren te Luik. Na het behalen van het diploma van hoger secundair onderwijs aan het Nieuwen-Bosch college te Gent, startte zij met de studies Diergeneeskunde te Gent. Het diploma van Dierenarts werd in 1998 behaald met grote onderscheiding.

In juli 1998 trad de schrijfster in dienst bij de Vakgroep Geneeskunde en Klinische Biologie van de Kleine Huisdieren van de Faculteit Diergeneeskunde te Merelbeke. Zij werkte eerst als wetenschappelijk medewerker in de afdeling anesthesiologie en interne geneeskunde (1998-1999). Vanaf 1999 werd ze assistent bij de Vakgroep en ontwikkelde een bijzondere interesse in de endocrinologie en neurologie. In het jaar 2000 werden, in samenwerking met Prof. Dr. A. Rijnberk, Dr. H.S. Kooistra en Dr. J.A. Mol van het Departement Geneeskunde van Gezelschapsdieren van de Faculteit der Diergeneeskunde te Utrecht en Dr. L.M.L. Van Ham van de Vakgroep Geneeskunde en Klinische Biologie van de Kleine Huisdieren van de Faculteit Diergeneeskunde te Merelbeke, de eerste stappen gezet die geleid hebben tot dit proefschrift. In januari 2004 werd het getuigschrift van de Doctoraatsopleiding van de Faculteit Diergeneeskunde te Merelbeke verkregen.

Sofie Bhatti was meerdere malen spreker op postuniversitaire studiedagen zowel in België als in Nederland. Zij is (co-)auteur van verscheidene publicaties in internationale en nationale tijdschriften en nam deel aan verscheidene nationale en internationale congressen. In het najaar van 2001 ontving ze de "Intervet Award" voor de beste wetenschappelijke orale presentatie op het 11de "ESVIM (European Society of Veterinary Internal Medicine) congress" te Dublin. In juni 2002 werd de auteur uitgenodigd om te spreken op het jaarlijks congres van de "ACVIM (American College of Veterinary Internal Medicine)" in Dallas, Verenigde Staten.

De schrijfster is in 2003 getrouwd met Sarne De Vliegher en in 2006 moeder geworden van Tuur.

Sofie Bhatti was born in Liége, Belgium, on November 24, 1973. After finishing her secondary school education (Nieuwen-Bosch college, Ghent), she started to study veterinary medicine at the Faculty of Veterinary Medicine at Ghent University (1992-1998).

After graduation the author started to work at the Department of Small Animal Medicine and Clinical Biology at the Faculty of Veterinary Medicine in Merelbeke as full time scientific contributor in anaesthesiology and internal medicine (1998-1999). In 1999 the

author became assistant at the same department and developed a special interest in endocrinology and neurology. In the year 2000 the author started with the studies described in this PhD thesis, in collaboration with Prof. Dr. A. Rijnberk, Dr. H.S. Kooistra, and Dr. J.A. Mol from the Department of Clinical Sciences of Companion animals, Utrecht University, and Dr. L.M.L. Van Ham from the Department of Small Animal Medicine and Clinical Biology, Ghent University.

Sofie Bhatti has been invited to speak on post university lectures in Belgium and The Netherlands. She is (co-)author of a number of publications in national and international peer-reviewed journals and participated in several different national and international congresses. In 2001 the author received the "Intervet Award" for the best oral presentation at the 11th "ESVIM (European Society of Veterinary Internal Medicine) congress" in Dublin. In June 2002 she was invited speaker at the annual congress of the "ACVIM (American College of Veterinary Internal Medicine)" in Dallas, United States.

In 2003 the author married to Sarne De Vliegher and in 2006 she became mother of Tuur.

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