Recent developments in canine Cushing's syndrome

Nieuwe ontwikkelingen met betrekking tot
het syndroom van Cushing bij de hond
(met een samenvatting in het Nederlands)

Novejša dognanja o Cushingovem sindromu pri psu
(z izvlečkom v slovenščini)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op dinsdag 22 juni 2010 des middags te 12.45 uur

door

Sara Galac

geboren op 16 januari 1968
te Slovenj Gradec, Slovenië

Promotor:	Prof.dr. J. Rothuizen
Co-promotoren:	Dr. H.S. Kooistra
	Dr.ir. J.A. Mol

The studies described in this thesis were conducted at and financially supported by the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Publication of this thesis was made possible by generous support of:

AUV Dierenartsencoöperatie
Boehringer Ingelheim B.V.
Dechra Veterinary Products
Eurovet Animal Health
J.E. Jurriaanse stichting
Novartis Animal Health
Royal Canin
Virbac

Ontwerp en grafische begeleiding: Kees van Veenendaal, Haarlem
Omslagillustratie: Ruby de Goede, Heemstede
Druk: Drukkerij de Bij, Amsterdam

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Galac, Sara
Recent developments in canine Cushing`s syndrome
Sara Galac, Utrecht
Universiteit Utrecht, Faculteit Diergeneeskunde
Thesis Universiteit Utrecht. – With ref. – With summary in Dutch and Slovene.
ISBN 978-90-393-5347-9
Subject headings: hypercortisolism, adrenocortical tumor, dogs

Contents

Chapter 1

Aims and scope of the thesis

Hypercortisolism or Cushing`s syndrome is one of the most common endocrinopaties in dogs. In about 80-85% of cases, hypercortisolism in dogs arises from hypersecretion of adrenocorticotropic hormone (ACTH) by a pituitary corticotroph adenoma and is called ACTH- or pituitary-dependent hypercortisolism (PDH). In the remaining cases canine hypercortisolism is ACTH-independent and results from excessive secretion of glucocorticoids by a benign or malignant adrenocortical tumor (AT).

In the general introduction of this thesis (**Chapter 2**) an overview is given of pituitary and adrenal morphology and their regulation. Furthermore, symptoms and signs, the diagnostic approach, and treatment options in dogs with hypercortisolism are described. In addition, less common forms of hypercortisolism in humans, such as ACTH-independent hypercortisolism due to aberrant expression of adrenocortical hormone receptors and ectopic ACTH secretion are introduced.

The mechanisms by which cortisol is produced in ACTH-independent hypercortisolism were previously classified as autonomous. However, recent research in humans has revealed that steroidogenesis in some cortisol-producing adenomas is stimulated by aberrant expression of adrenocortical hormone receptors. The presence of either ectopic or overexpressed eutopic G-protein coupled hormone receptors regulates steroidogenesis by mimicking cellular events that are triggered normally by the ACTH receptor. This leads not only to hypercortisolemia, but most likely also to hyperplasia, proliferative advantage, and tumor development. To provide the answer whether aberrant adrenocortical hormone receptors play a role in the pathogenesis of ATs in dogs with hypercortisolism, the mRNA expression of gastric-inhibitory polypeptide (GIP) receptor (GIPR), luteinizing hormone receptor (LHR), and vasopressin (V_{1a}, V_{1b}, and V_2) receptors was studied by quantitative PCR (QPCR) and the protein expression and localization of these receptors were studied by immunohistochemistry (**Chapter 3**).

Alterations in steroidogenesis due to upregulation of genes encoding for steroidogenic enzymes of the cortisol-pathway could also provide an explanation for hypercortisolemia in ACTH-independent hypercortisolism. This supposition seems especially at-

tractive because in dogs with cortisol-secreting ATs the plasma ACTH concentration is suppressed, which suggests that the steroidogenesis is not initiated by binding of ACTH to its receptor (ACTH-R), like under physiological circumstances. In an attempt to obtain more information about the steroidogenesis in dogs with ACTH-independent hypercortisolism due to AT, the expression of mRNA encoding steroidogenic enzymes needed for cortisol biosynthesis, steroidogenic acute regulatory protein (StAR), and ACTH-R were studied (**Chapter 4**).

Differentiation between PDH and adrenal hypercortisolism is mandatory to allow selection of the best treatment. The intravenous high-dose dexamethasone suppression test (HDDST) is often used to differentiate between these two forms of the disease. In the intravenous HDDST a decrease of more than 50% from baseline plasma cortisol concentration confirms pituitary dependency, whereas a decrease of less than 50% points to dexamethasone resistance. Whether this 50% criterion for dexamethasone resistance can also be adopted when the urinary corticoid:creatinine ratio (UCCR) is used for the diagnosis of hypercortisolism was explored in **Chapter 5**.

Trilostane has become the medical treatment of choice for canine PDH. Trilostane is a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (HSD3B), an essential enzyme system for the synthesis of cortisol, aldosterone (ALD), and androstenedione. In dogs with PDH, trilostane has the potential to induce a substantial decrease in basal and ACTH-stimulated plasma cortisol concentrations. Based on the mechanism of action, trilostane treatment is expected to affect both the pituitary-adrenocortical axis and the renin-ALD system. To test this assumption, measurement of the plasma concentrations of ACTH and cortisol and their ratio was used to assess the pituitary-adrenocortical axis and measurement of the plasma ALD concentration and plasma renin activity and their ratio was performed to assess the renin-ALD system in dogs with PDH receiving the optimal trilostane dose and in few dogs that received a trilostane overdose (**Chapter 6**).

The effectiveness of trilostane therapy is judged by resolution of the clinical signs associated with glucocorticoid excess and results of an ACTH stimulation test. The aim of performing the ACTH stimulation test in dogs on trilostane therapy is to evaluate whether sufficient adrenocortical reserve is present at the time of maximal effect of trilostane, which is 2-3 hours after its administration. The main disadvantages of this approach are that it only provides information about suppression of cortisol production during a short interval and that the post-ACTH cortisol concentration thought to indicate optimal dosage of trilostane is still arbitrary. Measuring the UCCR might be a more appropriate indicator of the therapeutic efficacy of trilostane, since the UCCR represents an integrated measure of glucocorticoid production. The results

of UCCR determination before and during trilostane therapy in dogs with PDH are presented in **Chapter 7**.

Ectopic ACTH secretion syndrome accounts for about 15% of cases of ACTH-dependent hypercortisolism in humans and comprises a spectrum of tumors from undetectable isolated lesions to wide-spread metastatic and aggressive malignancies. It is often associated with severe hypercortisolemia causing hypokalemia, diabetes mellitus, and general infections. Localizing the source of ectopic ACTH hypersecretion in humans is essential to stage the disease and to adopt optimal treatment modalities. In **Chapter 8** the first documented case of a dog with hypercortisolism due to ectopic ACTH secretion is presented.

Bilateral adrenocortical hyperplasia is a form of ACTH-independent hypercortisolism in humans as a result of aberrant expression of adrenocortical hormone receptors. Adrenal hormonal secretion in these cases is induced by the binding of the specific ligand to these aberrantly expressed adrenocortical hormone receptors. As a result, the conventional endocrine diagnostic tests may be negative for hypercortisolism. To date, GIP- or food-dependent hypercortisolism seems to be the most common cause of ACTH-independent bilateral adrenocortical hyperplasia in man. Low fasting plasma cortisol concentrations, which increase following a meal despite suppression of ACTH, are the result of ectopic expression of nonmutated GIP receptors in zona fasciculata cells. In **Chapter 9**, the first documented case of food-dependent hypercortisolism in a dog is presented.

Chapter 10 is a summarizing discussion with a list of conclusions.

Chapter 2

General introduction

1. Morphology and regulation of the canine pituitary gland

The pituitary gland or hypophysis (*glandula pituitaria or hypophysis cerebri*) is attached to the diencephalon on the ventral midline. Although small, it plays a major regulatory role in the entire endocrine system. The close anatomical relation of its glandular and nervous parts is symbolic of its function in interrelating the nervous and endocrine systems. So extensive are the influences of the hypophysis upon cells, tissues, and organs that it is often referred to as the "master gland" of the body. Its location as an appendage of the brain also points to its significance as the relay between the nervous and humoral mechanisms that jointly control multiple key body functions (Dyce et al. 2010).

The mature pituitary gland consists of two major parts, the *adenohypophysis* (AH) and the *neurohypophysis* (NH). The canine hypophysis is suspended from the midline of the hypothalamus by a short cylindrical stalk. This stalk is the proximal portion of the NH and is called the *pars proximalis NH* or *infundibulum*. In most dogs the third ventricle continues as an invagination, the *recessus NH*, into the infundibulum. The pars proximalis NH is continuous with the distal enlargement, the *pars distalis NH*, which is the major portion of the neurohypophysis. The pars distalis NH is in direct contact with the inner part of the AH, the *pars intermedia (PI),* its name referring to its location between the two major parts of the hypophysis. The largest portion of the AH remains separated from the PI by the hypophyseal cleft and forms the distal portion of the AH, the *pars distalis AH* or *anterior lobe* (AL). The AH also extends as a cuff or collar around the pars proximalis NH to envelop part of the median eminence. This is the *pars infundibularis AH* (Hullinger 1993) (Figure 1).

The pars distalis AH is populated by at least five highly differentiated types of endocrine cells, classified according to the tropic hormones they produce: somatotropic cells secreting growth hormone (GH), lactotropic cells secreting prolactin, thyrotropic cells secreting thyroid-stimulating hormone (TSH), gonadotropic cells secreting luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and corticotropic cells synthesizing the precursor molecule pro-opiomelanocortin (POMC), which gives rise to adrenocorticotropic hormone (ACTH) (Meij et al. 1997).

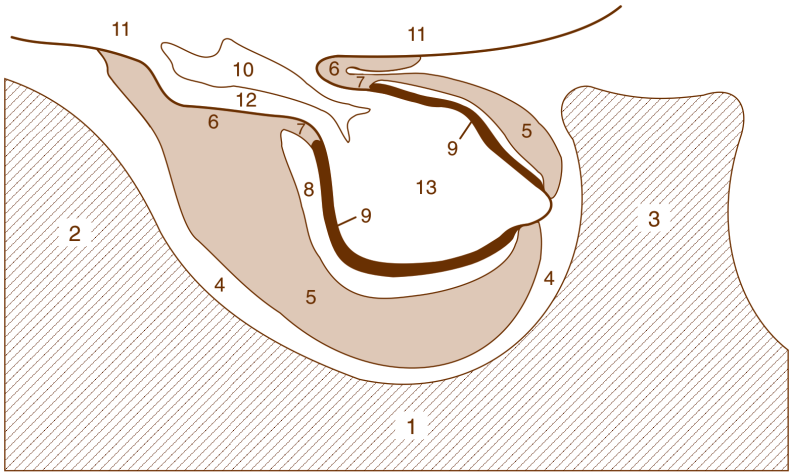

Figure 1
Schematic illustration of an median sagittal section through the canine pituitary gland (With permission, Meij et al. 1997).
Left is rostral, right is caudal. 1 = sphenoid bone, 2 = tuberculum sellae, 3 = dorsum sellae, 4 = pituitary fossa, 5 = pars distalis adenohypophysis, 6 = pars infundibularis 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.

POMC is also synthesized in cells of the PI, where two types of POMC-producing cells have been identified. One type is similar to the corticotroph cells of the AL in that it reacts with anti-ACTH in immunohistochemical staining. In the other type, ACTH is cleaved into $ACTH_{1\text{-}14}$ (precursor of α-melanocyte stimulating hormone (α-MSH)) and corticotropin-like intermediate-lobe peptide (CLIP or $ACTH_{18\text{-}39}$) (Figure 2). The PI is poorly vascularized and is directly innervated by predominantly dopaminergic nerve fibers from the hypothalamus. This direct neural control is mainly inhibitory in nature. Although there are high levels of bioactive ACTH in the canine PI, its main secretory product is α-MSH (Halmi et al. 1981, Kooistra et al. 1997a).

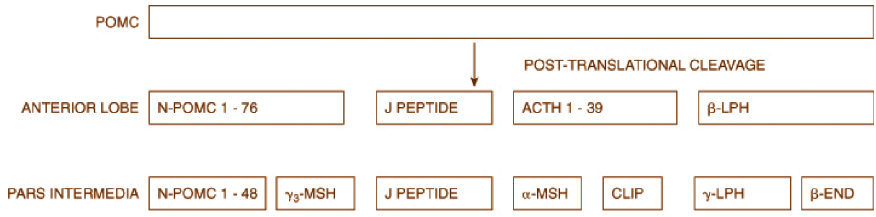

Figure 2
Posttranslational cleavage of pro-opiomelanocortin (POMC) in the anterior lobe (AL) and pars intermedia (PI) of the pituitary gland. ACTH = adrenocorticotropic hormone, J PEPTIDE = joining peptide, β-LPH = β-lipoprotein, α-MSH = α-melanocyte-stimulating hormone, CLIP = corticotropin-like intermediate lobe peptide, β-END = β-endorphin.

The pars distalis NH consists of cells (pituicytes) and unmyelinated nerve fibers derived from neurosecretory neurons in the hypothalamus. These nerve fibers contain oxytocin or vasopressin in transit for secretion from the end of the axon into the systemic circulation. They are stored in secretory granules within the nerve terminals in the NH and are released by exocytosis into the bloodstream in response to appropriate stimuli (Galac et al. 2010).

Hormone secretion from the pars distalis AH is regulated by the intimate contact between the hypothalamus and pituitary gland maintained via the hypophysial portal system. In the median eminence the hypothalamic releasing hormones (HRH) are secreted from the axon terminals of the hypothalamic neurons into fenestrated capillaries. Since the portal blood flow to the pituitary is not compartmentalized, the HRH gain access to all types of endocrine cells in the AL. Selectivity rests on the expression of specific receptors for HRH on the five different types of cells in the AL. However, the classic concept that hormone secretion from each cell type is regulated by a specific HRH has become modified by the finding of multiresponsive cells. These individual AH cells express multiple HRH receptors and are involved in critical endocrine events, such as lactation and adaptation to stress and low temperatures (Senovilla et al. 2008).

Hormone release from the AH is subject to a negative feedback (closed-loop) system. In a long-loop feedback, the hormones produced in the target endocrine organs, such as the thyroids, adrenals, and gonads, act on both the pituitary and the hypothalamus. In a short-loop feedback, some pituitary hormones, such as prolactin, regulate their own secretion by acting on the hypothalamus (Fitzgerald and Dinan 2008). Additionally, there is evidence of an ultra-short loop, by which the hormone acts within the hypophysis through autocrine and paracrine communication (Prummel et al. 2004). Superimposed on these blood-borne regulatory mechanisms there are other signals, mediated by neurotransmitters and hypophysiotropic hormones, that represent the influence of environment, stress, and intrinsic rhythmicity (Reichlin et al. 1992).

2. Morphology of the canine adrenal gland

The adrenal gland (glandula suprarenalis) is located near the craniomedial border of the kidney. The topographic relation of the adrenal to the kidney in humans and other primates in the standing position led to use of the term suprarenal gland. The term glandula adrenalis, introduced in the sixth edition of the Nomina Anatomica, is more appropriate in dogs.

The adrenal gland is composed of a cortex and a medulla, two structurally and functionally different tissues having different developmental histories. The cortex is of meso-

dermal origin and is derived from the patch of celomic epithelium close to the gonadal fold. The medulla arises from neural crest cells, which migrate from their point of origin into the developing mesodermal mass (Hullinger 1978). The adrenal capsule (capsula adrenalis) develops as a condensation of the mesenchyme at the periphery of the cortex. The outer portion, pars fibrosa, becomes a supportive fibrous stroma. The inner portion of the capsule at birth and in the young remains quite cellular and during this period is termed the pars cellulosa. Until the development of the adult cortex, this inner cellular layer can serve as the stem cell population for the generation of additional adrenal cortical parenchyma (Hullinger 1978). The outermost zone of the adrenal cortex is the zona arcuata or zona glomerulosa (ZG). It is composed of cells arranged in arches and nestled in a stromal template provided by the inner surface of the capsule. The next cortical zone, the zona fasciculata (ZF), is the thickest. Its narrow outer surface is called zona intermedia corticalis. This small region comprises less than 5% of the total cortex and functions as a blastemic region for replacement cells of the adult cortex (Nussdorfer 1986, Hullinger 1987). The major part of ZF is composed of anastomosing plates or muralia of cells that radiate toward the periphery (Elias and Pauly 1956). The cells of the outer one-third of this zone contain more lipid and are somewhat larger than those of the inner two-thirds. The innermost cortical layer is applied to all surfaces of the undulating contour of the medulla. The parenchyma of this inner zone is disposed in a relatively random and loose network and is termed the zona reticularis (ZR). The medulla, the central core of the gland, is separated from the cortex by a delicate network of reticular and loose collagenous connecting tissues, the septum corticomedullae (Figure 3)

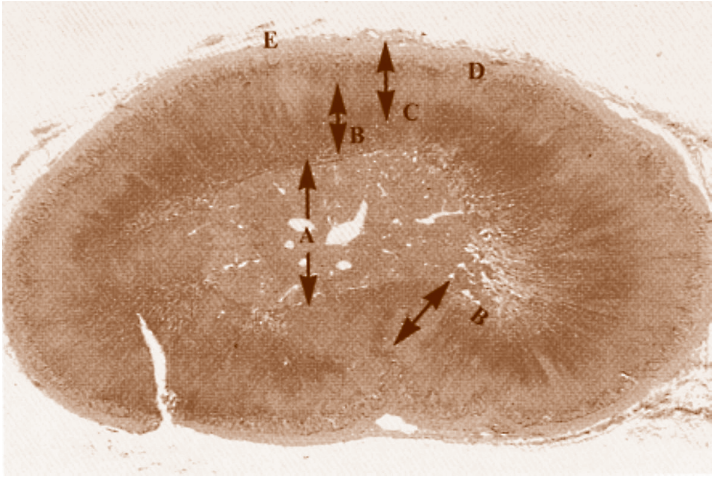

Figure 3
Histological section of the adrenal gland of a healthy dog stained with hematoxyline and eosin. A = medulla, B = zona reticularis (ZR), C = zona fasciculata (ZF), D = zona glomerulosa (ZG), E = capsule (C).

The adrenal cortex is a major steroid-producing organ. The ZG of the canine adrenal cortex secretes mineralocorticoids (primary aldosterone, ALD) and is deficient in 17α-hydroxylase activity (CYP17), which renders it incapable of synthesizing cortisol or androgens. In contrast, only cells in the ZG contain the enzymes necessary to synthesize ALD. The ZF and ZR function as a unit, secreting glucocorticoids (mainly cortisol) and small amounts of sex steroids (estrogens, progestins, and androgens). The adrenal medulla secretes catecholamines (adrenaline and noradrenaline) (O'Brien 2001).

3. Regulation of adrenal gland steroid secretion

3.1. Glucocorticoids

The secretion of glucocorticoids is under the control of the hypothalamic-pituitary-adrenocortical axis (HPA), which represents a complex set of direct influences and feedback interactions between the hypothalamus, the pituitary gland, and the adrenal gland. The main pituitary hormone regulating adrenal steroid secretion is ACTH.
ACTH is synthesized in both the AL and the PI from the well-characterized precursor molecule, POMC. The PI is directly innervated by predominantly dopaminergic nerve fibers from the hypothalamus; this direct neural control is largely a tonic (dopaminergic) inhibitory influence. The AL is regulated by the hypothalamus and central nervous system via neurotransmitters that cause the release of hypophysiotropic hormones, such as corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP), into capillaries of the hypothalamic-hypophyseal portal system. The AVP in portal blood is derived primarily from CRH-containing parvocellular neurons that originate in the paraventricular nucleus and project to the median eminence, thereby being fully separated from the vasopressin involved in water homeostasis (Dyce 2010).
In the neuroendocrine control of ACTH secretion the following mechanisms can be distinguished: episodic secretion, response to stress, feedback inhibition of cortisol, and immunological factors (Galac et al. 2010).
Like the other hormones of the pituitary, ACTH is secreted in a pulsatile fashion (as consequently is cortisol). Central nervous system events regulate both the number and magnitude of ACTH bursts, ranging in the dog from six to twelve per 24h period (Kooistra et al. 1997a). There seems to be no circadian rhythm in cortisol secretion in dogs (Kemppainen and Sartin 1984, Kooistra et al. 1997a, Koyama et al. 2003).
Stress responses originate in the central nervous system and increase the release of CRH and AVP. ACTH and cortisol are secreted within minutes following the onset of

stress (Benson et al. 2000, Devitt et al. 2005). Using urinary cortisol as an integrated measure of cortisol production, stress such as exposure to veterinary procedures is reflected in elevated urinary corticoid:creatinine ratios (UCCR) (Van Vonderen et al. 1998).

Feedback inhibition is another major regulator of ACTH and thus cortisol secretion. The inhibitory action of glucocorticoids is exerted at multiple target sites, of which two have been unequivocally identified: neurons in the hypothalamus that produce CRH and AVP, and corticotropic cells in the AL. The feedback actions of glucocorticoids are exerted through at least two structurally different receptor molecules: a mineralocorticoid-preferring receptor (MR) and a glucocorticoid-preferring receptor (GR). The MR has a 20-fold higher affinity for cortisol than does the GR. There is evidence that inhibition of basal ACTH secretion by glucocorticoids is mediated via occupancy of the MR. The dog brain and pituitary contain very high levels of MR, the highest being in the septohippocampal complex and the pituitary AL (Reul et al. 1990). The GR is more evenly distributed in the brain, but the level in the AL is about twice as high as elsewhere, being mainly involved in the feedback effect of glucocorticoids released as a result of stress-induced ACTH secretion.

Challenges to the immune system by infection invariably activate the HPA. This response is mediated by proinflammatory cytokines, a group of polypeptides released from colonies of activated immune cells. Among the several cytokines, particularly interleukin (IL-6) activates the HPA (Dunn 2000). It is released from activated macrophages in the periphery and is also produced in the brain (Goshen et al. 2009). The regulatory actions of the cytokines are exerted predominantly at the level of the hypothalamus, where CRH is the major mediator of the hypothalamic response. Cytokine-mediated activation of the HPA is also subject to feedback regulation by glucocorticoids, which not only impair the hypothalamic response to cytokine activation but also block cytokine production and inhibit macrophages. Thus there is bidirectional communication between the neuroendocrine system and the immune system (John and Buckingham 2003).

3.2. Mineralocorticoids

ALD is the most potent mineralocorticoid and the three main mediators of its release are the renin-angiotensin system (RAS), potassium, and ACTH (Corry and Tuck 2001).

In turn, the most sensitive mediator of renin release is renal perfusion pressure, changes in which are sensed by myotransducers in the wall of the afferent arteriole. The resulting signals are transmitted to the juxtaglomerular apparatus to modify the level of renin release and, therefore, ALD secretion (Bader and Ganten 2000). Adjacent to

the afferent arteriole, cells of the macula densa in the distal tubules "sense" the composition of the tubular fluid. Signals from these cells may be responsible for a rapid change in circulating renin levels (Persson et al. 2004). Among the factors inhibiting renin release, angiotensin II and potassium play the most important roles. Angiotensin II directly suppresses renin release through a short feedback loop, independent of changes in blood pressure, ALD secretion, or renal blood flow. In humans, the adrenergic nervous system also modifies the release of renin, particularly in response to upright posture, with α and β stimulation having opposite effects (Williams 2005).

Potassium acts directly on the ZG cells by activating the conversion of cholesterol to pregnenolone (Corry and Tuck 2001). It suppresses renal renin release by directly inhibiting the release of renin-containing granules. Furthermore, potassium is a very potent stimulus for ALD secretion and small increases in plasma potassium concentration produce an immediate and significant increase in plasma ALD concentration (Williams 2005).

ACTH is a potent acute ALD secretagogue but in both humans and experimental animals, the effect of ACTH infusion is short-lived unless it is pulsatile (Seely et al. 1989). During continuous ACTH stimulation, glomerulosa cell function appears to convert to fasciculata cell function and thus long-term ACTH stimulation paradoxically decreases ALD secretion.

An interesting feature of ALD secretion is the apparent likelihood that it is produced in other tissues. Although this is still controversial, studies have suggested that ALD production occurs in tissues such as the vasculature and the heart, but under the same control as ALD production by the adrenal gland cortex (Delcayre et al. 2000, Lijnen and Petrov 2003)

4. Adrenal gland steroid biosynthesis

Steroid hormones cannot be stored in steroidogenic cells but are secreted immediately after biosynthesis. Cortisol, 11-deoxycortisol, corticosterone, 11-deoxycorticosterone, and ALD are derived almost exclusively from adrenal secretion, whereas most other steroids are derived from a combination of adrenal and gonadal sources (O`Brien 2001).

4.1. Glucocorticoids

In the adrenocortical cell, glucocorticoid biosynthesis is initiated by binding of ACTH to its receptor (ACTH-R), which is a seven-transmembrane domain receptor belonging

to a G-protein-coupled receptor subfamily. ACTH-R is a member of the melanocortin receptor subfamily, together with several other melanotropin receptors (Mountjoy et al. 1992, Siegrist and Eberle 1995). These receptors are characterized by short NH_2-terminal extracellular domains, short intracellular COOH-terminal domains, short fourth and fifth transmembrane-spanning domains, and a small hydrophobic second extracellular loop (Cone et al. 1993). Until now, ACTH-R is the shortest known G-protein-coupled receptor, consisting of 297 amino acid residues with a predicted molecular mass of 33 kDa. It is mainly expressed in the adrenal cortex but has also been identified in human and canine skin (Slominski et al. 1996, Kidney et al. 2004) and rodent adipocytes (Boston and Cone 1996). There is no evidence of ACTH-R expression in other tissues, which implies the existence of an effective mechanism to restrict expression of this gene. The regulation of the ACTH-R gene is unique in that it is upregulated by its own ligand in a time- and dose-dependent manner (Mountjoy et al. 1994, Lebrethon et al. 1994). This unusual positive feedback loop might be an important adaptive process directed towards optimizing adrenal responsiveness to ACTH in the context of physiological stress and the maintenance of metabolic homeostasis in which the adrenals play a pivotal role (Mountjoy et al. 1992).

All steroid hormones are derived from cholesterol. The adrenocortical cells can synthesize cholesterol *de novo* from acetate, mobilize intracellular cholesterol ester pools, or import lipoprotein cholesterol from plasma. About 80% of the cholesterol is usually provided by circulating plasma lipoproteins (Gwynne and Hess 1982) Adrenal tissue *in vitro* utilizes low-density-lipoprotein (LDL) cholesterol via a specific receptor-mediated pathway (Brown et al. 1979). Specific cell surface receptors for LDL bind it and internalize it by receptor-mediated endocytosis. Under normal conditions, cholesterol *de novo* synthesis from acetyl coenzyme A represents about 20% of steroidogenic capacity.

The amount of free intracellular cholesterol available for adrenal steroidogenesis is metabolically regulated, and negative feedback is exerted via the LDL pathway to control the amount of free intracellular cholesterol in adrenocortical cells. LDL uptake reduces the activity of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting step in cholesterol biosynthesis. ACTH increases the number of LDL receptors on the cell surface and the cholesterol esterase activity that liberates cholesterol from esters delivered by LDL or stored in lipid droplets, and thus the amount of free intracellular cholesterol (Barrett 2005). ACTH does not stimulate HMG-CoA reductase activity or alter the ability of LDL to suppress it.

When stimulation occurs by the binding of ACTH to its receptor, a Gs protein activates adenyl cyclase, causing the formation of cAMP. Protein kinase A is then activated and phosphorylates cholesterol ester hydrolase, thereby increasing its activity. Consequently, more free cholesterol is formed from stored cholesterol and there is increased uptake from plasma lipoproteins, as well as increased cholesterol synthesis in the

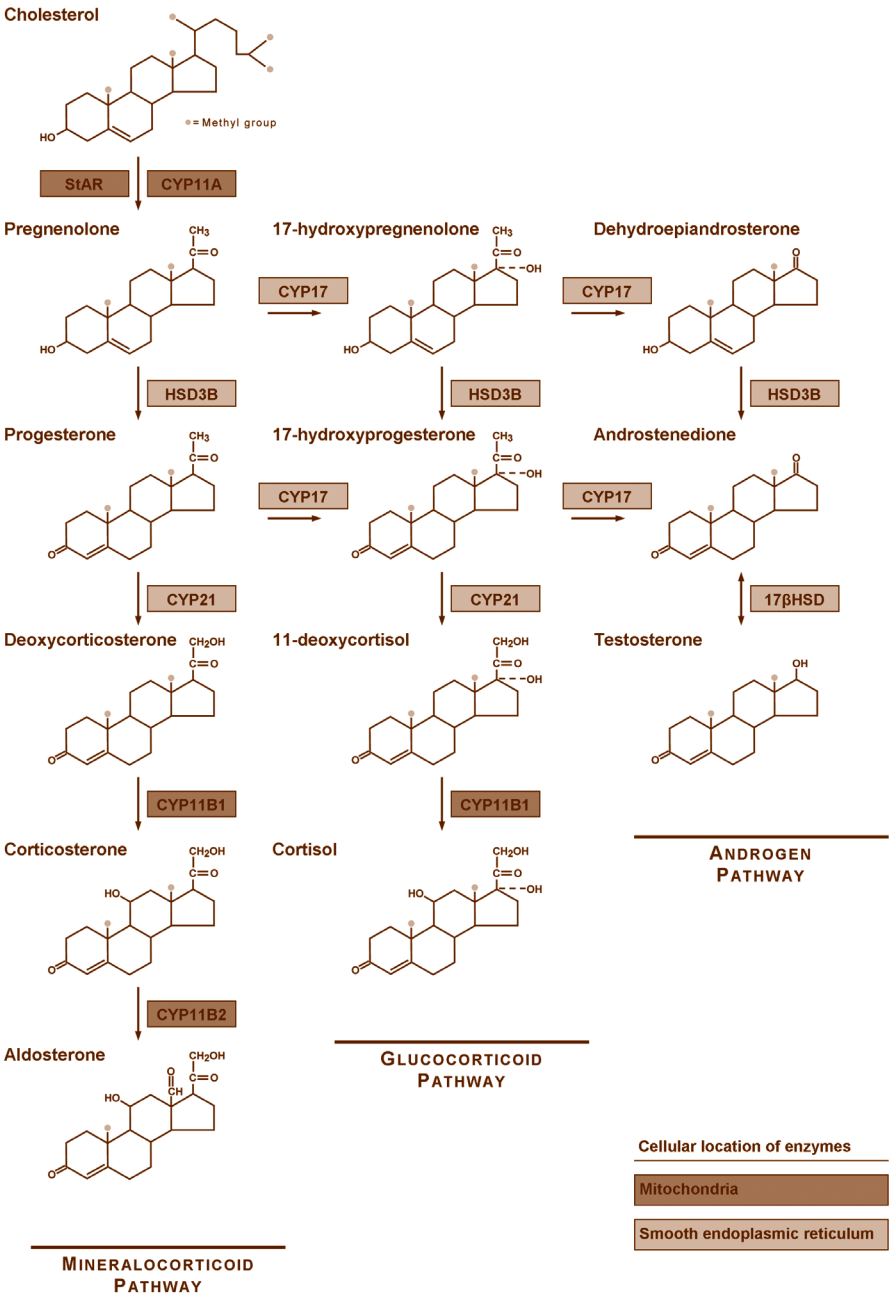

Figure 4

Major pathways of adrenocortical steroid biosynthesis. StAR = steroidogenic acute regulatory protein, CYP11A = cholesterol side-chain cleavage enzyme, HSD3B = 3β-hydroxysteroid dehydrogenase, CYP17 = 17β-hydroxylase/17,20-lyase, CYP21 = 21-hydroxylase, CYP11B1 = 11β-hydroxylase type 1, 17βHSD = 17β-hydroxysteroid dehydrogenase. Cellular location of enzymes (mitochondria or smooth endoplasmic reticulum) is depicted.

adrenals. The acute response to a steroidogenic stimulus is mediated by steroidogenic acute regulatory protein (StAR). This mitochondrial phosphoprotein enhances cholesterol transport from the outer to the inner mitochondrial membrane (Miller et al. 2007, Manna et al. 2009). The conversion of cholesterol to pregnenolone is the first and rate-limiting step in the cortisol steroidogenic pathway. It occurs in the mitochondria and involves the action of the cholesterol-side-chain cleavage enzyme (CYP11A). The newly synthesized pregnenolone is returned to the cytosolic compartment, where in a series of steps, microsomal enzymes convert it to 11-deoxycortisol. The first step is conversion of pregnenolone to progesterone by 3β-hydroxysteroid dehydrogenase-type 2 (HSD3B), the only non-cytochrome P450 (CYP) enzyme in adrenal steroidogenesis. The following step is catalyzed by CYP17, which has both 17α-hydroxylase activity and 17,20-lyase activity (Zuber et al. 1986). This dual function allows the enzyme to direct steroid precursors along several different pathways: 17α-hydroxylated substrates with the side chain are glucocorticoid precursors (17-OH progesterone and 17-OH pregnenolone), whereas both 17α-hydroxylase and 17,20-lyase direct substrate toward androgen and estrogen synthesis (dehydroepiandrosterone and androstenedione). Both progesterone and its 17α-hydroxylated derivate undergo 21-hydroxylation by a single 21-hydroxylase (CYP21) in the smooth endoplasmic reticulum. The last step in cortisol biosynthesis is 11β-hydroxylation of 11-deoxycortisol, catalyzed by 11β-hydroxylase-type 1 (CYP11B1). For this step, 11-deoxycortisol is transferred from the smooth endoplasmic reticulum back to the mitochondria (Aron 2001) (Figure 4).

4.2. Mineralocorticoids

The secretion of ALD is restricted to the ZG. The biosynthetic pathway of ALD is initially similar to that of cortisol except that 17-hydroxylation does not occur, because of the lack of CYP17. In ALD synthesis progesterone is metabolized in the endoplasmic reticulum to 11-deoxycorticosterone. This is transferred to the mitochondria where the zone-specific 11β-hydroxysteroid dehydrogenase-type 2 (CYP11B2 or ALD synthase) converts it to corticosterone and progressively oxidizes it to ALD (O`Brien 2001).

5. Hypercortisolism

Spontaneous hypercortisolism is characterized by physical and biochemical changes resulting from chronic exposure to elevated circulating glucocorticoid concentrations. This disorder is often called Cushing's syndrome, after Harvey Cushing, the neurosurgeon who first described the syndrome in humans in 1932.

About 80-85% of cases of hypercortisolism in dogs are ACTH-dependent, most often the result of excessive secretion of ACTH by a pituitary corticotroph adenoma. In the remaining cases canine hypercortisolism is ACTH-independent, the result of excessive secretion of glucocorticoids by benign or malignant adrenocortical tumors (Galac et al. 2010). In humans, hypercortisolism may also result from ectopic secretion of either ACTH or CRH (Newel-Price et al. 1998). In addition, ACTH-independent hypercortisolism in humans may be due to bilateral macronodular adrenal hyperplasia and primary pigmented nodular adrenocortical disease (Lacroix et al. 2001).

5.1. ACTH-dependent hypercortisolism

5.1.1. Pituitary-dependent hypercortisolism

Pituitary-dependent hypercortisolism (PDH) is one of the most common endocrine disorders in dogs. Exact incidence figures are lacking because there is no formal registration system, but some epidemiological studies have estimated the incidence to be 1 to 2 cases/1,000 dogs/year (Willeberg and Priester 1982). In humans, PDH is a rare disorder with an estimated incidence of 1.2–2.4 new cases/million/year (Etxabe and Vazquez 1994, Lindholm et al. 2001). The pituitary lesions producing excess ACTH range from small nests of hyperplastic corticotrophs to adenomas and large pituitary tumors (Peterson et al. 1982a). The tumor may originate from the AL or the PI, both of which contain cells that can synthesize POMC, albeit with different posttranslational processing. In about 20-25% of cases there is a tumor in the PI. This is of clinical interest not only because tumors of the PI tend to be larger than those of the AL (Kooistra et al. 1997b, Bosje et al. 2002), but also because of the difference in hypothalamic control of hormone synthesis in the two lobes (Kemppainen and Sartin 1987), which is of importance in diagnosis.

5.1.2. Hypercortisolism due to ectopic ACTH secretion

The ectopic ACTH syndrome accounts for about 15% of cases of ACTH-dependent hypercortisolism in humans. The majority of the ectopic tumors that secrete ACTH originate from cells of the diffuse neuroendocrine system and are highly malignant. They include small lung carcinomas, thymic and bronchial carcinoids, pancreatic islet cell tumors, medullary carcinomas of the thyroid, and pheochromocytomas (Newell-Price et al. 1998, Terzolo et al. 2001). The rapidly developing physical changes are usually associated with extremely high plasma concentrations of ACTH and cortisol and often severe hypokalemia. In humans circulating levels of cortisol are usually higher in the ectopic ACTH syndrome than in other forms of hypercortisolism. The high cortisol concentration saturates the renal protective tubular enzyme CYP11B2. This allows cortisol access to type I MR, resulting in cortisol-induced mineralo-

corticoid effects such as arterial hypertension and hypokalemia (Stewart et al. 1995). Hypercortisolism due to the ectopic ACTH syndrome is often difficult to distinguish from PDH. Tumors causing the ectopic ACTH syndrome are frequently small and difficult to visualize (Trainer and Grossman 1991, Newell-Price et al. 1998). In addition, incidental nonsecreting pituitary tumors occur in up to 10% of the human population, giving a false-positive finding by pituitary imaging. On the other hand, pituitary imaging may fail to demonstrate a microadenoma causing PDH (Aron and Howlet 2000). Consequently, the differentiation between PDH and hypercortisolism due to ectopic ACTH syndrome relies heavily on biochemical testing.

Although circulating ACTH concentrations tend to be higher in the ectopic ACTH syndrome than in PDH, there is a large overlap (Howlett et al. 1986). Dynamic noninvasive testing of the pituitary-adrenocortical axis is the most reliable means of differentiating the causes of ACTH-dependent hypercortisolism (Newel-Price et al. 2002, Salgado et al. 2006). Surgical resection of the primary lesion results in complete remission in up to 80% of cases. It is therefore mandatory to localize the source of ectopic ACTH secretion in order to stage the disease and determine the best treatment. Modern cross-sectional imaging techniques can identify the majority of the ACTH-secreting lesions, either initially or at follow-up reassessment (Isidori et al. 2005). However, in 10-20% of patients with ectopic ACTH syndrome the source of ACTH hypersecretion remains occult, in spite of extensive investigation and prolonged follow-up. In such cases, medical treatment is applied to control hypercortisolemia and the diagnostic imaging is repeated after some time (Grossman et al. 2006).

5.2. ACTH-independent hypercortisolism

5.2.1. Cortisol-secreting adrenocortical tumors

ACTH-independent hypercortisolism in dogs is usually caused by cortisol-secreting adrenocortical tumors (ATs). Most ATs are unilateral solitary lesions, the two glands being affected about equally. Bilateral tumors occur in about 10% of cases (Ford et al. 1993, Hoerauf et al. 1999). Histologically, ATs are classified as adenomas or carcinomas, although the distinction can be difficult. Microscopic examination of a seemingly benign tumor may reveal its expansion into blood vessels (Labelle et al. 2004). While expansion of tumor tissue into blood vessels is generally considered to be a hallmark of malignancy, ATs may be an exception to this rule (Van Sluijs et al. 1995). In humans a scoring system which takes into account not only the results of histopathological examination but also the clinical picture and follow-up has been introduced to improve the reliability of the differential diagnosis of AT (Weiss et al. 1989). Such a classification may also have to be introduced in veterinary medicine. In addition, reliable molecular and immunohistochemical markers have the potential to make

assessment of adrenal malignancy much easier (Wachenfeld et al. 2001, De Fraipont et al. 2005). The most frequent modulation in human ATs is overexpression of the IGF-II gene, observed in 85% of carcinomas (Gicquel et al. 2001, Fottner et al. 2003) and associated with paternal isodisomy at the 11p15 locus (Gicquel et al. 1997). Another remarkable feature of human adrenocortical carcinomas is low expression of the ACTH-R gene and loss of heterozygosity (LOH) (Reincke et al. 1997, 1998). One of the major characteristics of carcinomas is expansive growth, which is negatively correlated with steroid secretion (Mendonca et al. 1995). Furthermore, carcinomas have been found to be unable to carry steroidogenesis to completion, resulting in plasma patterns of steroid precursors typical of enzymatic blockage of cortisol synthesis with resulting hypersecretion of adrenal sex hormones (Symme et al. 2001, Hill et al. 2005). Mixed cortisol- and aldosterone-producing ATs have also been reported in dogs (Behrend et al. 2005, Machida et al. 2008). Simultaneous occurrence of an AT and a pheochromocytoma (Von Dehn et al. 1995) or/and pituitary adenoma (Greco et al. 1999, Thuróczy et al. 1998) has also been described in dogs.

5.2.2. Hypercortisolism due to aberrant expression of hormone receptors

In humans, ACTH-independent hypercortisolism can result from aberrant expression of adrenocortical receptors. Either ectopic or overexpressed eutopic adrenocortical hormone receptors can stimulate steroidogenesis and lead to the development of bilateral macronodular adrenal hyperplasia or an AT (Lacroix et al. 2001, 2004). The ectopic hormone receptors that have been identified include the gastric-inhibitory polypeptide (GIP) receptor (GIPR), the renal vasopressin (V_2) receptor (V_2R), the pituitary-specific vasopressin (V_{1b}) receptor ($V_{1b}R$), the β-adrenergic receptor, and the angiotensin II receptor (Lacroix 2009). The adrenocortical eutopic hormone receptors involved in hypercortisolism in humans include the vascular type vasopressin (V_{1a}) receptor ($V_{1a}R$), the LH receptor (LHR), and the serotonin 5-HT4 receptor. Most of these receptors belong to the superfamily of G protein-coupled receptors that have become aberrantly coupled to steroidogenesis (Lacroix et al. 2001). The presence of these receptors places adrenal cells under stimulation by a hormone that escapes the cortisol-mediated feedback system and leads to increased function and possibly to hyperplasia, proliferative advantage, and tumor development (Bordeau et al. 2001).

In veterinary medicine, LH-dependent hypercortisolism has been described in a pet ferret with a unilateral AT (Schoemaker et al. 2008), and the presence of vasopressin receptors on ATs has been postulated by the finding of non-ACTH-mediated cortisol responses to systemic lysine vasopressin administration in dogs with ACTH-independent hypercortisolism (Van Wijk et al. 1994).

6. Clinical manifestations of hypercortisolism in dogs

Spontaneous hypercortisolism is a disease of middle-aged and older dogs, although very rarely it has occurred as early as 1 year of age. There is no gender predilection. It occurs in all dog breeds, with a slight predilection for small breeds such as dachshunds and miniature poodles. Many of the clinical signs can be related to the biochemical effects of glucocorticoids, namely, gluconeogenesis and lipogenesis at the expense of protein. In dogs, the cardinal physical features are centripetal obesity and atrophy of muscles and skin. Polyuria and polyphagia are also dominating features. The polyuria is known to be due to impaired osmoregulation of vasopressin release and interference by the glucocorticoid excess with the action of vasopressin in the kidney. Abdominal palpation may reveal hepatomegaly (Galac et al. 2010).

Increased plasma alkaline phosphatase (AP) activity is a frequent finding in dogs with hypercortisolism. This is mainly due to the induction of an isoenzyme, which, having greater stability at 65 °C than other AP isoenzymes, is easily measured by a routine laboratory procedure (Teske et al. 1989). In about 50% of dogs with hypercortisolism, plasma thyroxine (T4) concentration is decreased as a consequence of altered transport, distribution, and metabolism of T4 rather than hyposecretion.

Clinical changes associated with the pituitary origin of the disease are only observed when the pituitary tumor becomes large enough to cause neurological symptoms. These are often vague, consisting of lethargy, inappetence, and mental dullness (Wood et al. 2007).

Clinical findings linked exclusively to an AT may be related to the adrenal mass and/or caused by metastases or nonspecific features of malignancy such as weight loss and anorexia. A palpable abdominal mass, obstruction of the caudal vena cava by tumor thrombi (Jaffe et al. 1999), or hemoperitoneum secondary to rupture of the tumor are rare consequences of an AT (Vandenbergh et al. 1992, Whittemoore et al. 2002).

7. Diagnosis of hypercortisolism

The biochemical diagnosis of hypercortisolism depends on the demonstration of two characteristics: (1) increased production of cortisol and (2) decreased sensitivity to glucocorticoid feedback (Miller and Crapo 1994). A single measurement of plasma cortisol concentration has little diagnostic value because the pulsatile secretion of ACTH results in a varying plasma cortisol concentration that may at times be within the reference range. There are two ways to overcome this problem: (1) by testing the integrity of the feedback system, and (2) by measuring urinary corticoid excretion.

The first approach, testing the sensitivity of the pituitary-adrenocortical system to sup-

pression, was introduced in 1960 by Grant Liddle. He demonstrated that when given a potent glucocorticoid such as dexamethasone in a dose too small to contribute to glucocorticoid measurements, patients with Cushing's syndrome could be separated from those with normal adrenocortical function. This discovery continues to be applied and is the basis of the low-dose dexamethasone suppression test (LDDST) used to diagnose hypercortisolism in dogs. In normal dogs, intravenous administration of 0.01 mg dexamethasone per kg body weight suppresses plasma cortisol concentration to 40 nmol/L or less at 8 hours after dexamethasone administration (Greco et al. 1993, Kemppainen and Peterson 1993). In dogs with hypercortisolism there is little or no suppression at 8 hours (Feldman 1996). The LDDST is a reliable screening test with a reported sensitivity of 85% (Rijnberk et al. 1988) or even 100% (Kaplan et al. 1995) and a specificity of 73% (Rijnberk et al. 1988, Kaplan et al. 1995).

The disadvantage of the LDDST is that it is rather time consuming and invasive, requiring blood collection at least twice. Hence is has been largely replaced by measurement of urinary corticoids. Because urine accumulates in the bladder for several hours before collection, its corticoid concentration is an integrated measure of corticoid production over this interval, smoothing out the effects of short-term fluctuations in plasma cortisol concentration. The urinary corticoids (largely cortisol) are related to the creatinine concentration in the urine, resulting in the urinary corticoid:creatinine ratio (UCCR). This test requires little time (from the veterinarian or the owner), is not invasive (no blood collection), and has a high diagnostic accuracy. In addition, the test procedure has the advantage of combining a test for basal adrenocortical function and a dynamic test for differential diagnosis (see below). To avoid the influence of stress, urine for measurement of the UCCR is collected at home, with an interval of at least one day after the visit to the veterinary clinic. The owner collects a sample of the first morning urine on two consecutive days and the UCCRs in these two samples are averaged. In our laboratory the basal UCCR in healthy pet dogs varies from 0.8 to 8.3 x 10^{-6} (Van Vonderen et al. 1998). In dogs the predictive value of a positive test result is 0.88 and that of a negative test result is 0.98 (Rijnberk et al. 1988). In some dogs there is considerable day-to-day variation in the UCCR, which in mild forms of hypercortisolism occasionally leads to UCCRs just within the reference range, whereas urine collection on other days might reveal one or two elevated UCCRs.

In dogs in which results of the UCCR and/or the iv-LDDST have been inconclusive or negative but in which there is still suspicion of hypercortisolism, an oral LDDST can be performed. The owner collects urine at 8.00 h (at home) for measurement of the basal UCCR and then administers 0.01 mg dexamethasone per kg body weight orally. The dog is walked at 12.00 h and 14.00 h to empty its bladder and then urine is collected at 16.00 h to measure the effect of the low dose of dexamethasone on the UCCR. In 7 healthy pet dogs the UCCR at 16.00 h was $<1.0 \times 10^{-6}$ (Vaessen et al. 2004) and in dogs with mild PDH it was $>1.0 \times 10^{-6}$ (Cerundolo et al. 2007),

The ACTH stimulation test has also been used in the diagnosis of hypercortisolism in dogs. In principle it is a test of adrenocortical reserve capacity, used to diagnose primary and secondary adrenocortical insufficiency, and thus it can be used to diagnose iatrogenic hypercortisolism, which via feedback suppression results in secondary adrenocortical insufficiency. However, the ACTH stimulation test was used more often in the past as a primary test to diagnose hypercortisolism. An exaggerated response, i.e., a greater elevation of plasma cortisol concentration than in healthy dogs, occurs in about 85% of dogs with PDH compared to about 55% of dogs with hypercortisolism due to AT (Peterson et al. 1982b). The main advantages of the ACTH stimulation test are its simplicity and short duration. However, its diagnostic accuracy for hypercortisolism is less than that of the UCCR and the LDDST. Hence it is no longer recommended for this purpose (Feldman 2005).

7.1. Differentiation between PDH and hypercortisolism due to AT

Once the diagnosis of hypercortisolism has been confirmed, it is necessary to distinguish between the different forms of the disease. Despite decreased sensitivity to suppression by glucocorticoids, which is a hallmark of hypercortisolism, in most dogs with PDH due to an adenoma in the AL, ACTH secretion can still be suppressed by a high-dose of dexamethasone (Feldman et al. 1996). In contrast, PDH of PI origin is resistant to suppression by dexamethasone. The tonic dopaminergic inhibition, which is the principal neural control of the PI, suppresses the expression of glucocorticoid receptors in the PI. However, this is not an absolute difference from AL lesions, as pituitary lesions causing hypercortisolism do not always maintain the regulation characteristics of the lobe of origin. In PDH, the resistance to glucocorticoid feedback is significantly correlated with the size of the pituitary gland (Kooistra et al. 1997b, Bosje et al. 2002).

The impaired sensitivity to glucocorticoid feedback in PDH due to an AL tumor can be demonstrated by performing an intravenous high-dose dexamethasone suppression test (HDDST). Blood for measurement of plasma cortisol concentration is collected immediately before and 3-4 h after intravenous administration of 0.1 mg dexamethasone per kg body weight. A decrease of more than 50% from the baseline value confirms PDH (Stolp et al. 1983). When the suppression is less than 50%, the hypercortisolism may still be pituitary-dependent (due to either a PI tumor or a resistant AL tumor) or be due to an AT. Further differentiation requires measurements of plasma ACTH concentration. In animals with PDH, plasma ACTH concentration is not completely suppressed despite high plasma cortisol concentrations (Gould et al. 2001).

After the diagnosis of hypercortisolism has been established, diagnostic imaging of the

pituitary gland and adrenals is of great assistance to determine the best treatment and objectively evaluate the prognosis. The pituitary can be visualized by computed tomography (CT) or nuclear magnetic resonance imaging (MRI). In dynamic contrast-enhanced CT, the absence of the pituitary flush indicates atrophy of the neurohypophysis and the adenohypophysis. Displacement of the pituitary flush in the early phase of the dynamic CT can be used to identify and localize microadenomas originating from the AL or PI in dogs (Van der Vlught-Meijer et al. 2002, 2003). The adrenal glands can also be visualized by CT or MRI (Voorhout et al. 1990a) but because ultrasonography is less expensive, requires less time, and does not require anesthesia, it is often used first (Voorhout et al. 1990b, Hoerauf et al. 1999). It can provide a good estimate of the size of an AT and may reveal its expansion into blood vessels and possible metastasis in the liver. If nodular structures are revealed in the liver, ultrasound-guided needle aspiration biopsy can be performed. Thoracic radiographs or a CT scan of the thorax should be made to determine whether there are metastases in the lungs.

8. Treatment

The goal of treatment of hypercortisolism is to eliminate the clinical signs related to glucocorticoid excess. Depending on the cause, this may be achieved by transsphenoidal hypophysectomy, adrenalectomy, or medical treatment with o,p'-DDD (mitotane) or trilostane.

8.1. Surgical treatment

In hands of a skilled neurosurgeon transsphenoidal hypophysectomy is an effective treatment for canine PDH (Meij et al. 1998, 2002). It is the only treatment that can eliminate the causative pituitary adenoma, but in the dog this requires complete removal of the pituitary. Following hypophysectomy, hormone replacement therapy consists of lifelong administration of cortisone acetate and thyroxine and temporary administration of desmopressin, a synthetic vasopressin analogue. The major complications are postoperative mortality, hypernatremia due to acute AVP deficiency, prolonged central diabetes insipidus, keratoconjunctivitis sicca, and residual or recurrent hypercortisolism (Meij et al. 1998). A recent study of the 10-year follow-up results in 150 dogs with PDH confirmed that it is effective, especially in the long term, with remission for up to seven years (Hanson et al. 2005). However, the survival and disease-free periods decrease and the incidence of central diabetes insipidus increases with increasing

pituitary size. Hence, transsphenoidal hypophysectomy can be expected to have the best outcome as the primary treatment in dogs with nonenlarged or only moderately enlarged pituitaries (Hanson et al. 2007).
The treatment of choice for AT is adrenalectomy, because the successful removal of the affected adrenal gland eliminates the tumor and thus the clinical signs related to glucocorticoid excess, without the need for lifelong medication. Because of the atrophy of the cortex of the nontumorous contralateral adrenal gland, due to the longstanding glucocorticoid excess, glucocorticoid substitution is needed temporarily (Galac et al. 2010).
Adrenalectomy can be performed in dogs via a ventral midline celiotomy, with a paracostal extension of the incision when needed, or via a paracostal approach (Van Sluijs et al. 1995, Anderson et al. 2001, Kyles et al. 2003). Regardless of the surgical technique used, adrenalectomy is associated with a perioperative mortality of about 20% (Anderson et al. 2001, Schwartz et al. 2008). This can be explained in part by the relatively high risk of postoperative complications such as renal failure, pneumonia, pancreatitis, and pulmonary thromboembolism, but it also reflects the level of difficulty of the surgical procedure. In dogs with hypercortisolism the adrenals are usually poorly accessible due to central obesity and hepatomegaly. Furthermore, ATs are friable, lie in close proximity to the vena cava, and tend to invade blood vessels. As for hypophysectomy (Meij et al. 1998), the skill of the surgeon will affect the outcome.
In humans, laparoscopic adrenalectomy has been reported to have lower perioperative morbidity and mortality than open transabdominal surgery (Imai et al. 1999). It may also become the surgical procedure of choice in veterinary medicine (Jiménez Peláez et al. 2008), but as in humans, the size of the AT may be a limitation of this technique.

8.2. Medical treatment

Selective (Kintzer and Peterson 1991) or nonselective (Den Hertog et al. 1999) destruction of the adrenal cortex with **o,p'-DDD (mitotane)** has long been the medical treatment of choice for PDH in dogs. Some treatment schedules aim at selective destruction of the ZF and ZR, sparing the ZG. However, in 5-6% of dogs in which this is attempted, the ZG is also destroyed to such an extent that iatrogenic hypoadrenocorticism develops. On the other hand, in more than half of the cases in which selective destruction is the aim, there are one or more relapses of hypercortisolism during treatment. In order to avoid these complications, a treatment schedule with the aim of complete destruction of the adrenal cortices and substitution for the induced hypoadrenocorticism (Rijnberk and Belshaw 1988) was developed. This nonselective destruction has been reported to result in fewer recurrences than does selective destruction (Den Hertog et al. 1999). However, o,p'-DDD is no longer regularly used for treatment of PDH, but rather for treatment of inoperable and/or metastasized ATs, with the aim

of complete destruction of all adrenocortical cells, including metastases (Kintzer and Peterson 1994).

A few years ago, **trilostane** was reported to be a safe and effective alternative for o,p'-DDD in dogs with PDH (Neiger et al. 2002, Ruckstuhl et al. 2002). Trilostane is a competitive inhibitor of HSD3B, an essential enzyme system for the synthesis of cortisol, ALD, and androstenedione (Potts et al. 1978). In dogs with PDH, treatment with trilostane has the potential to significantly decrease basal and ACTH-stimulated plasma cortisol concentration (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). This results in loss of negative feedback and thus increased plasma ACTH concentration (Witt and Niger 2004, Sieber-Ruckstuhl et al. 2006). Consistent with its competitive inhibitory effect on HSD3B, trilostane also causes a slight decrease in plasma ALD concentration in dogs with PDH, although ALD usually remains within the reference range (Ruckstuhl et al. 2002, Wenger et al. 2004). In humans, plasma renin activity rather than the plasma ALD concentration is considered to reflect mineralocorticoid deficiency (Loriaux et al. 2001). No detailed information is available on the effects of trilostane treatment on the RAS in the dog.

Trilostane is absorbed rapidly from the gastrointestinal tract. Administration with food significantly increases the rate and extent of absorption. There is marked variation in the optimal dose and to avoid adverse effects due to overdosage, treatment is started at a relatively low oral dose of 2 mg/kg body weight once daily.

The effectiveness of trilostane therapy is judged by resolution of clinical signs associated with glucocorticoid excess and results of an ACTH stimulation test (Neiger et al. 2002, Ruckstuhl et al. 2002). The aim of performing an ACTH stimulation test in a dog on trilostane therapy is to determine whether there is sufficient adrenocortical reserve at the time of maximal effect of trilostane, which is 2-3 hours after administration. The disadvantages of the test are that: (1) it only provides information about suppression of cortisol production during a short interval, (2) it is invasive, and (3) the post-ACTH cortisol concentration thought to indicate optimal dosage of trilostane is still arbitrary. Another way to evaluate trilostane therapy would seem to be measurement of the UCCR. This has been used to detect persisting cortisol secretion after hypophysectomy or nonselective destruction of the adrenal cortex by mitotane therapy (Meij et al. 1998, Den Hertog et al. 1999). Because the UCCR is an integrated measure of glucocorticoid production (Stolp et al. 1983), it might be a more appropriate indicator of the therapeutic efficacy of trilostane than an ACTH stimulation test.

Within about a week on an appropriate dose of trilostane, there is a clear reduction in water intake, urine output, and appetite, followed by improvement in the coat and skin, reduction of central obesity, and increased physical activity (Neiger et al. 2002, Ruckstuhl et al. 2002).

Overdosage of trilostane results in cortisol deficiency and sometimes even mineralocorticoid deficiency (Chapman et al. 2004, Ramsey et al. 2008). In addition, necrosis,

apoptosis, and hemorrhage in the ZF and ZR may cause life-threatening hypocortisolism (Reusch et al. 2007). If this occurs, trilostane must be stopped immediately and corticosteroid substitution started. In most cases there is sufficient recovery of adrenocortical function within a few weeks and substitution can be stopped (Wenger et al. 2004, Perez-Alenza et al. 2006).
The median survival time for dogs with PDH treated with trilostane once daily (662 days), is similar to that for selective adrenocorticolysis with o,p'-DDD (798 days) (Barker et al. 2005). The median survival time of dogs with PDH for treatment with trilostane twice daily (900 days) is comparable to that for the nonselective adrenocorticolysis with o,p'-DDD (720 days) (Clemente et al. 2007).
If there is metastasis of a functional AT or if neither adrenalectomy nor adrenocortical destruction with o,p'-DDD is an option, trilostane therapy can be used as a palliative treatment (Eastwood et al. 2003, Benchekroun et al. 2008).

9. References

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Chapter 3

Expression of receptors for luteinizing hormone, gastric-inhibitory polypeptide and vasopressin in normal adrenals and cortisol-secreting adrenocortical tumors in dogs

S. Galac[a], V.J. Kars[a], S. Klarenbeek[b], K.J. Teerds[c], J.A. Mol[a], H.S. Kooistra[a]

Domest Anim Endocrinol, accepted

[a] *Department of Clinical Sciences of Companion Animals,*
[b] *Department of Pathobiology,*
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[c] *Human and Animal Physiology, Department of Animal Sciences,*
Wageningen University, Wageningen, The Netherlands

Abstract

Hypercortisolism due to an adrenocortical tumor (AT) results from adrenocorticotropin (ACTH)-independent hypersecretion of glucocorticoids. Studies in humans demonstrate that steroidogenesis in ATs may be stimulated by ectopic or overexpressed eutopic G protein-coupled receptors. We report on a screening of 23 surgically removed cortisol-secreting ATs for the expression of receptors for luteinizing hormone (LH), gastric-inhibitory polypeptide (GIP) and vasopressin (V_{1a}, V_{1b} and V_2). Normal adrenal glands served as control tissues. Abundance of mRNA for these receptors was quantified using QPCR and the presence and localization of these receptors were determined by immunohistochemistry. In both normal adrenal glands and ATs mRNA encoding for all receptors was present, although the expression abundance of the V_{1b} receptor was very low. The mRNA expression abundance for the GIP and V_2 receptors in ATs were significantly lower (P=0.03 and 0.01, respectively) than in normal adrenal glands. The zona fasciculata (ZF) of normal adrenal glands stained immunonegative for GIP receptor. In contrast, islands of GIP receptor-immunopositive cells were detected in about half of the ATs. The ZF of normal adrenal glands and AT tissue were both immunopositive for LH receptor; in ATs in a homogenous or heterogeneous pattern. In normal adrenal glands, no immunolabeling for V_{1b}R and V_2 receptor was present, but in ATs V_2 receptor-immunopositive cells were detected. In conclusion, QPCR analysis did not reveal overexpression of LH, GIP, V_{1a}, V_{1b} and V_2 receptors in the ATs. However, the ectopic expression of GIP and V_2 receptor protein in tumorous ZF tissue may play a role in the pathogenesis of canine cortisol-secreting ATs.

Introduction

Spontaneous hypercortisolism is a relatively common condition in dogs and can be defined as the complex of physical and biochemical changes resulting from chronic glucocorticoid excess. In about 85% of cases, hypercortisolism is adrenocorticotropin

(ACTH)-dependent and mostly due to ACTH hypersecretion by a pituitary corticotroph adenoma (Galac et al. 2010). Ectopic ACTH secretion syndrome is rare in dogs (Galac et al. 2005). In the remaining cases, hypercortisolism is ACTH-independent and results from hypersecretion of glucocorticoids by an adrenocortical adenoma or carcinoma (Galac et al. 2010). In humans, ACTH-independent hypercortisolism can also be caused by bilateral macronodular adrenal hyperplasia (AIMAH) and primary pigmented nodular adrenocortical disease (Lacroix et al. 2001).

The mechanisms by which cortisol is produced in ACTH-independent hypercortisolism were previously classified as autonomous. However, recent research in humans has revealed that steroidogenesis in some cortisol-producing AIMAH and unilateral adenomas is stimulated by either ectopic or overexpressed eutopic adrenocortical hormone receptors (Christopoulos et al. 2005). The ectopic hormone receptors that have been identified include the gastric-inhibitory polypeptide (GIP) receptor (GIPR), the renal vasopressin (V_2) receptor (V_2R), the pituitary-specific vasopressin (V_{1b}) receptor ($V_{1b}R$), the β-adrenergic receptor, and the angiotensin II receptor (Lacroix et al. 2001). The adrenocortical eutopic hormone receptors involved in hypercortisolism in humans include the vascular type vasopressin (V_{1a}) receptor ($V_{1a}R$), the luteinizing hormone (LH) receptor (LHR), and the serotonin 5-HT4 receptor. Most of these receptors belong to the superfamily of G protein-coupled receptors that have become aberrantly coupled to steroidogenesis (Schorr and Ney 1971). The presence of these receptors places adrenal cells under stimulation of a hormone that escapes the cortisol-mediated feedback system and leads to increased function and possibly to hyperplasia, proliferative advantage and tumor development (Bordeaux et al. 2001, Li et al. 2005).

In veterinary medicine, LH-dependent hypercortisolism has been described in a pet ferret (Schoemaker et al. 2008) and aberrant adrenocortical expression of GIPR was probably the cause of food-dependent hypercortisolism in a dog (Galac et al. 2008). In addition, the presence of vasopressin receptors on adrenocortical tumors (ATs) has been postulated by the finding of non-ACTH-mediated cortisol responses to systemic lysine vasopressin administration in dogs with ACTH-independent hypercortisolism (Van Wijk et al. 1994).

Identifying (over)expression of these hormone receptors in the adrenal cortex of dogs with ACTH-independent hypercortisolism may provide new insights in the pathogenesis of the disease and offer the potential for novel pharmacological therapies. Therefore, the aims of the present study were: to investigate the mRNA expression of GIPR, LHR, and vasopressin (V_{1a}, V_{1b}, and V_2) receptors in cortisol-secreting ATs and normal adrenal glands by quantitative PCR (QPCR), and to demonstrate the protein expression and localization of these receptors by immunohistochemistry (IHC).

Materials and methods

Animals and tissues

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Utrecht University. The owners gave permission to use the adrenal gland tissue for the study. The ATs were obtained from 23 dogs with ACTH-independent hypercortisolism that underwent unilateral adrenalectomy at the Department of Clinical Sciences of Companion Animals. Their ages, at the time of surgery, ranged from 6 to 14 yr (mean, 10 yr). Nine dogs were of mixed breeding and the others were from 11 different breeds. There were 13 males (four neutered) and 10 females (seven neutered).

Suspicion of hypercortisolism was based on the history, physical examination, and routine laboratory findings. The diagnosis of hypercortisolism was considered to be confirmed by finding of an urinary corticoid:creatinine ratio (UCCR) above the upper limit of the reference range (>8.3 x 10^{-6}) in two consecutive morning urine samples collected at home (Van Vonderen et al. 1998). After collection of the second urine sample the dogs received three doses of 0.1 mg dexamethasone per kg body weight orally at 8 h intervals and the third urine sample was collected on the following morning. ACTH-independent hypercortisolism was diagnosed when the third UCCR sample was suppressed by less than 50% of the mean of the first two samples and basal plasma ACTH concentration was less than 15 ng/L, i.e., suppressed. In all dogs a unilateral AT was visualized by abdominal ultrasonography or computed tomography.

After unilateral adrenalectomy part of the tumor tissue was snap frozen in liquid nitrogen and stored at -70 °C until it was used for QPCR analysis. The remaining tumor tissue was fixed in formalin for histopathology and IHC. The diagnosis of AT was confirmed by histopathological examination in all dogs.

Seventeen adrenals (whole tissue explants) of healthy beagle dogs served as control tissues.

Histopathology

All tissues were fixed in 4% buffered formalin. After at least 24 h and maximally 48 h of fixation the tissues were embedded in paraffin, cut into 5 µm sections and mounted on SuperFrost Plus microscope slides (Menzel-Gläser, Braunsweig, Germany). One section per tissue was stained for histological examination and the other sections were used for IHC.

An AT was considered carcinoma when the tumor size exceeded 2 cm in diameter and when there was histological evidence of invasion of neoplastic cells into blood vessels, peripheral fibrosis, capsular invasion, a trabecular growth pattern, hemorrhage, necrosis,

and single cell necrosis. Typical histological characteristics for adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization (Labelle et al. 2004). Based on these criteria there were 14 carcinomas and nine adenomas.

Total RNA extraction and reverse transcription

Total RNA was extracted from the normal adrenal glands and ATs with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. For the elution, 50 µL of RNase and DNase free water (MilliQ) was used. The concentration of the nucleic acid samples was measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity and quality was performed with the 2100 bioanalyzer (Aligent Technologies, Palo Alto, CA, USA). The RNA Integrity Number (RIN) values of the RNA used varied from 9.9 to 7.8, all well above the recommended 6.0 for QPCR use.

Synthesis of cDNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) using 1 µg RNA input for a 20 µL reaction. After removing 2 µL of each sample to form the pool for the standard dilution series, the remaining cDNA was diluted twice.

Primer design

The mRNA sequences for the genes of interest were obtained from the NCBI (National Center for Biotechnology Information) GenBank database (http://www.ncbi.nlm.nih.gov). Primers (Table 1) for QPCR of GIPR, LHR, $V_{1a}R$, $V_{1b}R$, and V_2R were developed using the Oligo Explorer v1.1.0 software (http://www.genelink.com/tools/gl-oe.asp) and PerlPrimer v1.1.10 (http://perlprimer.sourceforge.net) according to the parameters in the Bio-Rad iCycler manual. The primer products were checked for secondary structure formation with mfold web server v3.1 (http://www.bioinfo.rpi.edu/applications/mfold) (Zucker 2003). QPCR reactions for each primer set were optimized by performing reactions under gradually increasing annealing temperatures. Primer specificity was confirmed by melting curve analysis and sequence analysis of the products. For sequencing, the products were purified by means of QIAquick PCR Purification Kit (Qiagen) and amplified with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). They were filtrated with Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom) and sequenced using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with the NCBI database with BLAST (Basic Local Alignment Search Tool).

Target gene	Sequence (5' → 3')	Ta (°C)	Position	Length of product (bp)
LHR				
For	AAT GGA GCC TTC TGG GG	60.0	658-674	187
Rev	TGG GGT AAG TCA GTG TGG C		844-826	
GIPR				
For	TAC TGC TGC TCT CGT CG	60.0	29-45	119
Rev	TGT CTC CTG GCA CTC TC		147-131	
$V_{1a}R$				
For	CTT TCG TGA TCG TGA CCG	59.0	1958-1975	136
Rev	GAA GCC AGT AAT GCC GTG		2093-2076	
$V_{1b}R$				
For	AAT CTG ACT GTG CTG CTG ACC C	67.0	154-175	105
Rev	GAA TAG TGC CAC GCC CAG GT		258-239	
V_2R				
For	CTC TTC ATC TTT GCC CAG CGT GAC	59.0	553-576	126
Rev	TGC CAC AAA GAC CAT TAG GGC GA		678-656	
RPS5				
For	TCA CTG GTG AGA ACC CCC T	62.5	366-384	141
Rev	CCT GAT TCA CAC GGC GTA G		506-488	
RPL8				
For	CCA TGA ATC CTG TGG AGC	55.0	731-748	64
Rev	GTA GAG GGT TTG CCG ATG		794-777	
HPRT				
For	AGC TTG CTG GTG AAA AGG AC	56.0	484-503	114
Rev	TTA TAG TCA AGG GCA TAT CC		587-568	

Table 1
Primer pairs for LH receptor (LHR), gastric-inhibitory polypeptide receptor (GIPR), three vasopressin receptors (V_{1a} R, V_{1b}R and V_2R), and the reference genes ribosomal protein S5 (RPS5), ribosomal protein L8 (RPL8), and hypoxanthine phosphoribosyltransferase (HPRT) used in the QPCR analysis of this study. All positions are based on the canine sequences of the genes as published on the NCBI GenBank database.
Accession numbers used: LHR: XM_538486, GIPR: XM_541554, V_{1a}R: XM_538263,V_{1b}: XM_545695, V_2R: NM_001003177, RPS5: XM_533568, RPL8: XM_532360, HPRT: AY_283372.
For = forward primer; Rev = reverse primer; Ta = annealing temperature; bp = base pairs.

QPCR

Quantitative-PCR analyses were performed on total RNA to determine and compare the levels of expression of LHR, GIPR, $V_{1a}R$, $V_{1b}R$, and V_2R in cortisol-secreting ATs and normal adrenal glands. To correct for efficiency problems in the reaction and/or for input differences of the sample, the expression levels were normalized against non-regulated reference genes expression levels. Because ribosomal protein S5 (RPS5), ribosomal protein L8 (RPL8) and hypoxanthine phosphoribosyltransferase (HPRT) have been proven stable in other canine tissues (Brinkhof et al. 2006) they were chosen to serve as reference genes in the adrenal gland tissue. The primers for these genes as described by Brinkhof et al. (2006) were used.

QPCR reactions were carried out utilizing a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each PCR reaction consisted of 1.0 µL cDNA, 1.0 µL (10 µM) of both the forward and the reverse primers and 11.0 µL iQ SYBR Green Supermix (Bio-Rad), filled up with 11.0 µL MilliQ to a 25 µL final volume. The cycling settings used were as follows: initial denaturation for 1s at 95 °C followed by 40 cycles each consisting of 20s denaturation at 95 °C, 30s primer annealing at Ta °C (see Table 1) and 30s extension at 72 °C (three-step reactions). The melting curve program consisted of 65 steps of 15s with a temperature increment of 0.5 °C per step, starting at 60 °C. For primers with Ta>58 °C a two-step protocol was used, in which annealing and extension were combined in 30s at Ta. All reactions were performed in duplicate. On every plate a four-fold reference standard dilution series was repeated and negative controls were run to assess the specificity and to identify any potential contamination. Data were analyzed using MyiQ Real-Time PCR detection system software (Bio-Rad).

Since $V_{1b}R$ mRNA expression was inconsistent, reliable quantification or comparison of the expression levels was impossible. The consistency of the results was not improved by purifying the cDNA product with the QIAquick PCR Purification Kit (Qiagen, Venlo, The Netherlands) before performing the QPCR for $V_{1b}R$. However, the results became consistent when the cDNA input was increased from 1 to 4 µL per reaction (reaction proportions were preserved by adding 8 instead of 11 µL MilliQ to each reaction), the effect being that $V_{1b}R$ mRNA was detectable in all investigated samples: 13 normal adrenal glands and 12 cortisol-secreting ATs (four adenomas and eight carcinomas). With these date, expression ratios were calculated and compared.

Hormone Measurements

Plasma ACTH concentration was measured by an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands) validated for the dog (Javadi et al. 2006). The intra-

and inter-assay coefficients of variation were 3.2% and 7.8%, respectively, and the sensitivity was 0.2 ng/L.
Urinary corticoid concentration was measured by a radioimmunoassay described by Rijnberk et al (1988). The intra- and inter-assay coefficients of variation were 6% and 8%, respectively, and the sensitivity was 1 nmol/L. The urinary creatinine concentration was determined by the Jaffé kinetic method (initial rate reaction). From these, the UCCR was calculated (Stolp et al. 1983).

Immunohistochemistry

LHR

For immunohostochemical staining of the LHR the sections were deparaffinized in xylene (twice for 10 min) and dehydrated in 100% ethanol (twice for 5 min). Endogenous peroxidase was blocked by immersion in 1% H_2O_2 in methanol for 30 min. The slides were washed 6 times for 5 min with 0.01 M PBS (pH 7.4) and immersed for 30 min in 0.075% glycine in PBS to demask epitopes. Subsequently, the slides were washed 6 times for 5 min in PBS and blocked with 10% normal goat serum in PBS for 30 min. The slides were incubated overnight at 4 °C with the LHR P1B1 monoclonal antibody at a dilution of 1:1000 in PBS to which 0.05% acetylated BSA (BSA-c, Aurion, Wageningen, The Netherlands) was added. The next day, the slides were washed again with PBS and incubated with a biotinylated horse-anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) for 60 min at a 1:200 dilution in PBS with 0.05% BSA-c at room temperature. The slides were washed thoroughly with PBS and incubated with the avidin-biotin enzyme complex (ABC) of the ABC staining kit (Vector Laboratories) for 60 min in a dilution of 1:1000 in PBS with 0.05% BSA-c. The ABC solution was prepared at least 15 min before use to allow formation of complexes. The slides were washed again 6 times for 5 min in PBS. Bound antibody was visualized using a 0.6 mg/ml 3,3'-diaminobenzidine tetrachloride solution (DAB, Sigma-Aldrich, St. Louis, MO, USA) in PBS to which 0.03% H_2O_2 was added. Sections were incubated with the DAB solution for approximately 1 min and counterstained with Mayers's hematoxylin for 15 sec. The slides were rinsed for 10 min with tap water and mounted with Aquatex (Merck, Whitehouse Station, NJ, USA).
The LHR was detected with murine LHR monoclonal antibody (P1B1), which was a gift from Dr. Wimalasena (Department of Obstetrics and Gynecology, University of Tennessee, Knoxville, TN, USA). The antibody had been raised against purified rat LH receptors (Indrapichate et al. 1992). The antibody binds specifically to LH receptors in various tissues of different species (Peters et al. 2001, Schoemaker et al. 2002).
For negative LHR controls, primary antibody was replaced with normal mouse serum in the same dilution.

GIPR, V_{1a}, V_{1b} and V_2 receptors

For immunohistochemical staining of GIPR, V_{1a}, V_{1b}, and V_2 receptors the sections were deparaffinized with xylene (2 x 5 min) and rehydrated with 100%, 96% and 70% alcohol twice for 3 min each. The sections were soaked in MilliQ twice for 2 min. The 0.01 M citrate buffer (pH 6.0) was preheated in the microwave for 10 min at 1000W and the sections were incubated with it in a microwave for 15 min at 700W. After cooling down at the room temperature for 30 min they were rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) once for 5 min. Endogenous peroxidase activity was inhibited by immersion in 1% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature. Subsequently, the sections were rinsed thrice for 5 min in PBS-Tween. The non-specific antibody binding was blocked with 10% normal goat serum in PBS for 15 min at room temperature. The sections were incubated overnight at 4˚C in a humid chamber with the rabbit antibody for GIPR (ab13004, Abcam, Cambridge, UK) at a 1:200 dilution or rabbit antibody for V_{1b}R (ab13147, Abcam) at a 1:800 dilution or rabbit antibody for V_2R (ab13148, Abcam) at a 1:200 dilution. The next day, after washing with PBS-Tween thrice for 5 min, the sections were incubated with a goat-anti-rabbit secondary antibody for 45 min at room temperature The sections were then rinsed with PBS thrice for 5 min and incubated for 10 min with the freshly made 0.6 mg/ml DAB solution (Sigma-Aldrich) in 0.05M Tris/HCL pH 7.8 with H_2O_2. Thereafter, the sections were rinsed twice for 5 min with tap water and the nuclei were stained with Mayers's haematoxylin for 45 sec. The sections were rinsed again with running tap water for 10 min. Subsequently, they were dehydrated through graded alcohol series (70%-96%-100%) twice for 3 min each and xylene twice for 5 min and coverslipped with Eukitt (EMS, Hatfield, PA, USA)

The primary antibodies AVP1A12-A for V_{1a}R (Alpha Diagnostics Intl, San Antonio, Texas, USA), ab13147 antibody for V_{1b}R, ab13148 antibody for V_2R and ab13004 antibody for GIPR (all Abcam) have never been tested for the dogs before. Their compatibility with the dog was judged based on the sequence homology.

The following positive tissue controls for IHC were used: canine and rat liver and lung for V_{1a}R, canine kidney for V_2R, canine pituitary for V_{1b}R, canine ovary for LHR and canine pancreas for GIPR.

The negative GIPR, V_2R and V_{1b}R controls the primary antibody was replaced with normal rabbit serum diluted at the same protein concentrations of the primary antibody (1:200 for GIPR and V_2R and 1:800 for V_{1b}R).

IHC analysis was performed using light microscopy. The intensity and localization of staining such as membranous, cytoplasmic or nuclear, and its pattern (diffuse, granular) were recorded. Normal adrenal glands of healthy dogs (n=9) served as controls.

Statistical analyses

The relative expression software tool REST 2008 for the comparison and statistical analysis of relative expression results at the 5% significance level.

Results

QPCR

No difference was observed between the tumor samples and the controls in the expression of HPRT, RPS5 and RPL8 (Table2). Expression of HPRT, RPS5, and RPL8 remained constant under the reaction conditions, justifying their use as reference genes.

Gene	Normal Cts ± SE	Adenomas Cts ± SE	Carcinomas Cts ± SE
LHR	32.2 ± 0.3	31.6 ± 0.8	31.6 ± 0.6
GIPR	31.7 ± 0.4	33.7 ± 0.8	32.6 ± 0.5
$V_{1a}R$	19.9 ± 0.2	19.7 ± 0.5	20.8 ± 0.7
$V_{1b}R$	34.6 ± 0.4	36.1 ± 0.4	35.6 ± 0.4
V_2R	31.2 ± 0.3	32.6 ± 0.6	32.1 ± 0.4
RPS5	16.1 ± 0.3	16.0 ± 0.1	16.1 ± 0.3
RPL8	17.9 ± 0.2	17.8 ± 0.2	18.0 ± 0.2
HPRT	22.3 ± 0.2	21.9 ± 0.2	22.3 ± 0.2

Table 2
The mean non-normalized threshold cycles (Cts) of luteinizing hormone receptor (LHR), gastric-inhibitory polypeptide receptor (GIPR), three vasopressin receptors ($V_{1a}R$, $V_{1b}R$ and V_2R), and the reference genes ribosomal protein S5 (RPS5), ribosomal protein L8 (RPL8), and hypoxanthine phosphoribosyltransferase (HPRT) used in the QPCR analysis of this study. QPCR analysis revealed mRNA encoding for LHR, GIPR, $V_{1a}R$, $V1_bR$, and V_2R in all normal adrenal glands and adrenocortical adenomas and carcinomas.

QPCR analysis revealed mRNA encoding for LHR, GIPR, $V_{1a}R$, $V_{1b}R$, and V_2R in all normal adrenal glands and ATs (Table 2). The mRNA expression abundance of $V_{1b}R$ was very low and near the detection limit of the QPCR technique. Differences in receptor mRNA expression abundance between ATs versus normal adrenal glands, adenomas versus normal adrenal glands, and carcinomas versus normal adrenal glands are presented in Table 3. The mRNA expression abundance of GIPR and V_2R in adenomas was significantly lower (P=0.01 and P=0.02, respectively) than in normal adrenal glands. Comparison of the adenomas with the carcinomas did not reveal differences in receptor mRNA expression abundance (data not shown).

Gene	ATs Expression	P	95% C.I.	Adenomas Expression	P	95% C.I.	Carcinomas Expression	P	95% C.I.
LHR	1.40	0.27	0.14-14.75	1.24	0.54	0.22-15.91	1.51	0.21	0.10-13.44
GIPR	0.38	0.03	0.01-13.46	0.22	0.01	0.01-9.01	0.54	0.17	0.02-14.97
$V_{1a}R$	0.69	0.35	0.02-5.18	1.03	0.92	0.06- 6.07	0.54	0.13	0.01-4.04
$V_{1b}R$	0.44	0.07	0.04-1.89	0.41	0.15	0.04-3.63	0.53	0.18	0.04-5.80
V_2R	0.46	0.01	0.01-4.81	0.34	0.02	0.01-2.98	0.55	0.08	0.06-5.55

Table 3
Relative expression ratios (with 95% confidence interval (C.I.) and P value) of the luteinizing hormone receptor (LHR), gastric-inhibitory polypeptide receptor (GIPR) and vasopressin receptors ($V_{1a}R$, $V_{1b}R$ and V_2R) normalized against the expression of the reference genes. The QPCR of all receptors, except for $V_{1b}R$, was performed on tissues of 23 cortisol-secreting adrenocortical tumors (ATs), including nine adenomas and 14 carcinomas. Gene expression levels in these groups were compared to the expression levels in 17 normal adrenal glands. The QPCR of $V_{1b}R$ was performed on 12 cortisol-secreting ATs, including four adenomas and eight carcinomas. Gene expression levels of $V_{1b}R$ were compared to the expression levels of $V_{1b}R$ in 13 normal adrenal glands

Immunohistochemistry

LHR

The anti-LHR antibody stained positively with the theca cells and cells of the corpus luteum in the ovary of a healthy control (Figure 1A). In the normal adrenal glands there was positive staining for LHR in the zona glomerulosa (ZG) and a less intense staining in the zona fasciculata (ZF). In both zones, a cytoplasmic granular pattern was observed. In the zona reticularis (ZR) and the medulla no staining was present. The capsule was also negative, except for the vessels included, which sometimes stained positive for the LHR (Figure 1B).

In ATs, the LHR-positive cells of the ATs demonstrated cytoplasmic granular pattern. The staining varied from a homogeneous, with only slight differences in staining intensity (Figure 1E), to a more heterogeneous pattern, with highly positive areas close to areas in which no staining could be detected (Figure 1F). This heterogeneous pattern was observed mainly in carcinomas. The remnants of normal adrenal tissue that were visible in some tumor sections showed the same immunopositive cells of ZG cells as in the normal adrenal gland tissue.

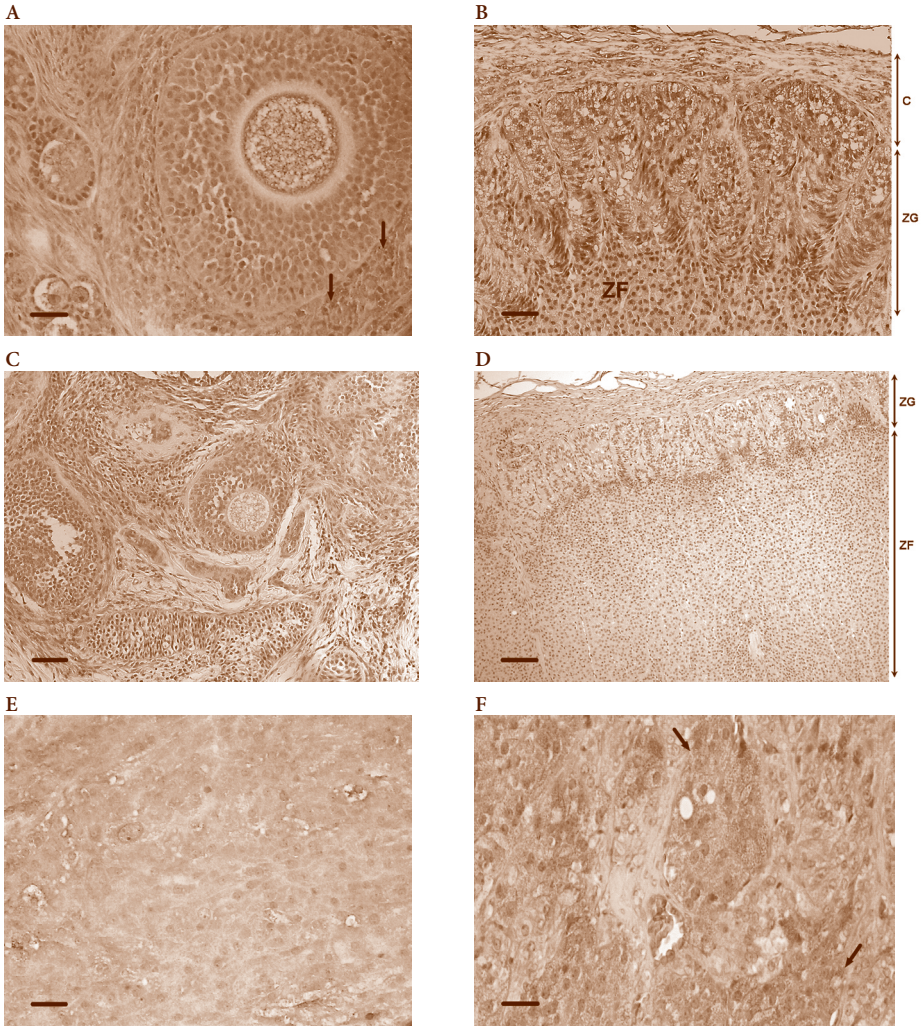

Figure 1
Histological sections of a normal canine ovarium (A), normal canine adrenal gland (B) and two cortisol-secreting adrenocortical adenomas (E and F) stained with the P1B4 luteinizing hormone receptor (LHR) antibody. Negative serum controls of a normal canine ovarium (C) and normal canine adrenal gland (D) are included.

A: Theca cells (arrowheads) and cells of the corpus luteum in the ovary stained positively with the LHR antibody. Bar = 30 µm.

B: LHR positive cells are present mainly throughout the entire zona glomerulosa (ZG) in a cytoplasmic granular pattern. Zona fasciculata (ZF) is less intense, capsule (C) is negative. Bar = 30 µm.

C: No brown background staining could be detected in normal canine ovarium. Bar = 60 µm.

D: No brown background staining could be detected in normal canine adrenal gland. Bar = 170 µm.

E: A homogeneous staining pattern of LHR in a cortisol-secreting adrenocortical adenoma. Bar = 30 µm.

F: A heterogeneous staining pattern of LHR in a cortisol-secreting adrenocortical carcinoma. LHR immunopositive cells (arrowheads) are surrounded by LHR negative regions. Bar = 30 µm.

GIPR

The anti-GIPR antibody stained positively with the islets of the pancreas (Figure 2A). In normal adrenal glands the ZG cells were diffusely positive for GIPR in a cytoplasmic granular pattern. In addition, about 20% of the cells in the medulla of normal adrenal glands stained intensely positive for GIPR in a cytoplasmic granular pattern (Figure 2B). There was no immunolabeling for GIPR in the ZF of normal adrenal glands.

In ATs islands of immunopositive cells, scattered throughout the tumor tissue, were detected in a cytoplasmic granular pattern in about half of the adenomas and carcinomas (Figure 2C).

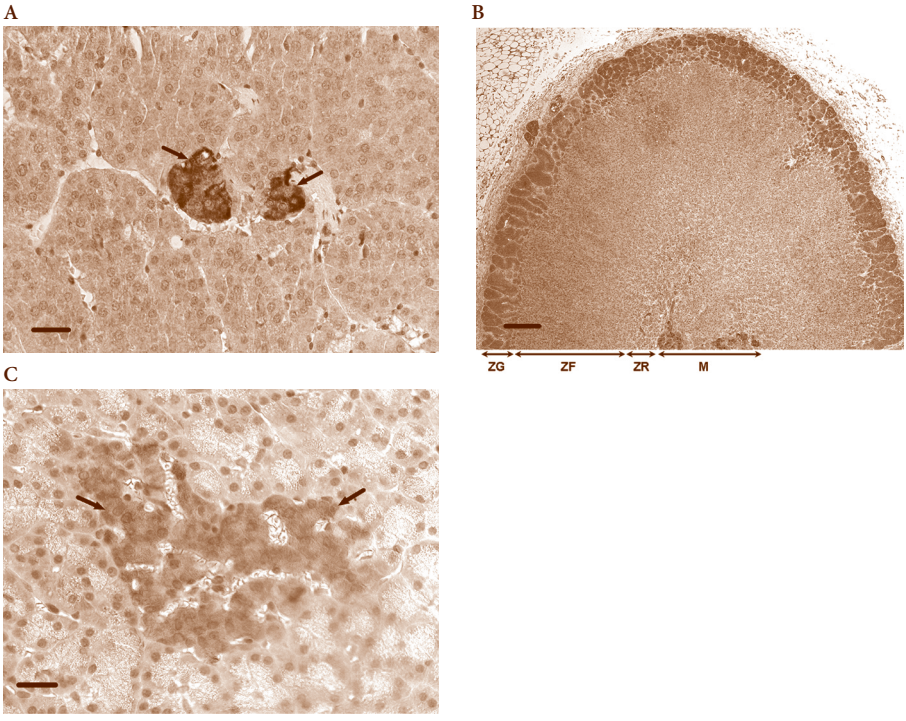

Figure 2

Histological sections of a normal canine pancreas (A), normal canine adrenal gland (B) and a cortisol-secreting adrenocortical carcinoma (C) stained with the ab13004 antibody for gastric-inhibitory polypeptide receptor (GIPR).

A: Langerhans eilands stain positive for GIPR (arrowheads) in a cytoplasmic granular pattern. Bar = 30 µm.

B: The zona glomerulosa (ZG) cells are diffusely positive for GIPR in a cytoplasmic granular pattern. The zona fasciculata (ZF) and zona reticularis (ZR) are negative. In addition, 10% to 20% of the cells in the medulla stain intensely positive for GIPR in a cytoplasmic granular pattern. The capsule is negative. Bar = 340 µm.

C: Two islands of positive GIPR immunoreactivity (arrowheads) in a cortisol-secreting adrenocortical carcinoma. Bar = 60 µm.

$V_{1a}R$

The anti-$V_{1a}R$ antibody utilized did not stain canine tissue in our experiments

V_2R

The anti-V_2R antibody stained positively with renal pelvic epithelium and distal collecting tubules (Figure 3A). In normal adrenal glands staining for V_2R was absent. In 6 of the 9 adrenocortical adenomas a heterogeneous V_2R staining was observed, with a cytoplasmic granular pattern in up to 5% of neoplastic cells. The same staining pattern, but in up to 15% of neoplastic cells, was observed in 12 of the 14 carcinomas (Figure 3B).

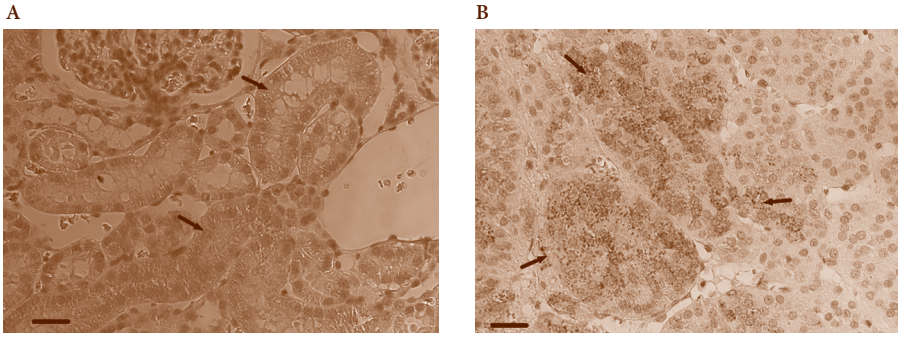

Figure 3
Histological section of a normal canine kidney (A) and cortisol-secreting adrenocortical adenoma (B) stained with the ab13148 antibody for renal vasopressin receptor (V_2R).
A: Distal collecting tubuli (arrowhead) stain immunopositive in a cytoplasmic granular pattern.
B: Clusters of V_2R positive immunoreactivity in a cytoplasmic granular pattern. Bar = 30 µm.

$V_{1b}R$

The anti-$V_{1b}R$ antibody stained positively with the pituitary gland (Figure 4A). Staining for $V_{1b}R$ was negative in normal adrenal glands as well as in ATs (Figure 4B).

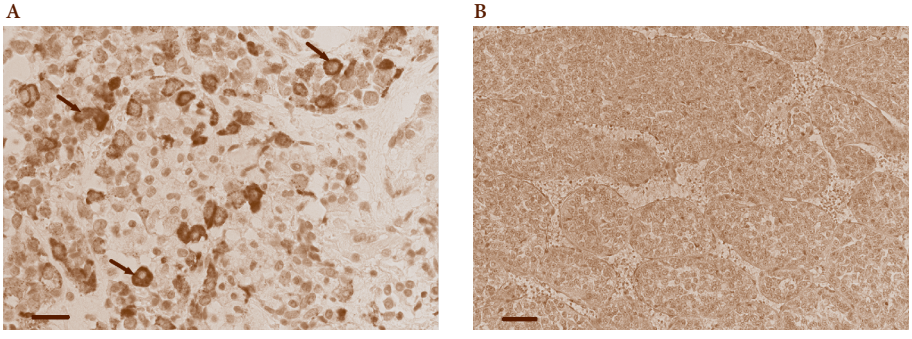

Figure 4
Histological section of a normal canine pituitary gland (A) and cortisol-secreting adrenocortical adenoma (B) stained with the ab13148 antibody for pituitary-specific vasopressin receptor ($V_{1b}R$).
A: Positive immunostaining in the anterior part of the pituitary gland (arrowheads) is detected in a cytoplasmic granulair pattern. Bar = 30 µm.
B: No immunostaining is detected. Bar = 40 µm.

Discussion

The results of the present study reveal that in dogs mRNA encoding for LHR, GIPR, $V_{1a}R$, $V_{1b}R$, and V_2R is detectable in both normal adrenal glands and cortisol-secreting ATs and mRNA expression levels for GIPR and V_2R in ATs are significantly lower in comparison with those in normal adrenal glands. In both normal adrenal tissue and ATs and there is a translation of LHR and GIPR mRNA into receptor protein, while V_2R immunoreactivity was found in some ATs, but not in normal adrenal glands.

The specific staining for the GIPR by IHC of the ZG in normal canine adrenals is similar to that in humans (Lampron et al. 2009). The role of GIPR in the normal human and canine adrenal cortex remains to be defined. In healthy humans a small increase of aldosterone during GIP infusion has been demonstrated, which suggests that the GIPR might be functional in the ZG (Lampron et al. 2009). Whether the same holds true for dogs requires confirmation.

In normal adrenal glands the ZF was immunonegative for GIPR. In contrast, in the cortisol-secreting ATs several GIPR-immunopositive cells were found, indicating ectopic expression of GIPR in tumorous ZF tissue. It may seem contradictory that the expression level of mRNA encoding for GIPR was downregulated in ATs compared to normal adrenal tissue. However, QPCR was performed exclusively in tumorous ZF tissue, while whole adrenal tissue, including ZG cells and medulla, served as control. The results of the QPCR analysis might have been different when only normal and tumorous ZF tissues would have been compared. In future studies, efforts should be made to compare the abundance of the mRNA of receptors in the AT tissue with its equivalent tissue from healthy dogs.

In general, the QPCR results of this study indicate that overexpression of GIPR was not responsible for hypercortisolism and tumorigenesis of the ATs. However, it may be hypothesized that the ectopic expression of the GIPR protein may play a role in the pathogenesis of the AT. Studies with genetically engineered primary bovine adrenocortical cells expressing the GIPR revealed that GIP activates the adenyl cyclase/cAMP signaling cascade normally triggered by the ACTH receptor (Mazzuco et al. 2006a). Transplantation of these modified cells under the renal capsule of adrenalectomized immunodeficient mice resulted in adenomatous adrenocortical tissue that hypersecreted glucocorticoids in an ACTH-independent manner (Mazzuco et al. 2006a, 2007). This indicates that aberrant expression of GIPR is sufficient to initiate the complete phenotypic alterations that ultimately lead to formation of a benign adrenocortical tumor.

In the normal canine adrenals LHR protein was present in the ZG and ZF, whereas there was no LHR staining in the ZR and the medulla. A similar immunohistochemical staining pattern has been reported in ferrets (Schoemaker et al. 2002). Normal human adrenal tissue also contains both LHR mRNA and LHR protein, located in the

ZF and ZR (Pabon et al. 1996). These data indicate that this receptor, normally associated with the pituitary-gonadal axis, is constitutively expressed in the adrenal gland of these species. The role of the LHR in steroidogenesis in normal adrenal glands is, however, not known so far.

The QPCR results of this study seem to indicate that overexpression of LHR was not responsible for hypercortisolism and adrenocortical tumorigenesis. However, as stated above for GIPR, mRNA expression levels of ATs, consisting almost completely of tumorous ZF tissue, were compared with those in normal adrenal glands, including the immunopositive ZG cells. In addition, in normal adrenal glands immunopositivity for LHR in the ZF tissue was strongest just beneath the ZG, whereas the tumorous ZF tissue of the ATs was more uniformly immunopositive. Thus, a role for aberrant expression of LHR in adrenocortical tumorigenesis can still not be excluded. Moreover, the known ability of the LHR to regulate steroid production in the gonads and the numerous reports of LHR-mediated hypercortisolism in humans (Lacroix et al. 2001, Christopoulos et al. 2005, Li et al. 2005), in a ferret (Schoemaker et al. 2008), and in mice (Bernichtein et al. 2009), are highly supportive of a role of LHR as a cause of adrenal disease. In ferrets (Schoemaker et al. 2002) and certain inbred strains of mice (Bernichtein et al. 2009) it is well-known that gonadectomy induces LHR-dependent adrenal hyperplasia and tumorigenesis. This response is apparently triggered by the elevated post-gonadectomy levels of luteinizing hormone. The functional link between LHR expression and tumor development has also been studied by a similar design as for the GIPR (Mazzuco et al. 2006b, 2007). The enforced expression of LHR in adrenalectomized immunodeficient mice leaded to the development of hypercortisolemia, adrenal hyperplasia, and benign transformation, indicating that aberrant LHR expression is a sufficient genetic event to trigger adrenocortical tumorigenesis.

Immunostaining of LHR in the canine ATs showed two patterns: a homogeneous and a heterogeneous staining. The heterogeneous pattern, characterized by clusters of immunopositive areas close to areas with no staining, was observed mainly in carcinomas. The loss of LHR protein expression in some of the carcinoma cells may be a consequence of dedifferentiation, which is a characteristic feature of malignant tumors.

In humans, mRNA encoding for $V_{1a}R$ is normally present in the adrenal gland (Mune et al. 2002). The functional role of this receptor has been postulated in vivo and in vitro. Administration of arginine-vasopressin (AVP), which binds to $V_{1a}R$, leads to a cortisol response in healthy adrenals and cortisol-secreting adenomas (Perraudin et al. 1995, Joubert et al. 2008). Also in dogs with cortisol-secreting ATs intravenous administration of lysine-vasopressin resulted in a supranormal cortisol increase, probably caused by direct stimulation of adrenal steroidogenesis through $V_{1a}R$ activity (Van Wijk et al. 1994). Because the antibodies for $V_{1a}R$ were not effective in the present study, demonstration of $V_{1a}R$ protein in tumorous fasciculata tissue remains to be proven in dogs. In humans, IHC has revealed $V_{1a}R$- and AVP-immunoreactivity in a

subpopulation of cells scattered through the AT (Louiset et al. 2008). Based on these findings it may be hypothesized that vasopressin, locally released by intracortical cells, may act as a paracrine factor to stimulate adrenal steroidogenesis. Moreover, in vitro studies in rat adrenocortical cells revealed that overexpression of $V_{1a}R$ might posses a tumorigenic effect (Lagumdzija et al. 2004, Trejter et al. 2005). In the present study, there was no upregulation of $V_{1a}R$ in the ATs, which makes it unlikely that overexpression of this receptor is involved in adrenal steroidogenesis in pathologic conditions. However, a similar scenario as for the GIPR and LHR expression is possible. IHC of normal adrenal glands and ATs will be needed to detect possible ectopic expression of $V_{1a}R$.

Among all three vasopressin receptors studied, $V_{1a}R$ was found to have the highest mRNA expression levels in both normal adrenal glands and ATs. These values may overestimate the quantity of $V_{1a}R$ mRNA expressed in AT cells, because this receptor type is also expressed in vessels (Birmbauer 2000). Immunohistochemistry will be needed to distinguish the cell type from which the detected mRNA originated.

In contrast to humans, in whom V_2R mRNA could not be demonstrated in normal adrenal gland cortex (Mune et al. 2002, Louiset et al. 2009) mRNA encoding for V_2R was detectable by QPCR in normal canine adrenals. These data may be ambiguous, since whole adrenal gland tissue was used in the present study and -at least in humans- the mRNA encoding for V_2R could also originate from adrenal chromaffin cells and blood vessels (Aldasoro et al. 1997, Grazzini et al. 1999). However, this could not be confirmed by IHC, since no V_2R-immunopositive cells were detected in the normal canine adrenal gland. The lack of V_2R protein in normal adrenal tissue could be a result of decreased mRNA stability. Various molecular partners, such as RNA-stabilizing proteins and small RNA`s, may potentially be involved in this process (Chu and Rana 2007, Eberhardt et al. 2007). The V_2R mRNA in the AT group was significantly downregulated, however IHC demonstrated that V_2R mRNA present was translated to V_2R protein in the tumors. Islands of V_2R-immunopositive cells were detected throughout the AT tissue, a pattern observed also in AIMAH causing Cushing`s syndrome in humans (Louiset et al 2009). The V_2R is classically coupled to adenyl cyclase (Birmbaumer 2000), a cascade normally triggered by the ACTH receptor. The V_2R ectopic expression may potentially lead to excessive steroidogenesis and mitogenesis based on the same principles as described for the GIPR and LHR (Mazzuco et al. 2006a, 2006b, 2007). Since in dogs the V_2R protein was expressed in ATs but not in healthy adrenals, it may be hypothesized that its ectopic expression may play a role in pathogenesis of the ATs.

In the present study, the quantity of $V_{1b}R$ mRNA in normal adrenal glands and ATs was very low. The positive Ct values for $V_{1b}R$ were near to template control of the QPCR technique, which lies around a Ct of 38. In concordance with these results it was not surprising that the IHC failed to detect any $V_{1b}R$-like immunoreactivity in normal

canine adrenals and ATs. In humans, the role of $V_{1b}R$ in cortisol-secreting ATs has been reported to be very limited (Louiset et al. 2009). This may hold true for the dogs as well. In line with this supposition, it has been demonstrated that administration of desmopressin, which binds specifically to $V_{1b}R$, failed to induce a cortisol response in dogs with ATs (Zeugswetter et al. 2008).

A limitation of this study is that none of the antibodies used was species specific, only the anti-LHR antibody has been validated for use in dogs before (Peters et al. 2001). The GIPR and vasopressin antibodies were chosen based on sequence homology and because the immunostaining of positive controls satisfied our expectations. However, if the specificity of GIPR and vasopressin antibodies would have been verified by Western Blot, an additional evidence that the proper protein has been stained would be provided. Another concern is the patchy pattern of the of the GIPR and V_2R immunostaining in ATs. A similar staining pattern could be a result of a detrimental effect of too long fixation on preservation of markers for IHC. However, in all tissues the same fixation procedure with a maximum duration of 48 h was followed. According to the literature (Webster et al. 2009), the immunohistochemical reactivity of most commonly used antigens should not be affected during this period of fixation. Although we did not study the effect on formalin fixation on the antigen detection of the receptors studied, it seems unlikely that the staining pattern would be a result of harmful effect of too long fixation on immunoreactivity.

It is important to mention that in none of the dogs included in the present investigation aberrant expression of receptors was suspected and, consequently, no clinical screening for the presence of the receptors was performed before surgery. In dogs, aberrant regulation of cortisol secretion in ACTH-independent hypercortisolism has only been suspected once in a dog with food-dependent hypercortisolism (Galac et al. 2008), but unfortunately adrenal tissue of this dog was not available. Human patients with Cushing`s syndrome as a result of aberrant expression of ectopic or overactive eutopic receptors in adrenal glands comprise a minority of cases of adrenal hypercortisolism (Lacroix et al. 2001). In addition to the low incidence, the condition is also difficult to diagnose due to the subtle clinical signs. Therefore, clinical screening for the presence of aberrant receptors is conducted in all patients with AIMHA or subclinical Cushing`s syndrome (Christopoulos et al. 2005, Dall`Asta et al. 2004). In dogs with ACTH-independent hypercortisolism, similar screening protocols as in humans would be useful, especially in those cases in which diagnostic imaging reveals adrenal nodular lesions or bilateral AT. There are at least two reasons to include screening protocols in dogs with ACTH-independent hypercortisolism: the evidence of functional expression of one or even more adrenocortical receptors in dogs with ACTH-independent hypercortisolism would confirm their contribution to the pathophysiology of the disease, and it would provide a potential for novel pharmaceutical therapies by suppressing the endogenous ligands or blocking the receptor with specific antago-

nists. In humans, pharmacological treatment clearly improves the clinical manifestations of hypercortisolism, but does not induce any measurable regression of adrenal tumor size (Reznik et al. 1992, De Herder et al. 1996, Lacroix et al. 1997). In a ferret with LH-dependent hypercortisolism, however, treatment with a depot gonadotropin releasing hormone-agonist implant resolved not only the clinical signs but resulted also in a reduction of the size of the AT (Schoemaker et al. 2008).

Differentiating between adrenocortical adenoma and carcinoma is very difficult. In order to make the pathological diagnosis as reliable as possible, all of the ATs in this study were judged by a single pathologist using always the same criteria. Despite this approach, misjudges are possible. A scoring system which accounts not only the pathology, but also the clinical picture and follow-up, may be more reliable. Such a classification is used to judge the nature of ATs in humans and may have to be introduced in veterinary medicine as well (Weiss et al. 1989).

In conclusion, the potential functional role of LH, GIP and vasopressin receptors in normal adrenal glands and ATs remains to be elucidated. Aberrant expression of these hormone receptors, such as the ectopic expression of GIPR and V_2R protein, in tumorous ZF tissue suggests that they may play a role in the pathogenesis of canine cortisol-secreting ATs.

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Chapter 4

Expression of the adrenocorticotropic hormone receptor, steroidogenic acute regulatory protein, and steroidogenic enzymes in canine cortisol-secreting adrenocortical tumors

S. Galac[a], M.M.J. Kool[a], E.C. Naan[a], S. Daminet[b], J.A. Mol[a], H.S. Kooistra[a]

Submitted

[a] *Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[b] *Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Ghent, Belgium*

Abstract

Studies of human adrenocortical tumors (ATs) causing Cushing's syndrome suggest that hypersecretion of cortisol is due to altered expression of steroidogenic enzymes and that steroidogenesis can only be maintained when there is expression of the adrenocorticotropin receptor (ACTH-R). Here, we report on a screening of 38 surgically removed ATs in dogs with Cushing's syndrome for the mRNA expression of steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A), 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20-lyase (CYP17), 21-hydroxylase (CYP21), 11β-hydroxylase type 1 (CYP11B1) and the ACTH-R. Abundances of mRNA were quantified using quantitative PCR (QPCR) and were compared with those in normal canine adrenal glands. In addition, protein expression of ACTH-R was demonstrated by Western blot analysis. In both normal adrenal glands and cortisol-secreting ATs, mRNA encoding StAR, steroidogenic enzymes and the ACTH-R was present. Abundances of mRNA encoding StAR and enzymes of the steroidogenic cluster needed for cortisol production, did not differ significantly between adenomas, carcinomas, and normal adrenal glands. The abundances of mRNA encoding ACTH-R in carcinomas were significantly lower (P=0.008) than those in normal adrenal glands. In agreement with the QPCR results, Western blot analysis revealed less protein expression of the ACTH-R in carcinomas. In conclusion, QPCR analysis did not reveal overexpression of StAR and steroidogenic enzymes in canine cortisol-secreting ATs. In carcinomas, significant downregulation of the ACTH-R may contribute to the malignant character of this tumor.

Introduction

Adrenal or adrenocorticotropin (ACTH)- independent hypercortisolism represents about 15% of cases of spontaneous hypercortisolism in dogs (Galac et al. 2010). The mechanisms leading to the autonomous hypersecretion of cortisol have not been

defined yet. Under physiological circumstances, steroidogenesis in an adrenocortical cell is initiated by binding of ACTH to its receptor (ACTH-R) (Mountjoy et al. 1994). This will lead to protein kinase A activation and intracellular cAMP accumulation, which in turn increases the transcription of the cholesterol side-chain cleavage enzyme (CYP11A), the rate limiting step in steroidogenesis (Miller 1988, Mountjoy et al. 1992). In humans and mice it has been demonstrated that the regulation of the ACTH-R gene is unique in that it is upregulated by its own ligand in a time- and dose dependent manner (Mountjoy et al. 1994). In case of cortisol-secreting adrenocortical tumors (ATs) the plasma ACTH concentration is suppressed. Therefore, in cortisol-secreting ATs the expression of the ACTH-R is expected to be low and the role of ACTH and its receptor, as the initiators of steroidogenesis, is supposed to be small. Indeed, in human cortisol-secreting carcinomas and non functional ATs, the expression of ACTH-R has been reported to be significantly downregulated (Reincke et al. 1997a, Zwerman et al. 2004). In cortisol-secreting adenomas, however, the ACTH-R mRNA expression did not differ from that in normal adrenals and was independent of the plasma ACTH concentration (Reincke et al. 1997b). Administration of ACTH to patients with cortisol-secreting adenomas resulted in a significant increase of the plasma cortisol concentration (Imai et al. 2001). This and the finding that in adenomas the expression of the ACTH-R correlates with the expression of the CYP11A, led to the conclusion that the ACTH-R appears to be functional in cortisol-secreting adenomas (Reincke et al. 1997b, Imai et al. 2001).

A recent study on the expression of enzymes in the cortisol-pathway of steroidogenesis, provides evidence that hypersecretion of cortisol by human ATs can be explained by alterations in steroidogenesis. Human cortisol-secreting adenomas overexpressed mRNA encoding CYP11A, 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20-lyase (CYP17) and 11β-hydroxylase type 1 (CYP11B1), when compared to normal adrenals (Bassett et al. 2005).

In dogs, so far there is no information about the expression of the ACTH-R and enzymes of the steroidogenic cluster needed for cortisol production in ATs. However, there is indirect evidence about the expression and functionality of the ACTH-R in canine ATs. In approximately 60% of dogs with hypercortisolism due to an AT there is an exaggerated cortisol response to ACTH administration (Peterson et al. 1982).

The aim of this study was to investigate 1) the mRNA expression of steroidogenic acute regulatory protein (StAR) and the steroidogenic enzymes of the cortisol pathway CYP11A, HSD3B, CYP17, 21-hydroxylase (CYP21) and CYP11B1 (Figure 1) by QPCR and 2) the mRNA expression of the ACTH-R by QPCR and its protein expression by Western blot analysis, in canine cortisol-secreting ATs.

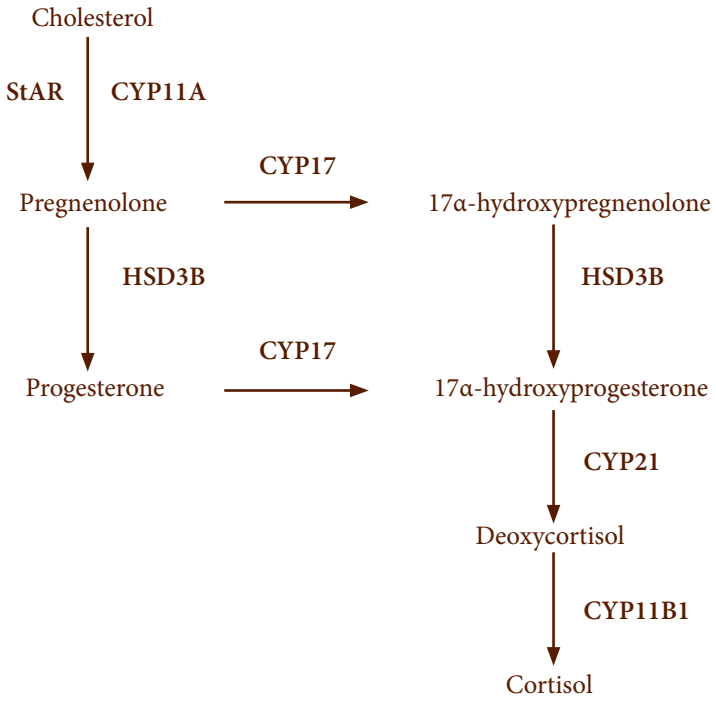

Figure 1
Steroidogenic acute regulatory protein (StAR) and steroidogenic enzymes involved in the cortisol pathway: steroidogenic cholesterol side-chain cleavage enzyme (CYP11A), 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20-lyase (CYP17), 21-hydroxylase (CYP21) and 11β-hydroxylase type 1 (CYP11B1).

Materials and methods

Animals and tissues

The study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of Utrecht University. Permission to use the adrenal gland tissue in this study was obtained from all patient owners. The ATs were obtained from 38 dogs with ACTH-independent hypercortisolism that underwent unilateral adrenalectomy at the Department of Clinical Sciences of Companion Animals. Their ages, at the time of surgery, ranged from 6 to 14 yr (mean, 9 yr). Twelve dogs were mongrels and the others were from 10 different breeds. There were 18 males (8 castrated) and 20 females (15 neutered).

Suspicion of hypercortisolism was based on the history, physical examination, and

routine laboratory findings. The diagnosis of hypercortisolism was considered to be confirmed by finding of a urinary corticoid:creatinine ratio (UCCR) above the upper limit of the reference range (>8.3 x 10^{-6}) in two consecutive morning urine samples collected at home (van Vonderen et al. 1998). After collection of the second urine sample the dogs received three doses of 0.1 mg dexamethasone per kg body weight orally at 8 h intervals and the third urine sample was collected on the following morning. ACTH-independent hypercortisolism was diagnosed when the third UCCR sample was suppressed by less than 50% of the mean of the first two samples and basal plasma ACTH concentration was less than 15 ng/L, i.e., suppressed. In all dogs a unilateral AT was visualized by abdominal ultrasonography or computed tomography.

After unilateral adrenalectomy part of the tumor tissue was snap frozen in liquid nitrogen and stored at -70 °C until it was used for QPCR analysis. The remaining tumor tissue was fixed in formalin for histopathology and IHC. The diagnosis of AT was confirmed by histopathological examination in all dogs.
Fifteen adrenals (whole tissue explants) of healthy beagle dogs served as control tissues.

Histopathology

Formalin-fixed, paraffin-embedded tissues were cut and stained with hematoxylin and eosin for histopathological evaluation. An AT was considered a carcinoma when the tumor size exceeded 2 cm in diameter and when there was histological evidence of invasion of neoplastic cells into blood vessels, peripheral fibrosis, capsular invasion, a trabecular growth pattern, hemorrhage, necrosis, and single cell necrosis. Typical histological characteristics for adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization (Labelle et al. 2004). Based on these criteria there were 26 carcinomas and 12 adenomas.

Total RNA extraction and reverse transcription

Total RNA was extracted from the normal adrenal glands and ATs with the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase (Qiagen) according to the manufacturer's instructions. For the elution, 50 µl of RNase and DNase free water (MilliQ) was used. The concentration of the nucleic acid samples was measured with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity and quality was verified with the 2100 bioanalyzer (Aligent Technologies, Palo Alto, CA, USA). The RNA Integrity Number (RIN) values of the RNA used varied from 9.9 to 7.8, all well above the recommended 6.0 for QPCR use.

Target gene	Sequence (5' → 3')	Ta (°C)	Position	Length of product (bp)
CYP11A				
For	CAC CGC CTC CTT AAA AAG TAA CAA G	63.3	904-927	129
Rev	GCT GCG TGC CAT CTC GTA G		1032-1014	
ACTH-R				
For	TCATGTGGTTTTGCCGGAAGAGAT	58.5	66-89	138
Rev	AATGGCCAGGCTGCAAATGAAA		204-183	
StAR				
For	CTCTGCTTGGTTCTCGG	62.5	238-254	125
Rev	CCTTCTTCCAGCCTTCC		363-347	
CYP17				
For	CCT GCG GCC CCT ATG CTC	60.0	1075-1092	134
Rev	GGC CGG TAC CAC TCC TTC TCA		1208-1188	
HSD3B				
For	CAG GAG GGT TTC TGG GTC AG	56.5	158-178	186
Rev	AGG CTC TCT TCA GGC ACT GC		343-324	
CYP21				
For	AGC CCG ACC TTC CCC TCC ACC TG	64.5	185-207	152
Rev	TCT GCC GGC GAA GTC CAC CCA TTT		336-313	
CYP11B1				
For	GGA GGC CCC GGA GGA GGA AGA GAC	59.0	1176-1199	172
Rev	TCG TGC GGT GAG GGG GAT GAA GG		1347-1325	
RPS5				
For	TCA CTG GTG AGA ACC CCC T	62.5	366-384	141
Rev	CCT GAT TCA CAC GGC GTA G		506-488	
RPS19				
For	CCT TCC TCA AAA AGT CTG GG	61.0	482-501	95
Rev	GTT CTC ATC GTA GGG AGC AAG		576-556	
HPRT				
For	AGC TTG CTG GTG AAA AGG AC	56.0	484-503	114
Rev	TTA TAG TCA AGG GCA TAT CC		587-568	

Table 1
Primer pairs for adrenocorticotropin receptor (ACTH-R), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A), 17α-hydroxylase/17,20-lyase (CYP17), 3β-hydroxysteroid dehydrogenase (HSD3B), 21-hydroxylase (CYP21) and 11β-hydroxylase type 1 (CYP11B1) and the reference genes ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and hypoxanthine phosphoribosyltransferase (HPRT), used in the QPCR analysis of this study. All positions are based on the canine sequences of the genes, as published on the NCBI GenBank database.
Accession numbers used: StAR: NM_001097542, ACTH-R: XM_541098, CYP11A: XM_535539.2, CYP17: XM_858257.1, HSD3B: AY739720.1, CYP21: NM_001003335.1, CYP11B1: XM_846295.1, RPS5: XM_533568, RPS19: XM_533657, HPRT: AY_283372.
For = *forward primer*; Rev = *reverse primer*; Ta = *annealing temperature*; bp = *base pairs*.

Synthesis of cDNA was done with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) using 1 µg RNA input for a 20-µl reaction. After taking out 2 µl of each sample to form the pool for the standard dilution series, the remaining cDNA was diluted two times.

Primer design

The mRNA sequences for the genes of interest were obtained from the NCBI (National Center for Biotechnology Information) GenBank database (http://www.ncbi.nlm.nih.gov). Primers (Table 1) for QPCR of ACTH-R, StAR, CYP11A, CYP17, HSD3B, CYP21 and CYP11B1 were developed using the Oligo Explorer v1.1.0 software (http://www.genelink.com/tools/gl-oe.asp) and PerlPrimer v1.1.10 (http://perlprimer.sourceforge.net) according to the parameters in the Bio-Rad iCycler manual. The PCR products were checked for secondary structure formation with mfold web server v3.1 (http://www.bioinfo.rpi.edu/applications/mfold) (Zucker 2003).
QPCR reactions for each primer set were optimized by performing reactions under gradually increasing annealing temperatures. Primer specificity was confirmed by melting curve analysis and sequence analysis of the products. For sequencing, the products were purified by means of QIAquick PCR Purification Kit (Qiagen) and amplified with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). They were filtrated with Sephadex G-50 Superfine (Amersham, Buckinghamshire, United Kingdom) and sequenced using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with the NCBI database with BLAST (Basic Local Alignment Search Tool).

Quantitative polymerase chain reaction (QPCR)

QPCR analyses were performed on total RNA, to determine and compare the levels of expression of ACTH-R, StAR, CYP11A, CYP17, HSD3B, CYP21 and CYP11B1 in cortisol-secreting ATs and normal adrenal glands. To correct for efficiency problems in the reaction and/or for input differences of the sample, the expression levels were normalized against non-regulated reference gene expression levels. Because ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19) and hypoxanthine phosphoribosyltransferase (HPRT) have been proven stable in other canine tissues (Brinkhof et al. 2006) they were chosen to serve as reference genes in the adrenal gland tissue. The primers for these genes have been described by Brinkhof et al. (2006).
QPCR reactions were carried out utilizing a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Each PCR reaction consisted of

1.0 µl cDNA, 1.0 µl (10 µM) of both the forward and the reverse primers and 11.0 µl iQ SYBR Green Supermix (Bio-Rad), filled up with 11.0 µl MilliQ to a 25-µl final volume. The cycling settings used were as follows: initial denaturation for 1s at 95 °C followed by 40 cycles each consisting of 20s denaturation at 95 °C, 30s primer annealing at Ta °C (see Table 1) and 30s extension at 72 °C (three-step reactions). The melting curve program consisted of 65 steps of 15s with a temperature increment of 0.5 °C per step, starting at 60 °C. For primers with Ta>58 °C a two-step protocol was used, in which annealing and extension were combined in 30s at Ta. All reactions were performed in duplicate. On every plate a 4-fold reference standard dilution series was repeated and negative controls were run to assess the specificity of the reaction and to identify any potential contamination. Data were analyzed using iQ5 Real-Time PCR detection system software (Bio-Rad).

Gene expression levels of ACTH-R, StAR, CYP11A, HSD3B and CYP21 were determined in 12 adenomas and 26 carcinomas and were compared to the expression levels in 15 normal adrenals. Gene expression levels of CYP17 and CYP11B1 were determined in 12 adenomas and 16 carcinomas and were compared to the expression levels in 7 normal adrenals.

Western blot

The Mini-protean electrophoresis system (Bio-Rad) was used for Western blot analysis. The gel was a 10% SDS-PAGE running gel, with a 4% stacking gel. 30µg per sample was loaded onto the gel and as a standard the Precision plus protein standard (Bio-Rad) was used. The ACTH-R is expected to show a band at 33 kD. The gel-electrophoresis was done at 150 Volts for 1.5 h. Next, the blotting was done at a voltage of 100 Volts for one h. For blocking and staining of the blot the ECL Advance Western Blotting Detection kit Amersham (GE Healthcare, Diegem, Belgium) was used. The blot was incubated overnight with a goat polyclonal IgG anti-ACTH-R antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) dissolved in 0.2% TBST-buffer + 4% bovine serum albumin at a concentration of 1:500. The secondary antibody was a HRP-conjugated donkey anti-goat IgG antibody, dissolved at a concentration of 1:20.000 and pre-incubated with a blot to decrease background staining. All washing steps were done with a 0.2% TBST-solution at room temperature, also for decreasing background staining. After staining of the blot, it was examined with the ChemiDoc XRS (Bio-Rad).

As a reference, a β-actin staining was performed, to be able to compare the results of the adenomas with the carcinomas and the controls. Beta-actin has a molecular weight of 42 kD. The concentration of the primary antibody, also dissolved in 0.2% TBST-buffer + 4% bovine serum albumin, was 1:2000. The secondary antibody, a

HRP-conjugated anti-mouse IgG antibody (R&D Systems, Minneapolis, MN, USA), had a concentration of 1:20.000.

Hormone Measurements

Plasma ACTH concentration was measured by an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands) validated for the dog (Javadi et al. 2006). The intra- and inter-assay coefficients of variation were 3.2% and 7.8%, respectively, and the sensitivity was 0.2 ng/L.

Urinary corticoid concentration was measured by a radioimmunoassay described by Rijnberk et al (1988). The intra- and inter-assay coefficients of variation were 6% and 8%, respectively, and the sensitivity was 1 nmol/L. The urinary creatinine concentration was determined by the Jaffé kinetic method (initial rate reaction). From these, the UCCR was calculated (Stolp et al. 1983).

Statistical analyses

All values are expressed as mean ± SEM. The relative expression software tool REST 2008 was used for the comparison and statistical analysis of relative expression of ACTH-R, StAR and steroidogenic enzymes (CYP11A, CYP17, HSD3B, CYP21 and CYP11B1). In general, $P < 0.05$ was considered statistically significant, but in case of multiple comparison Bonferoni correction was applied and $P < 0.025$ was considered statistically significant.

Results

QPCR

No difference was observed between the AT samples and the controls in the expression of HPRT, RPS5 and RPS19 (Table 2). Expression of HPRT, RPS5, and RPS19 remained constant under the reaction conditions, justifying their use as reference genes.

QPCR analysis revealed mRNA encoding ACTH-R, StAR, CYP11A, CYP17, HSD3B, CYP21 and CYP11B1 in all normal adrenal glands and ATs (Table 2). Differences in mRNA abundance between ATs versus normal adrenal glands, adenomas versus carcinomas, adenomas versus normal adrenal glands, and carcinomas versus normal adrenal glands are presented in Table 3.

The expression abundances of mRNA encoding ACTH-R in cortisol-secreting carcinomas were significantly lower (P=0.008) when compared to those in normal adrenal

glands. The expression abundances of mRNA encoding HSD3B in cortisol-secreting adenomas tended to be higher (P=0.04) when compared to those in normal adrenal glands. Comparison of the adenomas with the carcinomas revealed no differences in mRNA expression abundances of the ACTH-R, StAR, and steroidogenic enzymes, although CYP21 tended to be higher in adenomas (P=0.04) compared to carcinomas.

A:

Gene	*Normal* C_ts ± SE	*Adenomas* C_ts ± SE	*Carcinomas* C_ts ± SE
ACTH-R	19.3 ± 0.2	19.5 ± 0.3	20.7 ± 0.3
StAR	13.9 ± 0.1	13.5 ± 0.2	13.0 ± 0.2
CYP11A	15.1 ± 0.2	14.5 ± 0.3	15.1 ± 0.2
HSD3B	14.1 ± 0.2	13.0 ± 0.3	14.3 ± 0.3
CYP21	14.5 ± 0.2	14.0 ± 0.3	14.9 ± 0.2
RPS5	15.1 ± 0.1	15.0 ± 0.2	14.8 ± 0.1
RPS19	14.3 ± 0.1	14.2 ± 0.2	14.5 ± 0.1
HPRT	19.7 ± 0.1	19.4 ± 0.2	20.0 ± 0.1

B:

Gene	*Normal* C_ts ± SE	*Adenomas* C_ts ± SE	*Carcinomas* C_ts ± SE
CYP17	14.0 ± 0.4	14.2 ± 0.3	14.8 ± 0.6
CYP11B1	18.7 ± 0.3	18.8 ± 0.3	19.4 ± 0.4
RPS5	15.1 ± 0.5	14.4 ± 0.1	15.0 ± 0.2
RPS19	14.6 ± 0.3	14.2 ± 0.1	16.4 ± 1.3
HPRT	21.3 ± 0.8	20.0 ± 0.2	20.1 ± 0.2

Table 2
QPCR analysis revealed mRNA encoding adrenocorticotropin receptor (ACTH-R), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A), 17α-hydroxylase/17,20-lyase (CYP17), 3β-hydroxysteroid dehydrogenase (HSD3B), 21-hydroxylase (CYP21) and 11β-hydroxylase type 1 (CYP11B1) in normal adrenal glands and adrenocortical tumors (ATs).

A: The mean non-normalized threshold cycles ((C_ts) ± SE) of ACTH-R, StAR, CYP11A, HSD3B, CYP21 and the reference genes ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19), and hypoxanthine phosphoribosyltransferase (HPRT) determined in 15 normal adrenal glands, 12 cortisol-secreting adenomas, and 26 cortisol-secreting carcinomas.

B: The mean C_ts (± SE) of CYP17 and CYP11B1, and the reference genes RPS5, RPS19 and HPRT determined in 7 normal adrenal glands, 12 cortisol-secreting adenomas, and 16 cortisol-secreting carcinomas.

A:

Gene	*Adenomas Expression*	*P*	*95% C.I.*	*Carcinomas Expression*	*P*	*95% C.I.*
ACTH-R	0.73	0.14	0.21 – 1.41	0.38	0.008	0.01 – 2.56
StAR	0.82	0.22	0.29 – 2.52	1.42	0.08	0.26 – 7.45
CYP11A	0.43	0.76	0.01 – 5.07	1.04	0.86	0.18 - 5.80
CYP17	0.53	0.14	0.06 – 3.47	0.50	0.15	0.04 – 5.05
HSD3B	1.70	0.04	0.31 – 9.32	0.94	0.86	0.05 – 8.85
CYP21	1.24	0.26	0.33 – 4.25	0.77	0.21	0.08 – 2.72
CYP11B1	0.97	0.26	0.06 – 8.37	0.49	0.15	0.04 – 8.15

B:

Gene	*AT Expression*	*P*	*95% C.I.*	*A versus C Expression*	*P*	*95% C.I.*
ACTH-R	0.47	0.21	0.01 – 3.03	0.52	0.13	0.01 – 4.64
StAR	1.28	0.21	0.18 – 6.03	1.23	0.35	0.21 – 5.56
CYP11A	0.78	0.69	0.07 – 5.66	0.71	0.21	0.09 – 5.57
CYP17	0.51	0.11	0.04 – 4.23	0.97	0.95	0.08 – 11.77
HSD3B	1.12	0.79	0.06 – 9.10	0.55	0.10	0.03 – 4.30
CYP21	0.88	0.50	0.14 – 3.28	0.61	0.04	0.08 – 3.33
CYP11B1	0.53	0.11	0.04 – 8.70	0.81	0.57	0.07 – 7.15

Table 3
Relative mRNA expression ratios (with 95% confidence interval (C.I.) and P value) of adrenocorticotropin receptor (ACTH-R), steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (CYP11A), 17α-hydroxylase/17,20-lyase (CYP17), 3β-hydroxysteroid dehydrogenase (HSD3B), 21-hydroxylase (CYP21) and 11β-hydroxylase type 1 (CYP11B1), normalized against the mRNA expression of the reference genes ribosomal protein S5 (RPS5), ribosomal protein S19 (RPS19), and hypoxanthine phosphoribosyltransferase (HPRT). Gene expression levels of ACTH-R, StAR, CYP11A, HSD3B and CYP21 were determined in 12 cortisol-secreting adenomas and 26 cortisol-secreting carcinomas and were compared to the expression levels in 15 normal adrenal glands. Gene expression levels of CYP17 and CYP11B1 were determined in 12 cortisol-secreting adenomas and 16 cortisol-secreting carcinomas and were compared to the expression levels in 7 normal adrenal glands.

A: Cortisol-secreting adenomas and carcinomas versus normal adrenal glands.

B: Cortisol-secreting adrenocortical tumors (ATs) versus normal adrenal glands and cortisol-secreting adenomas (A) versus cortisol-secreting carcinomas (C).

Western blot

Protein expression of the ACTH-R was present in normal adrenals and ATs. However, staining of the band at 33 kD, which corresponds with the ACTH-R, was more intense in adenomas and normal adrenals than in carcinomas (Figure 2).
There was a co-amplification of the β-actin fragment at 42 kD in all samples.

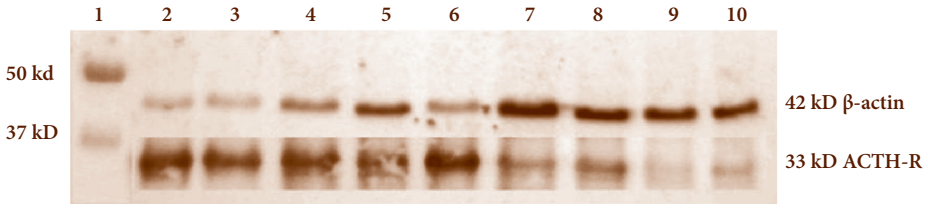

Figure 2
The results of Western blot analysis of the protein expression of the adrenocorticotropin receptor (ACTH-R) in 2 normal adrenal glands (samples 2 and 3), 3 cortisol-secreting adenomas (samples 4 - 6) and 4 cortisol-secreting carcinomas (samples 7 - 10). The protein standard is shown in the left column (sample 1). The upper band represents 50 kD and the lower band 37 kD. In samples 2 - 10, the upper band is a β-actin band (42 kD) and the lower is the ACTH-R band (33 kD). The intensity of the bands is more intense in normal adrenal glands and adenomas than in carcinomas.

Discussion

The results of the present study demonstrate that in dogs:
1) mRNA encoding StAR and enzymes of the steroidogenic cluster needed for cortisol production is detectable in both normal adrenal glands and cortisol-secreting ATs,
2) there is no significant difference in mRNA abundance of StAR and steroidogenic enzymes between normal adrenals and cortisol-secreting adenomas/carcinomas, and
3) the expression of the ACTH-R is significantly downregulated in cortisol-secreting adrenocortical carcinomas.
ACTH is the major peptide regulating adrenocortical steroidogenesis. Under physiological circumstances, the ACTH-R-cAMP-protein kinase A signaling cascade maintains a highly differentiated cellular phenotype, whereas proliferation of adrenocortical cells is also stimulated by peptides and receptors other than ACTH and its receptor (Estivariz et al. 1988, Reincke et al. 1997a, Fassnacht et al. 2003). In highly malignant and non-functional human ATs, low expression of the ACTH-R gene and loss of heterozygosity (LOH) have been described (Reincke et al. 1997a, 1998). It has also been suggested that deletion of the ACTH-R provides ATs with a growth advantage. In the canine cortisol-secreting carcinomas from this study, QPCR analysis also revealed significant downregulation of the gene encoding ACTH-R, which was substantiated at the transcriptional level with Western blot experiments. It may be hypothesized that loss of the

ACTH-R signal transduction cascade in canine adrenocortical carcinomas, leads to loss of differentiation and enhanced clonal expansion of AT cells, similar to what has been described in humans (Reincke et al. 1997a, 1998, Beuschlein et al. 2001).
In the canine adenomas ACTH-R expression did not differ from that in normal adrenal glands. Apparently, the suppressed plasma ACTH concentration in these patients did not affect the expression of its receptor, as would be expected based on the regulation mechanism of the ACTH-R gene in cultured normal human and bovine adrenocortical cells (Lebrethon et al. 1994, Penhoat et al. 1995). This implies a different regulatory mechanism of ACTH-R expression in adenomas (Morita 1995). It has been suggested, that expression of ACTH-R even in the absence of ACTH might result in increased basal adenyl cyclase activity, as observed in case of thyroid-stimulating hormone receptor, and thereby might play a role in autonomous production of cortisol (Imai et al. 2001).

Based on the data of the mRNA and protein expression of the ACTH-R, adenomas are more likely to respond to ACTH administration than carcinomas. Efforts to differentiate between adenomas and carcinomas based on the post-ACTH plasma cortisol concentration have been made in the past, but results were inconclusive (Peterson et al. 1982, Feldman 1983). One of the major problems in interpreting the results of the ACTH stimulation test in dogs with a cortisol-secreting AT is that the differentiation between adrenocortical adenoma and carcinoma by conventional histopathology is very difficult (Labelle et al. 2004, Weiss et al. 1989). The results of this study suggest that determination of the ACTH-R expression, and possibly also the outcome of an ACTH stimulation test, may provide the pathologist with additional information allowing a better differentiation between adrenocortical carcinomas and adenomas. A more reliable detection of adrenocortical carcinoma would allow post surgical adjuvant therapy only in patients that may really benefit from it.
Cholesterol is the basic substance for all steroid hormones. For acute steroidogenesis, cholesterol has to be mobilized from intracellular lipid droplets and delivered to CYP11A, which is associated with the inner mitochondrial membrane (Miller 2007, Manna et al. 2009). The presence of mRNA encoding StAR in normal canine adrenals and ATs, suggests intact transport of cholesterol from the outer to the inner mitochondrial membrane. In humans, high expression of CYP11A and StAR has been described in functional adenomas (Zenkert et al. 2000). In non-functional ATs the expression of StAR was normal and CYP11A was significantly reduced, which indirectly confirms a rate limiting role of CYP11A in steroidogenesis. In the present study, mRNA encoding StAR and CYP11A was present in all ATs. Since only cortisol-secreting ATs were studied, this is in accordance with the findings in human functional ATs.
Investigation of the mRNA expression of steroidogenic enzymes in canine ATs, revealed a trend to upregulation of HSD3B in adenomas compared to normal adrenal glands. The orchestration of adrenocortical enzyme levels depends on the regulating

mechanisms of a single steroidogenic enzyme. The expression of each steroidogenic enzyme is complex and the signaling pathways involve hormonal and transcriptional control (Sewer et al. 2007, Lavoie et al. 2009). It may be hypothesized that the differential regulation of HSD3B versus cytochrome P450 (CYP) enzymes is crucial for the relatively higher mRNA expression levels of only HSD3B in canine cortisol-secreting adenomas (Mason et al. 1997, Simard et al. 2005, Lavoie et al. 2009). A higher abundance of mRNA encoding HSD3B may result in more protein and more HSD3B protein may lead to increased plasma 17-OH progesterone concentration. In fact, increased circulating concentrations of 17-OH progesterone have been reported before in dogs with pituitary- and adrenal-dependent hypercortisolism (Ristic et al. 2003, Benitah et al. 2005). However, the diagnostic value of the circulating 17OH-progesterone concentration is limited, because there is an overlap between the basal and post-ACTH concentration of 17OH-progesterone in healthy dogs and dogs with hypercortisolism (Chapman et al. 2003). Whether the plasma 17OH-progesterone concentration could be used as a marker for adenomas once an AT has been diagnosed, has not been studied yet.

Dogs with hypercortisolism due to AT have been treated successfully with trilostane, a competitive inhibitor of HSD3B (Eastwood et al. 2003, Benchekroun et al. 2008). Because mRNA encoding HSD3B is expressed in relatively high abundance in tumourous zona fasciculata tissue, the results of the present study provide an explanation for the successfulness of trilostane treatment to control cortisol secretion and consequently clinical signs due to hypercortisolism in dogs with an (inoperable) adrenocortical adenoma.

Comparison between adenomas and carcinomas demonstrated a trend to higher mRNA expression of CYP21 in adenomas. In humans, aldosterone-secreting adenomas are characterized by significantly higher expression abundances of CYP21 and 11β-hydroxysteroid dehydrogenase type 2 (CYP11B2) than normal adrenals and cortisol-secreting adenomas (Bassett et al. 2005). The tendency towards upregulation of CYP21 in the adenomas in the present study may therefore suggest aldosterone secretion in these tumors. This seems interesting, because significantly higher basal plasma aldosterone concentrations have previously been reported in dogs with an AT, in comparison to healthy dogs and those with PDH (Javadi et al. 2003).

The results of the QPCR analysis in this study demonstrate that the mRNA expression of CYP17 and CYP11B1, essential enzymes for cortisol production (Enberg et al. 2001), did neither differ between ATs and normal adrenals nor between adenomas and carcinomas. In humans, similar findings have been reported for cortisol-secreting adenomas when analyzed with microarray, but the results were rejected when significantly higher transcript levels were demonstrated by real-time PCR (Bassett et al. 2005). Obviously, the data from the present study indicate that another explanation for hypercortisolism than upregulation of essential steroidogenic enzymes is required. In order to fully explain the steroidogenic potential of ATs not only the mRNA expression of steroido-

genic enzymes has to be taken into consideration but also the expression and activity of some regulatory proteins, which can modulate protein expression and/or enzymatic activity. Hypothetically, the enzymes of interest could be present in a more active form in AT cells, compared to normal adrenocortical cells. It is also possible that these proteins have a longer half-life. Taken together this may result in higher cortisol production, and thus hypercortisolemia, despite the fact that there is no significant upregulation of the genes encoding the steroidogenic enzymes needed for cortisol production. Furthermore, hypercortisolism in dogs with a cortisol-secreting AT may be explained as the result of an increase in the number of cortisol producing cells. This will also lead to an absolute increase in plasma cortisol concentration in ATs compared to healthy dogs.

Although carcinomas tend to be larger than adenomas, they usually secrete less cortisol and may even be unable to carry steroidogenesis efficiently to term (Mendonca et al. 1995). This results in plasma patterns of steroid precursors, typical of enzymatic blocks and a characteristic urinary steroid profile, which can be applied in the diagnostics of ATs (Kikuchi et al. 2000, Thomas and Otten 2000). The QPCR results of the present study demonstrate that mRNA encoding steroidogenic enzymes was present in equal amounts in adenomas and carcinomas. Therefore, inefficient steroidogenesis in carcinomas cannot be ascribed to altered expression of genes encoding for the steroidogenic enzymes.

The QPCR results of the present study demonstrate that in neither cortisol-secreting adenomas nor carcinomas, upregulation of the genes encoding for steroidogenic enzymes is responsible for hypercortisolemia. In carcinomas, significant downregulation of the ACTH-R may contribute to the malignant character of the tumor.

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Chapter 5

Urinary corticoid:creatinine ratios in the differentiation between pituitary-dependent hypercortisolism and hypercortisolism due to adrenocortical tumor in the dog

S. Galac, H.S. Kooistra, E. Teske, A. Rijnberk

Vet Q 1997;19:17-20.

Department of Clinical Sciences of Companion Animals,
Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Abstract

In a study on the differentiation between pituitary-dependent hypercortisolism (PDH) and hypercortisolism due to adrenocortical tumor (AT), two questions were addressed: 1. Do basal urinary corticoid:creatinine ratios (UCCRs) have any value in this respect, and 2. What is the reference percentage suppression of the UCCRs in the high-dose dexamethasone suppression test. Data obtained from 160 dogs with hypercortisolism were analyzed. In 49 dogs the diagnosis AT was confirmed by the finding of plasma adrenocorticotropic hormone (ACTH) concentrations <40 ng/L, by visualization of the tumor by ultrasonography and/or computed tomography, and by histological examination of the adrenal tissue obtained at surgery or autopsy. Among the 111 dogs with PDH, there were 31 animals with resistance to dexamethasone suppression, i.e., suppression <50%. The basal UCCRs of dogs with PDH and AT did not differ significantly, although UCCRs $>100 \times 10^{-6}$ almost exclusively occurred in association with PDH. Among the dogs with hypercortisolism, the positive predictive value of a basal UCCR $>100 \times 10^{-6}$ for the diagnosis of PDH was 0.90 (95% CI: 0.74-0.98). Of the 49 dogs with AT, 34 had a UCCR after dexamethasone administration higher than the basal UCCR. The maximum suppression of the basal UCCR in dogs with AT was 43.7%. It is concluded that in dogs with hypercortisolism basal UCCRs only have predictive value in the differentiation between AT and PDH when the ratio exceeds 100×10^{-6}. The generally accepted criterion of 50% suppression by dexamethasone in the differentiation between PDH and AT is also applicable to the UCCR.

Introduction

Canine hypercortisolism (canine Cushing's syndrome) can be defined by the physical and biochemical changes that are the result of chronic glucocorticoid excess. In >80% of dogs the disease is caused by the excessive secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland (pituitary-dependent hypercortisolism, PDH). This

results in bilateral adrenocortical hyperplasia and hypersecretion of cortisol. In the remaining dogs the disease is due to a benign or malignant adrenocortical tumor (AT) which autonomously secretes excessive amounts of cortisol (Kemppainen and Peterson 1994). A combination of PDH and AT may also occur (Cohen and Knieser 1980, Van Sluijs et al. 1995, Greco et al. 1999). Ectopic secretion of ACTH or corticotropin releasing hormone (CRH) has not yet been documented in the dog.

The low-dose dexamethasone suppression test, as introduced by Meijer et al. (1978), can be used to distinguish dogs with hypercortisolism from those without the disease (Peterson 1986). Another option is the measurement of the urinary corticoid:creatinine ratio (UCCR) (Stolp et al. 1983, Jones et al. 1990, Kaplan et al. 1995). This test has a high diagnostic accuracy, is cost effective, and is easy to perform (Rijnberk et al. 1988). Because of its high sensitivity and the high predictive value of a negative test result, the UCCR is used with increasing frequency (Smiley and Peterson 1993, Rijnberk et al. 1988).

For the differentiation between PDH and AT, the high-dose dexamethasone suppression test (HDDST) is commonly used. This test is based on the principle that in most dogs with PDH a high-dose of dexamethasone suppresses ACTH secretion and consequently the adrenocortical secretion of cortisol, whereas in dogs with AT cortisol secretion is resistant to suppression by dexamethasone (Meijer 1980). Two procedures can be used to assess the effect of dexamethasone on cortisol production: 1. the intravenous HDDST in which the plasma cortisol concentration is measured and 2. the urinary HDDST in which the UCCR is measured. It is generally accepted that in the intravenous HDDST, dexamethasone suppression of plasma cortisol concentrations in dogs with AT is <50% (Meijer 1980). Consequently, a decrease >50% from baseline values is assumed to indicate PDH. However, suppression of <50% may not only be due to AT but may also be encountered in some cases of PDH.

One aim of this study was to determine whether the 50% criterion for dexamethasone resistance of ATs could also be adopted for UCCR as a measure of adrenocortical function. Another aim of this study was to investigate whether the absolute value of the urinary UCCR helps to differentiate between PDH and AT, under the assumption that the magnitude of the cortisol excess may be different for hyperplastic adrenal cortices and ATs.

Materials and methods

Animals and tests

Ninety-one female (40 spayed) and 69 male (19 castrated) dogs with ages ranging from 4 to 14 years (median 9.5 years) were included in this study. Hypercortisolism was

suspected in all cases on the basis of the history, physical examination, and routine laboratory examinations. The diagnosis was based on the finding of elevated UCCRs in two consecutive morning urine samples collected at home (Stolp et al. 1983, Rijnberk et al. 1988). Ratios exceeding 10×10^{-6} were regarded as being compatible with hypercortisolism. Following the collection of the second morning urine sample, dexamethasone was administered orally three times in a dose of 0.1 mg per kg body weight at 8-hour intervals.

When the UCCR in the third urine sample was suppressed by >50% of the mean value of the first two ratios (basal ratio), PDH was diagnosed. When the suppression was <50%, i.e., in the dexamethasone-resistant dogs, the diagnosis of AT was confirmed by the finding of plasma ACTH levels <40 ng/L together with demonstration of an AT by ultrasonography (Voorhout et al. 1990) and/or by computed tomography (Voorhout et al. 1988), and histological examination of the adrenal tissue obtained at surgery or necropsy. In cases of tumor growth into the caudal vena cava demonstrable by ultrasonography, the diagnosis AT was accepted without histological confirmation. The diagnosis of PDH in the dexamethasone-resistant dogs was made when plasma ACTH levels were >90 ng/L and abdominal ultrasonography or CT scanning revealed the presence of two adrenal glands of approximately equal size.

Hormone determination

Urinary corticoid concentrations were measured by radioimmunoassay for cortisol as described previously (Meijer et al. 1978, Rijnberk et al. 1988). The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) and UCCR was calculated (Stolp et al. 1983).

Plasma ACTH concentrations were measured by radioimmunoassay without extraction, according to methods described previously (Arts et al. 1985).

Calculations and statistics

Basal UCCR and ages of the dogs are presented as medians with ranges. The suppression of the basal UCCR by dexamethasone (HDDST) is expressed as the mean per cent suppression ± SEM. Basal UCCRs and the ages of dogs in the two groups (PDH, AT) were compared by use of the Mann-Whitney test. $P \leq 0.05$ was considered significant. The 95% confidence intervals (CI) for the proportions (p) were calculated as $p \pm (1.96 \times SEM)$ (Bulpitt 1987).

Results

AT was diagnosed in 49 of the 160 dogs. Four of the dogs with AT had bilateral tumors. Of the 111 dogs with PDH, 31 were dexamethasone resistant, i.e., the suppression of the basal UCCR was less than 50%. With the exception of age, the dogs with AT had no physical characteristics that were significantly different from those of the dogs with PDH. The ages of dogs with AT (median 10 years; range 7-13 years) were significantly higher than the ages of the dogs with PDH (median 9.0 years; range 4-14 years; $P<0.001$).

The median of the basal UCCR in dogs with AT was 44×10^{-6} (range $14\text{-}158 \times 10^{-6}$). Basal UCCR exceeded 100×10^{-6} in only 3 of the 49 dogs with AT (Figure 1). All 3 dogs had a unilateral AT, with a maximal diameter ≤2.5 cm. In the 4 dogs with bilateral AT, the basal UCCRs were 15, 34, 45, and 50×10^{-6}.

The basal UCCRs in the 111 dogs with PDH ranged between 11 and 1475×10^{-6} with a median of 53×10^{-6} (Figure 1). In the 31 dogs with dexamethasone-resistant PDH, the basal UCCRs ranged from 14 to 921×10^{-6}, with a median of 65×10^{-6}. The basal UCCRs did not differ significantly among dogs with AT, PDH, or dexamethasone-resistant PDH.

Among the dogs with hypercortisolism the positive and negative predictive value of basal UCCRs exceeding 100×10^{-6} for the diagnosis of PDH were 0.90 (95% CI 0.74-0.98) and 0.35 (95% CI 0.27-0.43), respectively.

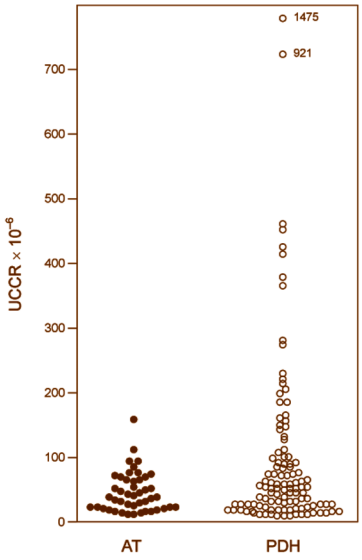

Figure 1
Basal urinary corticoid:creatinine ratios (UCCRs) in 49 dogs with hypercortisolism due to adrenocortical tumor (AT) and 111 dogs with pituitary-dependent hypercortisolism (PDH).

In the dogs with AT the mean % change of the UCCR from the basal values (set at 100%) in the HDDST was 116.2 ± 4.2% (range 56.3% - 201.5%) (Figure 2). Among the 49 dogs with AT, 34 had UCCRs after dexamethasone administration higher than the basal ratio. In dogs with PDH the mean per cent change in the UCCR in the HDDST ranged from 3.5 to 696.3% of the basal UCCR, with a mean of 44.4% ± 7.1% (Figure 2).

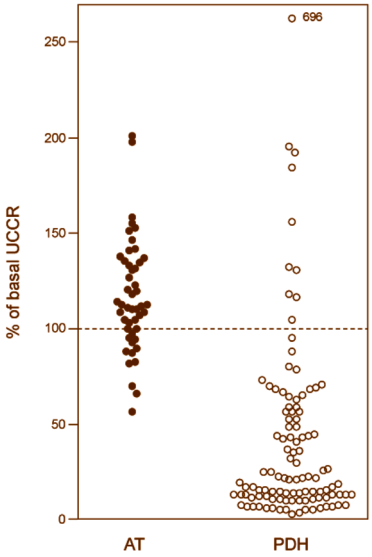

Figure 2
Changes of the urinary corticoid:creatinine ratios (UCCRs) in the high-dose dexamethasone suppression test (HDDST, 3 oral doses of 0.1 mg per kg body weight at 8-hour intervals), depicted as percentages of the basal UCCR (set at 100%).

Discussion

Once the diagnosis of hypercortisolism has been made it is necessary to distinguish between AT and PDH, because the treatment for these conditions is usually different. It may be tempting to try to decide on the basis of physical findings whether the dog has an AT or PDH. However, in the present study the only difference was that the dogs with AT were significantly older than those with PDH, but the overlap was such that this difference was of no practical value.

The basal UCCRs were somewhat different between the dogs with AT and PDH, although the difference in mean values was not significant. The highest UCCRs were found among the dogs with PDH. The calculated probability of PDH was 90% when basal UCCRs exceeded 100×10^{-6}. This indicates that the hyperplastic adrenal cortices in dogs with PDH have a greater capacity to produce cortisol than does neoplastic

adrenocortical tissue. It may be hypothesized that the dedifferentiation associated with neoplastic transformation leads to decreased hormone production per unit volume. In addition, calcification, hemorrhage, and/or necrosis within the AT may contribute to the diminished hormone production per unit of tissue mass.

In humans, it has been observed that small ATs usually have a greater hormone production than large tumors (Hamper et al. 1987). In agreement with this, the three dogs with AT and high UCCRs ($> 100 \times 10^{-6}$) had relatively small tumors, compared with previously reported sizes of ATs (Voorhout et al. 1990). The UCCRs of the four dogs with bilateral AT were relatively low.

In 1960 Liddle demonstrated that in humans with PDH, plasma cortisol levels could be suppressed by a high dose of dexamethasone. In humans, adrenocortical function is considered to be suppressible when the post-dexamethasone plasma cortisol value is less than 50% of the baseline value (Howlet et al. 1986, Miller and Crapo 1994). This 50% criterion has also been adopted for dogs (Meijer 1980, Owens and Drucker 1977). The present study reveals that the criterion of 50% suppression is also applicable when the UCCR is used. Because the maximum decrease in the basal UCCRs after a high dose of dexamethasone in dogs with AT was 43.7%, the criterion of 50% suppression may even be confined to 45%.

In this study the percentage of animals with dexamethasone resistance was relatively high. This is due to the fact that many dogs had been referred for further studies because the HDDST with measurement of UCCRs had revealed dexamethasone resistance.

Measurements of plasma ACTH concentrations are generally used in the differentiation between dexamethasone-resistant PDH and AT (Feldman 1983, Rijnberk 1996). In the case of AT, the plasma ACTH concentration will be low as a result of the negative feedback by the autonomous hypersecretion of cortisol on the unaffected pituitary corticotrophs. However, plasma ACTH concentrations in dogs with AT can be in the normal range or even elevated due to co-existing PDH (Van Sluijs et al. 1995). To exclude the likelihood of this combination, the diagnosis AT was substantiated not only by ultrasonography and/or CT-scanning of the adrenals, but also by the finding of plasma ACTH concentrations <40 ng/L.

In 69% of the dogs with AT, UCCRs after dexamethasone administration were higher than the basal ratios. In humans with AT, plasma cortisol values after administration of a high dose of dexamethasone are also often higher than the basal values (Flack et al. 1992). The reason for this remains unclear, but it may be that dexamethasone has a direct effect on the AT.

It is concluded that the basal UCCRs in dogs with hypercortisolism only have predictive value in the differentiation between AT and PDH when the ratio exceeds 100×10^{-6}. The generally accepted criterion of 50% suppression in distinguishing between AT and PDH is also applicable to the UCCR.

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Chapter 6

Effects of trilostane treatment on the pituitary-adrenocortical and renin-aldosterone axis in dogs with pituitary-dependent hypercortisolism

S. Galac, J.J.C.W.M. Buijtels, J.A. Mol, H.S. Kooistra

Vet J 2010;183:75–80.

Department of Clinical Sciences of Companion Animals,
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

Medical records of 63 dogs with pituitary-dependent hypercortisolism (PDH) before and during treatment with trilostane were retrospectively reviewed. The correct trilostane dosage in dogs with PDH was determined based upon resolution of clinical signs and results of an adrenocorticotropic hormone (ACTH) stimulation test. The mean (± SD) dosage of trilostane at the time of good control was 2.8 ± 1.0 mg/kg body weight. Trilostane treatment resulted in a significant decline of the basal plasma cortisol concentration. The median plasma ACTH concentration at the time the trilostane dosage was considered optimal (39 pmol/L, range 7–132 pmol/L, n=60) was significantly higher (P<0.001) than that before treatment (13 pmol/L, range 2–102 pmol/L). These values did not overlap with the plasma ACTH concentrations of five PDH dogs with trilostane-induced hypocortisolism (range 212–307 pmol/L). The median cortisol: ACTH ratio (CAR) in well-controlled dogs (0.23, range 0.03–2.5, n=46) was significantly lower (P<0.001) than before treatment (2.59, range 0.27–13.25). Trilostane treatment resulted in an insignificant decrease of the plasma aldosterone (ALD) concentration, but the median plasma renin activity (PRA) at the time the trilostane dosage was considered optimal (265 fmol/L/s, range 70–3280 fmol/L/s, n=18) was significantly higher (P<0.001) than before treatment (115 fmol/L/s, range 15–1330 fmol/L/s). Similarly, the median ALD:PRA ratio during trilostane treatment (0.16, range 0.003–0.92, n=17) was significantly lower (P<0.001) than before treatment (median 0.44, range 0.04–1.33). Trilostane affected both the pituitary-adrenocortical axis and the renin-ALD axis. The results also suggested that the basal plasma ACTH concentration may be used to detect trilostane overdosage.

Introduction

Spontaneous hypercortisolism can be defined as the complex of physical and biochemical changes resulting from chronic glucocorticoid excess. In dogs, there are two endogenous forms, namely, the adrenocorticotropic hormone (ACTH)-dependent

form, which accounts for 85% of the cases, and the ACTH-independent form (Rijnberk 1996, Galac et al. 2005). The goal of treatment of hypercortisolism is to eliminate the clinical signs related to glucocorticoid excess. Depending on the origin of the disease, this goal can be achieved by transsphenoidal hypophysectomy, adrenalectomy, or medical treatment. Selective (Kintzer and Peterson, 1991) or non-selective (Den Hertog et al. 1999) destruction of the adrenal cortex with o,p'-DDD (Lysodren, Brystol-Mayers Co.) has long been the medical treatment of choice for hypercortisolism in dogs. Recently, trilostane (Vetoryl, Dechra) has been reported to be a safe and effective alternative for o,p'-DDD in dogs with pituitary-dependent hypercortisolism (PDH) (Neiger et al. 2002, Ruckstuhl et al. 2002).

Trilostane is a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase (HSD3B), an essential enzyme system for the synthesis of cortisol, aldosterone and androstenedione (Potts et al. 1978). In dogs with PDH, treatment with trilostane has the potential to induce a significant decrease of the basal and ACTH-stimulated plasma cortisol concentration (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). As a result, there is a loss of negative feedback, leading to an increase of the plasma endogenous ACTH concentration (Witt and Niger 2004, Sieber-Ruckstuhl et al. 2006). Some studies have addressed changes in the plasma cortisol concentration during trilostane treatment, but information on the plasma ACTH concentration during trilostane treatment is scarce (Witt and Niger, 2004, Sieber-Ruckstuhl et al. 2006).

In line with its competitive inhibitory effect on HSD3B, trilostane lowers the plasma aldosterone (ALD) concentration in dogs with PDH, although plasma ALD concentration normally remains within the reference range (Ruckstuhl et al. 2002, Wenger et al. 2004). However, in humans it is the plasma renin activity (PRA) rather than plasma ALD concentration that is regarded as a guide to mineralocorticoid deficiency (Loriaux et al. 2001). Detailed information on the effects of trilostane treatment on the renin-ALD axis is lacking in the dog.

The objective of this retrospective study was to evaluate the effect of trilostane on the pituitary-adrenocortical axis and the renin-ALD axis in a large group of dogs with PDH. Blood samples were collected before and during trilostane treatment. Measurement of the hormone pair ACTH and cortisol and their ratio was used to assess the pituitary-adrenal axis. The renin-ALD axis was assessed by measuring plasma ALD concentration and PRA and their ratio (Javadi et al. 2003, 2006).

Material and methods

Animals, treatment and tests

Sixty-three client-owned dogs with PDH were investigated. The dogs comprised 25 neutered females and 38 males (25 intact and 13 castrated). The ages ranged from 6–16 years (median 10 years) and bodyweights from 4 to 46 kg (median 16 kg). The dogs were from 28 different breeds and there were 12 mongrel dogs.

Inclusion criteria for the dogs that entered the study were physical and biochemical changes suggestive of hypercortisolism and endocrine test results which indicated PDH. Suspicion of hypercortisolism was based on the dogs` history, physical examination and routine laboratory investigations.

The diagnosis of hypercortisolism was considered confirmed when the mean urinary corticoid:creatinine ratio (UCCR) in two consecutive morning urine samples collected at home exceeded 8.3×10^{-6} (van Vonderen et al. 1998). After collection of the second urine sample the dogs received three doses of 0.1 mg dexamethasone per kg body weight orally at 8-hour intervals. The next morning, a third urine sample was collected. PDH was diagnosed when the UCCR in the third sample was <50% of the mean of the first two samples (Galac et al. 1997). Basal UCCRs ranged from 9 to 352×10^{-6} (median 31×10^{-6}, n=63). In 58 of the dogs, oral dexamethasone suppressed the UCCR by >50% of the mean of the ratios of the previous two days. In the dogs in which the third UCCR was suppressed by <50% (n=5), plasma ACTH concentrations ranged from 10 to 39 pmol/L (median 24 pmol/L) and were interpreted as non-suppressed. Abdominal ultrasonography did not reveal any adrenocortical tumor. Together these findings indicated PDH in these five dogs.

Treatment with trilostane was started with an initial dose of 2–4 mg/kg body weight once daily. The first recheck was undertaken 3 weeks after the start of treatment and at 3.5 h (median, range 2–4 h) after administration of trilostane a blood sample was taken for routine hematological and biochemical analysis and determination of PRA and plasma concentrations of ALD, ACTH and cortisol. Immediately after blood sampling, 0.25 mg ACTH (Synacthen, Novartis Pharma BV) was administered intravenously, and a second blood sample for determination of the plasma cortisol concentration was taken 90 minutes later (Frank et al. 2004).

The dose of trilostane was adjusted based on the clinical signs and the results of the ACTH stimulation test. When post-ACTH plasma cortisol concentration was <190 nmol/L, the dose was considered appropriate if the owner also reported resolution of the clinical signs of hypercortisolism, such as polyuria, polydipsia, and polyphagia. When post-ACTH plasma cortisol concentration was >190 nmol/L and/or the clinical signs of hypercortisolism persisted, the dose of trilostane was increased and a new appointment was scheduled 3 weeks later.

For the increase in trilostane dose the following schedule was used: from 10 to 20 mg, from 20 to 30 mg, from 30 to 40 mg, from 40 to 60 mg, from 60 to 90 mg, from 90 to 120 mg, etc. When post-ACTH plasma cortisol concentration was <30 nmol/L and the clinical signs of hypercortisolism had disappeared completely, the dose of trilostane was decreased using the same schedule in reverse. Once the optimal dose had been achieved the following recheck was scheduled 3 months later. When satisfactory results were found at this time, further rechecks were scheduled at 6 monthly intervals. Dogs were reevaluated at least once after achieving the optimal dose. When post-ACTH plasma cortisol concentration was suppressed to <10 nmol/L and the owners reported bad appetite and dullness, hypocortisolism was diagnosed and trilostane treatment was stopped. These dogs were temporarily or permanently supplemented with cortisone acetate. The supplementation of cortisone acetate was discontinued when UCCR indicated sufficient endogenous production of cortisol (Meij et al. 2002).

Blood samples for ACTH, ALD and PRA measurements were collected from the jugular or cephalic vein and transferred immediately to ice-chilled EDTA-coated tubes. Samples were centrifuged at 4 °C for 10 min. Plasma was stored at –25 °C until assayed.

Hormone measurements

Plasma ACTH concentration was measured by an immunoradiometric assay (Nichols Institute) validated for the dog (Javadi et al. 2003). The inter-assay coefficient of variation was 7.8% and the sensitivity was 0.044 pmol/L.

Plasma cortisol concentrations were measured by a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation) validated for the dog (Javadi et al. 2006). The inter-assay coefficient of variation was 4 to 6.4% and the sensitivity was 1 nmol/L.

Urinary corticoid concentration was measured by a radioimmunoassay described by Rijnberk et al. (1988). The intra- and inter-assay coefficients of variation were 6% and 8%, respectively, and the sensitivity was 1 nmol/L. The urinary creatinine concentration was determined by the Jaffe kinetic method (initial rate reaction) and the UCCR was calculated (Stolp et al. 1983).

ALD was extracted from 1 mL plasma with dichloromethane. The extracts were evaporated, re-dissolved in assay buffer, and plasma ALD concentration was measured by radioimmunoassay (ICN pharmaceuticals Inc.), as described by Boer et al. (1983) and validated for the dog (Javadi et al. 2003). The intra- and inter-assay coefficients of variation were 6% and 14%, respectively. The reference range for plasma ALD concentration in our laboratory was 25–280 pmol/ L (Javadi et al. 2006).

Renin was assayed indirectly by measuring angiotensin I. The term PRA has been used throughout the text in agreement with the human literature (Boer et al. 1983, Loriaux

et al. 2001). PRA was measured by incubating 0.5 mL plasma at pH 6.0 for 1 h at 37 ^{0}C in the presence of inhibitors of angiotensinases and angiotensin I-converting enzyme. After incubation, the samples were de-proteinised with 4 mol/L of a 9:1 (v/v) mixture of acetone/ammonia and centrifuged. The supernatants were evaporated, re-dissolved in assay buffer, and angiotensin I was measured by a radioimmunoassay (antibody from Peninsula Laboratories Inc., and a tracer from NEN Life Sciences) as described by Boer et al. (1983) and validated for the dog (Javadi et al. 2003). The intra- and inter-assay coefficients of variation were 8% and 15%, respectively. The reference range for PRA in our laboratory is 65–547 fmol/L/s (Javadi et al. 2006).

Data analysis

Statistical analysis was performed using commercial statistical software (SPSS 13.1 for Windows, SPSS). The Q-Q plots and the Kolmogorov-Smirnov test were used to test the data for normal distribution. Differences in basal values of cortisol, ACTH, cortisol:ACTH ratio (CAR), ALD, PRA and ALD:PRA ratio before and during treatment with trilostane were assessed using Wilcoxon's signed ranks test. Results are expressed as median and range. $P<0.05$ was considered significant.
Results are graphically represented in box-and-whisker plots. In these figures the boxes represent the 25th to 75th percentiles. The dark horizontal line in each box is the median. The whiskers represent the main body of the data. Outliers (circles) and extreme values (star symbols) are also indicated.

Results

In 60/63 dogs with PDH, an appropriate dose of trilostane could be established, resulting in resolution of clinical signs of hypercortisolism and a post-ACTH plasma cortisol concentration <190 nmol/L. In 34/60 dogs, the initial dose of trilostane was the optimal dose. In 22 dogs the dose of trilostane had to be increased and four dogs needed a decrease of the dose at the first recheck. Twenty dogs had to return twice, four dogs three times and two dogs four times until satisfactory results were achieved. Of the 22 dogs in which the dose of trilostane had to be increased, 15 dogs had a post-ACTH plasma cortisol concentration between 100 and 190 nmol/L. None of the dogs showed improvement of the most common clinical signs of hypercortisolism if the post-ACTH plasma cortisol concentration exceeded 190 nmol/L. The mean (± SD) dose of trilostane at the re-check at which good control had been achieved was 2.8 ± 1.0 mg/kg body weight (range 0.8–5.8 mg/kg).

In the group of well-controlled dogs, the median basal plasma cortisol concentration after reaching the optimal dose was 32 nmol/L (range 2–101 nmol/L, n=60). In 46 dogs, basal plasma cortisol concentrations were available both before trilostane treatment and after reaching the optimal trilostane dose. In these 46 dogs, the median basal plasma cortisol concentration after reaching the optimal trilostane dose (37 nmol/L, range 2–101 nmol/L) was significantly lower (P<0.001) than that before treatment (median 164 nmol/L, range 24–1029 nmol/L). The median basal plasma ACTH concentration in well-controlled dogs (39 pmol/L, range 7–132 pmol/L, n=60) was significantly higher (P<0.001) than that before trilostane treatment (13 pmol/L, range 2–102 pmol/L). The median CAR in well-controlled dogs (0.23, range 0.03–2.5, n=46) was significantly lower (P<0.001) than that before trilostane treatment (2.59, range 0.27–13.25) (Fig. 1).
The median plasma ALD concentration before treatment with trilostane was 50 pmol/L (range 10–160 pmol/L, n=35). In 21 dogs plasma ALD concentrations were available both before trilostane treatment and after reaching the optimal trilostane dose. The median plasma ALD concentration in these 21 well-controlled dogs (40 pmol/L, range 10–110 pmol/L) did not differ significantly (P=0.078) from that before treatment (50 pmol/L, range 20–160 pmol/L). The median PRA before treatment with trilostane was 100 fmol/L/s (range 10–1330 fmol/L/s, n=31). In 18 dogs, the PRA concentration was available both before trilostane treatment and after reaching the optimal dose. The median PRA concentration in well-controlled dogs (265 fmol/L/s, range 70–3280 fmol/L/s, n=18) was significantly higher (P<0.001) than that before trilostane treatment (115 fmol/L/s, range 15–1330 fmol/L/s). The median ALD:PRA ratio before treatment with trilostane was 0.44 (range 0.04–2, n=31). The median ALD:PRA ratio after reaching the optimal trilostane dose (0.16, range 0.003–0.92, n=17) was significantly lower (P<0.001) than that before treatment (median 0.44, range 0.04–1.33) (Fig. 2).
Five dogs developed hypocortisolism during trilostane treatment. This group consisted of two dogs which were well regulated initially, but developed hypocortisolism 10 and 12 months later in the course of the treatment, and of three dogs which did not fulfil the inclusion criteria for the group with a optimal dose of trilostane. Two of the five dogs with trilostane-induced hypocortisolism had to receive supplementation of cortisone acetate permanently and three dogs only temporarily. The basal plasma ACTH concentrations in this group at the moment of presentation with hypocortisolism ranged from 212 to 307 pmol/L (median 271 pmol/L; n=5) and the CAR from 0.004 to 0.085 (median 0.009; n=5). Plasma ALD concentration and PRA were available in 2 dogs with trilostane-induced hypocortisolism and were 20 and 120 pmol/L, and 100 and 1400 fmol/L/s, respectively. The ALD:PRA ratios in these two dogs were 0.2 and 0.09, respectively.

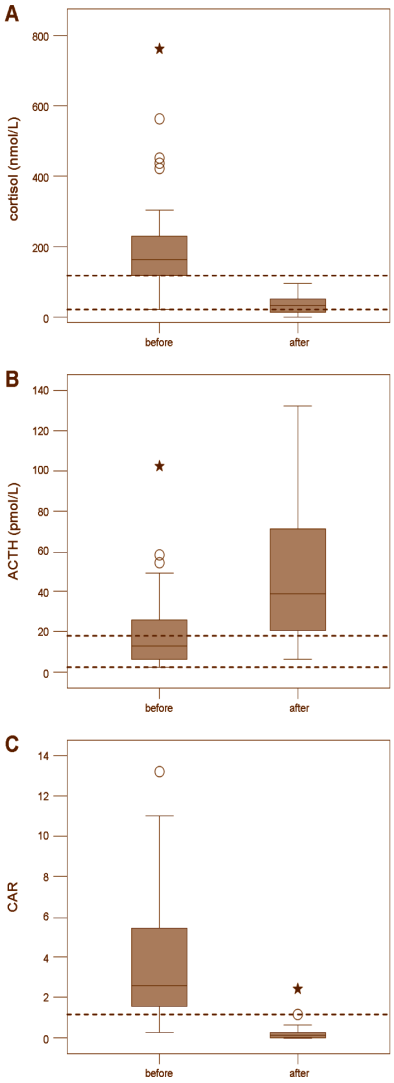

Figure 1.
Box-and-whisker plots of the plasma concentrations of cortisol (n=46) and adrenocorticotropin (ACTH, n=60), and the cortisol:ACTH ratio (CAR, n=46) in dogs with pituitary-dependent hypercortisolism (PDH) before treatment with trilostane (before) and after the optimal dose of trilostane was reached (after).
Reference ranges for cortisol and ACTH concentration and the lower level of the reference range of CAR have been added (------).
O indicates outlier;
* indicates extreme outlier.

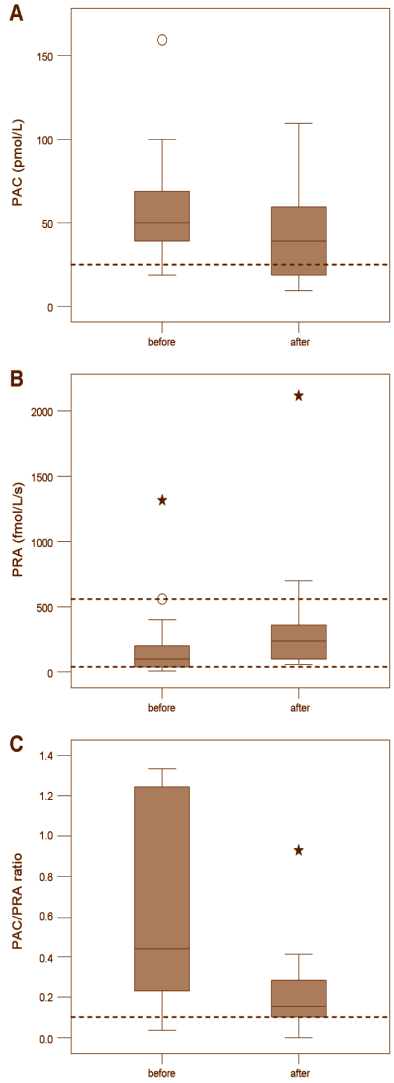

Figure 2
Box-and-whisker plots of plasma aldosterone (ALD) concentration (n=21), plasma renin activity (PRA, n=18) and ALD:PRA ratio (n=17) in dogs with pituitary-dependent hypercortisolism (PDH) before treatment with trilostane (before) and at the moment the optimal dose of trilostane was reached (after).
Reference ranges for PRA and the lower level of the reference rangeof plasma ALD concentration and AL:PRA ratio have been added (------).
O indicates outlier;
* indicates extreme outlier.

Discussion

The aim of the trilostane treatment in dogs with PDH is to suppress the plasma cortisol concentration to such a degree that the signs of hypercortisolism will disappear. In the present study, a large variation was found in the basal and post-ACTH plasma cortisol concentrations at the time a optimal dosage of trilostane was achieved. The upper limit of a post-ACTH plasma cortisol concentration of <190 nmol/L was chosen arbitrarily. Our results have shown that in some dogs the most common clinical signs of hypercortisolism disappeared only when the post-ACTH plasma cortisol concentration was <100 nmol/L. In humans, inter-individual differences in sensitivity to cortisol have been well documented (Vermeer et al. 2004, Russcher et al. 2006). There is no reason to assume that such differences in cortisol sensitivity do not exist in dogs and this may also explain why quite low levels of post-ACTH cortisol concentration are sometimes needed to control the clinical signs of hypercortisolism.

The optimal trilostane dose in the PDH dogs in the present study was lower than reported so far, i.e., 1–6 mg/kg body weight versus 4.4–16 mg/kg body weight (Ruckstuhl et al. 2002), 11 mg/kg body weight (Neiger et al. 2002) and 16–19 mg/kg body weight (Braddock et al. 2003). The low trilostane dosages needed may also explain the low percentage of side effects in the present study.

A main factor in determining the optimal trilostane dosage is the post-ACTH plasma cortisol concentration. In the present study the ACTH stimulation test was performed around 3.5 h after administration of trilostane, whereas in the study of Ruckstuhl et al. (2002) the ACTH stimulation test was run 2–6 h after trilostane. Braddock et al. (2003) recommended an ACTH stimulation test within 12 h after trilostane administration, while Neiger et al. (2002) performed the ACTH stimulation within few hours of giving the drug although the interval was not consistent due to non-specific instructions given to the owners. As demonstrated by Bell et al. (2006), the timing of the ACTH stimulation test relative to the trilostane administration must be consistent to obtain results that can be interpreted meaningfully.

As a response to the lowering of the plasma cortisol concentration, pituitary ACTH secretion increased, which induced significantly higher basal plasma ACTH concentration. Normally, trilostane suppresses the plasma cortisol concentration to low-normal levels for only a few hours, and consequently only a modest elevation of the plasma ACTH concentration would be expected. However, hyposecretion of cortisol, such as in primary hypoadrenocorticism, will induce a severe increase of the plasma ACTH concentration (Peterson et al. 1996, Javadi et al. 2003). Therefore, determination of the plasma ACTH concentration seems particularly relevant to detect trilostane overdosage. Indeed, in the five dogs with trilostane-induced hypocortisolism, plasma ACTH concentration was much higher than the highest plasma ACTH concentration in the well-controlled dogs, indicating that inclusion of the basal plasma ACTH concentration

may be worthwhile in the evaluation of the trilostane dosage needed to control PDH adequately.

In humans, intravenous administration of huge doses of exogenous ACTH is associated with the risk of adrenocortical hemorrhagic necrosis and hypocortisolism (Kornbluth et al. 1990, Levin and Morton 1993). It may be hypothesized that the elevated endogenous ACTH concentrations in dogs on excessive trilostane therapy can provoke a similar effect on the adrenal cortex, but the design of the present study does not permit conclusions with regard to this hypothesis. Chapman et al. (2004) published a case report on a dog with PDH that developed adrenocortical necrosis during treatment with trilostane, but unfortunately plasma ACTH concentrations were not reported.

Recently, necrosis and apoptosis of the adrenal glands were described in dogs treated with trilostane and the severity may have been related to the doses of trilostane used and the duration of treatment (Reusch et al. 2007). Whether the occurrence of hypocortisolism requiring treatment with cortisone acetate in some dogs in the present study was the result of (partial) adrenocortical necrosis due to ACTH hypersecretion (induced by a too high dosage of trilostane) remains to be elucidated. Furthermore, the interpretation of the plasma ACTH concentration must take into account when the trilostane is administered. Witt and Neiger (2004) demonstrated that the period between the administration of trilostane and when the circulating endogenous ACTH concentration was measured correlated significantly, with a higher endogenous ACTH level found in dogs which had received trilostane more recently.

The plasma ALD concentrations before treatment with trilostane in our dogs with PDH were similar to values reported previously for dogs with PDH and lower than values reported for healthy dogs (Javadi et al. 2003). In rats chronically treated with ACTH, transformation of zona glomerulosa cells into zona fasciculata-like cells has been reported (Pudney et al. 1984). Based on these findings it was hypothesized that the chronic ACTH excess in PDH dogs may lead to transformation of zona glomerulosa cells into zona fasciculata-like cells and therefore reduce the ALD secretory capacity (Javadi et al. 2003). Another explanation for the lower plasma ALD concentrations in dogs with PDH compared with healthy dogs, may be an increase in circulating volume as a result of the mineralocorticoid effect of glucocorticoids. Cortisol has the same affinity for the mineralocorticoid receptor (MR) as ALD, but normally 11β-hydroxysteroid dehydrogenase type 2 enzyme activity (CYP11B2) prohibits stimulation of the ALD receptor by cortisol, by converting cortisol into inactive cortisone. In case of chronic cortisol excess, such as occurs in PDH, the capacity of CYP11B2 becomes insufficient to convert all cortisol into cortisone. Consequently cortisol will stimulate the MR resulting in increased renal reabsorption of sodium and water and thus in an increase in circulating volume. Additionally, the increase in circulating volume will suppress renin secretion. In line with this supposition, PRAs before treatment with trilostane in our dogs with PDH were lower than PRAs reported in healthy

dogs (Javadi et al. 2003, 2006). The lower PRA may then explain the lower plasma ALD concentrations in our dogs with PDH.

The results of this study demonstrate that trilostane not only affects the pituitary-adrenocortical axis but also influences the renin-ALD axis. Although trilostane administration was not associated with a significant decreased plasma ALD concentration, PRA during trilostane treatment was significantly higher than before treatment. An increased PRA during trilostane therapy has also been described in humans (Komanicky et al. 1978). The higher PRA during treatment can be explained by a trilostane-induced decrease in ALD secretion. Subsequently, the decline in plasma ALD concentration resulted in less circulating volume due to a decrease in ALD-mediated renal reabsorption of sodium. The decrease in circulating volume stimulates renin secretion and the activation of the renin-angiotensin system results in a stimulus for ALD synthesis and secretion. This sequence of events indicates that PRA is a better guide to mineralocorticoid deficiency than plasma ALD concentration, as has been reported in humans (Loriaux et al. 2001).

To detect abnormalities in the renin-ALD system, the ALD:PRA ratio has been identified as a more sensitive screening test than plasma ALD concentration in humans (Lim et al. 1999), cats (Javadi et al. 2005) and dogs (Javadi et al. 2006). Therefore, interpretations of the effect of trilostane on the renin-ALD axis should not rely on basal circulating ALD concentrations, but must consider the ALD:PRA ratio. The significantly lower ALD:PRA ratio found in the present study during trilostane treatment reflected the effect of trilostane on the renin-ALD axis.

A limitation of the current study was that not all endocrine variables studied were available for all time points. This is a reflection of the retrospective nature of the study. For example, basal plasma cortisol concentrations before trilostane treatment were only available in 46 dogs, whereas basal plasma cortisol concentrations were available from all dogs after reaching the optimal trilostane dose. Only the results of 46 dogs were therefore used for paired statistical analysis.

In conclusion, trilostane treatment of dogs with PDH not only affected the pituitary-adrenocortical axis, but also the renin-ALD axis. To observe any disturbances of the renin-ALD axis, determination of the ALD:PRA ratio would be indicated. Furthermore, the results suggested that hypocortisolism induced by an excessive trilostane dosage may be detected by a clearly elevated plasma ACTH concentration.

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Chapter 7

Urinary corticoid:creatinine ratios in dogs with pituitary-dependent hypercortisolism during trilostane treatment

S. Galac, J.J.C.W.M. Buijtels, H.S. Kooistra

J Vet Intern Med 2009;23:1214-1219.

Department of Clinical Sciences of Companion Animals,
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

The adrenocorticotropic hormone (ACTH) stimulation test is used to evaluate trilostane treatment in dogs with hypercortisolism. The major disadvantage of this approach is that it only provides information about suppression of cortisol production during a short period of time. The urinary corticoid:creatinine ratio (UCCR) is an integrated measure of glucocorticoid production and might be a good alternative to the ACTH stimulation test to determine the optimal trilostane dose. In this prospective study of 18 dogs with pituitary-dependent hypercortisolism (PDH), the dose of trilostane was judged to be optimal on the basis of resolution of clinical signs of hypercortisolism and results of an ACTH stimulation test. The owners collected urine for determination of UCCR at 2-week intervals for at least 8 weeks after achieving the optimal trilostane dose. The UCCRs were significantly higher before treatment (11.5-202.0 x 10^{-6}; median, 42.0 x 10^{-6}) than at rechecks 2 months after optimal dosing, but they did not decrease below the upper limit of the reference range in the majority of dogs. The UCCRs of 11 dogs that initially were dosed insufficiently (range, 7.5-79.0 x 10^{-6}; median, 31.0 x 10^{-6}) did not differ significantly from UCCRs when the dosage was optimal (8.2-72.0 x 10^{-6}; median, 33.0 x 10^{-6}). Post-ACTH plasma cortisol concentrations did not correlate significantly with UCCRs at rechecks during trilostane therapy. Long-term follow-up indicated that a decrease of the UCCR below the upper limit of the reference was associated with development of hypocortisolism. The results of this study indicate that the UCCR cannot be used as an alternative to the ACTH stimulation test to determine the optimal dose of trilostane, but might be helpful in detecting dogs at risk for developing hypocortisolism during trilostane therapy.

Introduction

Trilostane has become the medical treatment of choice for pituitary-dependent hypercortisolism (PDH) in dogs. Trilostane is a competitive inhibitor of the 3β-hydroxysteroid

dehydrogenase (HSD3B), an essential enzyme system for the synthesis of cortisol, aldosterone, and androstenedione (Potts et al. 1978). In dogs with PDH, trilostane has the potential to induce a substantial decrease in basal and adrenocorticotropic hormone (ACTH)-stimulated plasma cortisol concentrations (Neiger et al. 2002, Ruckstuhl et al. 2002, Bell et al. 2006). Consequently, a loss of negative feedback occurs, leading to an increase in endogenous plasma ACTH concentration (Witt and Niger 2004, Siber-Ruckstuhl et al. 2006). Trilostane also affects aldosterone (ALD) synthesis (Sieber-Ruckstuhl et al. 2006, Galac et al. 2009).

The effectiveness of trilostane therapy is judged by resolution of clinical signs associated with glucocorticoid excess and results of an ACTH stimulation test (Neiger et al. 2002, Ruckstuhl et al. 2002). The aim of performing an ACTH stimulation test in a dog on trilostane therapy is to evaluate whether sufficient adrenocortical reserve is present at the time of maximal effect of trilostane, which is 2-3 hours after administration (Neiger and Campbel 2001). The disadvantages of the test are that: (1) it only provides information about suppression of cortisol production during a short interval, (2) it is invasive, (3) the post-ACTH cortisol concentration thought to indicate optimal dosage of trilostane is still arbitrary, and (4) there is concern about the availability and cost of the injectable ACTH (Peterson 2004) required for the test.

The urinary corticoid:creatinine ratio (UCCR) provides an integrated measure of corticoid production over a given interval, thereby overcoming the problem of fluctuations in plasma concentrations (Rijnberk and Wees 1988). It is useful not only in the initial diagnosis of hypercortisolism but also in detection of persisting cortisol secretion after hypophysectomy or nonselective destruction of the adrenal cortex by mitotane therapy (Meij et al. 2002, Den Hertog et al. 1999). Because the UCCR is an integrated measure of glucocorticoid production, it might be a more appropriate indicator of the therapeutic efficacy of trilostane than an ACTH stimulation test. In addition, it requires less time and is not invasive.

The aim of the present study was to determine whether the UCCR is a suitable alternative to the ACTH stimulation test for evaluating the dosage of trilostane and the efficacy of treatment of dogs with PDH.

Materials and Methods

Animals, Treatment, and Tests

Eighteen client-owned dogs with PDH were investigated. They consisted of 4 spayed females and 14 males (8 intact and 6 castrated). Their ages ranged from 8 to 17 years

(median, 12 years) and their body weights from 5 to 46 kg (median, 12 kg). Four dogs were mongrels and the others were from 11 different breeds.

Suspicion of hypercortisolism was based on the history, physical examination, and routine laboratory findings. An ACTH stimulation test demonstrated hyperresponsiveness (i.e., post-ACTH cortisol >552 nmol/L) (Frank et al. 2004) of the adrenal cortex in all but 1 dog. In the latter dog, the post-ACTH plasma cortisol concentration was 352 nmol/L. This dog was included in the study, because based on the history, clinical findings, laboratory investigation, increased UCCRs that could not be suppressed by high doses of dexamethasone, and diagnostic imaging there were no doubts about the correctness of the diagnosis of hypercortisolism.

The diagnosis of hypercortisolism was considered to be confirmed by finding an increased UCCR (> 8.3 x 10^{-6}) in 2 consecutive morning urine samples collected at home (Van Vonderen et al. 1998). After collection of the second urine sample, the dogs received 3 doses of 0.1 mg/kg body weight dexamethasone orally at 8-hour intervals and the third urine sample was collected on the following morning. PDH was diagnosed when the UCCR in the third sample was <50% of the mean of the first 2 samples (Rijnberk 1996, Galac et al. 1997). In 4 dogs in which the third UCCR was >50%, basal plasma ACTH concentrations were 35-144 ng/L (median, 49 ng/L) and were interpreted as nonsuppressed. Abdominal ultrasonography revealed no evidence of an adrenocortical tumor in any of these 4 dogs and in 3 of them a pituitary mass was detected by computed tomography. Treatment with trilostane (Vetoryl, Dechra, UK) was started at an initial dosage of 2-4 mg/kg body weight once daily at 8 a.m. The first recheck was at 3 weeks after the start of treatment. The history and physical examination were recor-ded and, at 3.5 hours (median, range 2-4 hours) after administration of trilostane, a blood sample was collected for routine hematological and biochemical analyses and determination of plasma concentrations of ACTH and cortisol (Galac et al. 2009). Immediately after blood collection, 0.25 mg ACTH (Synacthen, Novartis Pharma BV, The Netherlands) was administered IV and a second blood sample for measurement of plasma cortisol was collected 90 min later (Frank et al. 2004, Stolp et al. 1983). The dose of trilostane was adjusted on the basis of clinical signs and results of the ACTH stimulation test. When post-ACTH plasma cortisol concentration was <165 nmol/L, the dose was considered to be optimal if the owner also reported resolution of polyuria, polydipsia, and polyphagia (the most common signs of hypercortisolism). When post-ACTH plasma cortisol concentration was >165 nmol/L, the clinical signs of hypercortisolism persisted or both, the dose was increased and the examination was repeated 3 weeks later. The dose was increased in steps as follows (in mg): 10 to 20, 20 to 30, 30 to 40, 40 to 60, 60 to 70, 70 to 80, 80 to 90 and 90 to 120. When the dose appeared to be optimal, the examination was repeated 12 ± 1 weeks later and if the results were still satisfactory, examinations were repeated at 12- to 24-week intervals. The dog was examined in the clinic at least once after the dose was considered to be optimal. When

there were no clinical signs suggestive of hypocortisolism, but the post-ACTH plasma cortisol concentration was suppressed to <28 nmol/L, the dose was decreased. If the dog became lethargic and its appetite decreased and the post-ACTH plasma cortisol concentration was suppressed to <28 nmol/L, treatment with trilostane was stopped. Treatment was resumed, at a lower dose, when clinical signs of hypercortisolism re-occurred.

Blood samples were collected from the jugular or cephalic vein. Blood samples for measurement of ACTH were placed immediately in ice-chilled EDTA-coated tubes and centrifuged at 4 °C for 10 minutes. Plasma was stored at –25 °C until assayed.

The owners collected urine samples for determination of the UCCR at 2-week intervals from 2 weeks on after start of treatment and continued collecting for at least 8 weeks after the dose of trilostane was considered to be optimal. These samples were collected shortly before (basal sample) and 6 hours after trilostane administration (6h post-trilostane sample), all collected at home. Long-term follow-up was possible in 10 dogs, in all of which the UCCR was determined at least once every 6 months according to the above scheme. The longest follow-up was 2 years after the dose was judged to be optimal.

Hormone Measurements

Plasma ACTH concentration was measured by an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands) validated for the dog (Javadi et al. 2006). The intra- and inter-assay coefficients of variation were 3.2% and 7.8%, respectively, and the sensitivity was 0.2 ng/L.

Plasma cortisol concentration was measured by a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation, Los Angeles, CA) validated for the dog (Javadi et al. 2006). The inter-assay coefficient of variation was 4.5 to 6.3%, the intra-assay coefficient of variation was 4%, and the sensitivity was 1 nmol/L.

Urinary corticoid concentration was measured by a radioimmunoassay described by Rijnberk et al. (1988) The intra- and inter-assay coefficients of variation were 6% and 8%, respectively, and the sensitivity was 1 nmol/L. The urinary creatinine concentration was determined by the Jaffé kinetic method (initial rate reaction). From these, the UCCR was calculated (Stolp et al. 1983).

Statistical Analysis

Statistical analysis was performed using commercial statistical software (SPSS 16.1 for Windows, SPSS, Chicago, IL). The Q-Q plots and the Kolmogorov-Smirnov test were

used to test the data for normal distribution. Differences in basal values of plasma cortisol and ACTH concentration before and during treatment with trilostane were assessed using Wilcoxon's signed ranks test. Spearman`s rank correlations were performed between post-ACTH cortisol concentration when the dose was considered optimal and UCCRs (basal and 6h post-trilostane) at 2, 4, 6, and 8 weeks thereafter, described as the 1st, 2nd, 3rd and 4th UCCR recheck.

Differences between pretreatment UCCRs and the basal and 6h-post-trilostane UCCRs of urine samples collected after the dose of trilostane was optimal were compared with Wilcoxon's signed ranks test. Also, the basal and 6h post-trilostane UCCRs at 2, 4, 6, and 8 weeks after achieving the optimal dose (1st, 2nd, 3rd and 4th UCCR recheck) were compared with the same test. Finally, in 11 dogs in which adjustments of the trilostane dose were needed, the UCCRs while insufficiently dosed were compared with the UCCRs at 2 weeks after the optimal dose had been achieved with Wilcoxon`s signed ranks test. Results are expressed as median and range. $P<0.05$ was considered significant.

Results

The median dose of trilostane at the time of good control was 3.4 mg/kg body weight, once daily (range, 2-8 mg/kg). In 7 of the 18 dogs this was the initial dose, whereas in 5 dogs the dose had to be increased twice, in 2 dogs 3 times, in 3 dogs 4 times and in 1 dog 5 times.

The median basal plasma cortisol concentration at the optimal dose was 45 nmol/L (range, 21-114 nmol/L), which was significantly lower ($P<0.001$) than that before treatment (median, 165 nmol/L; range, 145-655 nmol/L). The median post-ACTH plasma cortisol concentration at the optimal dose was 106 nmol/L (range, 38-165 nmol/L), which was significantly lower ($P<0.001$) than that before treatment (median, 881 nmol/L; range, 352-1380 nmol/L) (Figure 1A).

The median plasma ACTH concentration in well-controlled dogs (88 ng/L; range, 25-401 ng/L) was significantly higher ($P<0.05$) than that before treatment (median, 46 ng/L; range, 10-144 ng/L) (Figure 1B). The median plasma ACTH concentration in 11 dogs that were insufficiently dosed (103 ng/L; range, 21-313 ng/L) did not differ significantly from that before treatment (median, 51 ng/L; range, 12-144 ng/L).

Basal UCCRs before treatment were 11.5-202.0 x 10^{-6} (median, 42.0 x 10^{-6}), which were significantly higher than basal UCCRs at 1st, 2nd, 3rd ($P = 0.001$) and 4th recheck ($P<0.05$) after the optimal dose of trilostane had been reached (Figure 2). The 6h post-trilostane UCCRs were significantly lower than the basal UCCRs on the same day ($P<0.05$) at each recheck.

The UCCRs of dogs (n=11) that initially were inadequately dosed ranged from 7.5 to

79.0 x 10^{-6} (median, 31.0 x 10^{-6}) and did not differ significantly from UCCRs when the dosage was considered optimal (8.2-72.0 x 10^{-6}; median, 33.0 x 10^{-6}). The 6h post-trilostane UCCRs of the 11 dogs that initially were dosed inadequately ranged from 5.1-97.0 x 10^{-6} (median, 24.0 x 10^{-6}) and did not differ significantly from 6h post-trilostane UCCRs of dogs receiving the optimal dose (4.4-46.0 x 10^{-6}; median, 13.0 x 10^{-6}). The post-ACTH cortisol concentrations did not correlate significantly with basal and 6h post-trilostane UCCRs at 1st, 2nd, 3rd and 4th recheck after the optimal dose had been achieved.

Basal UCCRs in 13 dogs and 6h post-trilostane UCCRs in 12 dogs did not decrease below the upper limit of the reference range within 8 weeks after the dose of trilostane was judged to be optimal. Among the 6 dogs in which the UCCR did decrease below the upper limit of the reference range within 8 weeks after the dose was judged to be optimal, in 2 the basal UCCRs were below the upper limit of the reference range 3 of 4 times and 6h post-trilostane UCCRs were below the upper limit of the reference range all 4 times. In the other 4 dogs, the basal UCCRs were below the upper limit of the reference range only once in 3 dogs, whereas the 6h post-trilostane UCCRs were below the upper limit of the reference range once (1 dog), twice (2 dogs), or 3 times (1 dog).

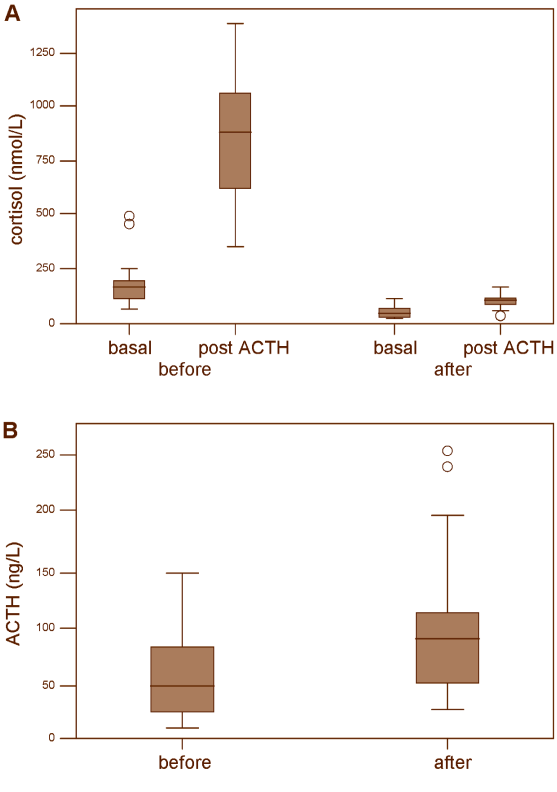

Figure 1

A: Box-and-whisker plots of basal and post-adrenocorticotropin (ACTH) plasma cortisol concentrations before trilostane therapy (basal before and post ACTH before) and when the dose of trilostane was judged to be optimal (basal after and post-ACTH after) in 18 dogs with pituitary-dependent hypercortisolism (PDH).
O indicates outlier.

B: Box-and-whisker plots of plasma ACTH concentrations before treatment (before) and when the dose of trilostane was judged to be optimal (after) in 18 dogs with PDH.
O indicates outlier.

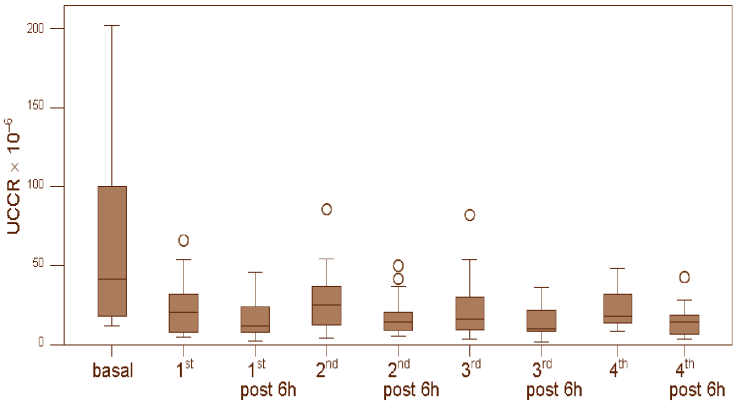

Figure 2
Box-and-whisker plots of urinary corticoid:creatinine ratios (UCCRs) before and during trilostane therapy in 18 dogs with pituitary-dependent hypercortisolism (PDH). Urine for determination of the UCCR was collected at the time of diagnosis (basal) and at 2-week interval rechecks after the dose of trilostane was judged to be optimal: shortly before trilostane administration (1st, 2nd, 3rd and 4th) and 6h after trilostane administration (1st post 6h, 2nd post 6h, 3rd post 6h and 4th post 6h). O indicates outlier.

Long term follow up

Dogs with UCCRs below the upper limit of the reference range within 8 weeks after reaching the optimal trilostane dose

In 2 dogs among the 6 dogs in which the UCCRs were below the upper limit of the reference range most of the times, the ACTH stimulation test revealed hypocortisolism at 15 and 22 weeks after the dose was judged to be optimal. Although neither of these 2 dogs had clinical signs of hypocortisolism, the dose was reduced.

In the other 4 dogs from this group, at 4, 6, and 21 months on the dose judged to be optimal 3 dogs developed anorexia, abdominal pain and were lethargic, which was interpreted as mild clinical signs of hypocortisolism. Unfortunately, in none of these 3 dogs an ACTH stimulation test was performed at that time. Trilostane treatment was stopped temporarily and resumed at a lower dose when the dogs again developed clinical signs of hypercortisolism. There were no long-term follow-up UCCRs in these 3 dogs.

In the fourth dog, the ACTH stimulation test indicated hypocortisolism 40 weeks after the dose was judged to be optimal. In addition, both the basal and 6h post-trilostane UCCRs were below the upper limit of the reference range (5.0 and 4.5 x 10^{-6}, respectively). Although there were no clinical signs of hypocortisolism, the dose of trilostane was decreased.

Dogs with UCCRs above the upper limit of the reference range within 8 weeks after reaching the optimal trilostane dose

Among the 12 dogs in which the UCCRs were above the upper limit of the reference range within 8 weeks after the dose was considered optimal, the ACTH stimulation test indicated hypocortisolism in 4 of them at 40 weeks, and 1, 1.2, and 2 years. The basal UCCRs (5.5, 4.7, 3.0, and 4.3 x 10^{-6}) and the 6h post-trilostane UCCRs (4.4, 2.7, 2.0, and 1.5 x 10^{-6}) were below the upper limit of the reference interval a few weeks to months before hypocortisolism was indicated by the ACTH stimulation test. One of these dogs developed clinical signs of complete Addison's disease with vomiting, diarrhea, dehydration, hyponatremia and hyperkalemia and needed supportive therapy with IV fluids, mineralocorticoids and glucocorticoids. When stable, temporary oral supplementation with cortisone acetate (Cortisoni acetas, Genfarma BV, Zaandam, The Netherlands), fludrocortisone acetate (Fludrocortison, Aesculap BV, Boxtel, The Netherlands) and salt (Natrii Chloridum 1000 mg, Genfarma BV, Zaandam, The Netherlands) was continued. When clinical signs of hypercortisolism reoccurred, supplementation was stopped and trilostane therapy was resumed at a lower dose. The basal and 6h post-trilostane UCCRs in this dog at the time of good control on a decreased dose of trilostane were 26 and 4.5 x 10^{-6}, respectively.

In 4 of the 12 dogs, it was not necessary to change the dose of trilostane during long-term follow-up. In 2 dogs at 1 year and in 1 dog at 2 years after starting treatment, basal UCCRs were above the upper limit of the reference range (23.0, 15.0, and 44.0 x 10^{-6}) whereas 6h post-trilostane UCCRs were close to the upper limit of the reference range (5.9, 9.9, and 8.8 x 10^{-6}).

In 1 of the remaining 4 dogs, the dose of trilostane had to be increased 6 months after it was first judged to be optimal. Before the dose was changed, both the basal and 6h post-trilostane UCCRs, were above the upper limit of the reference range (21.0 and 18.0 x 10^{-6}, respectively). After the dose was increased, the values were 23.0 and 5.9 x 10^{-6}. In another dog twice daily administration of trilostane was required 24 weeks after initial stabilization. The basal and 6h post-trilostane UCCRs before altering the dose were 17.0 and 12.0 x 10^{-6}, respectively, but long-term follow-up UCCRs were not available. Two of the remaining dogs died due to causes unrelated to trilostane treatment. One dog developed severe neurological problems due to pituitary macroadenoma and the second dog had severe congestive heart failure and was euthanized.

Discussion

The results of this study indicate that the UCCR cannot be used as an alternative to the ACTH stimulation test to determine the optimal dose of trilostane. In most dogs with PDH included in this study, UCCRs remained above the upper level of the reference range for at least 2 months after receiving the optimal dose of trilostane. The UCCRs also did not correlate with the post-ACTH plasma cortisol concentrations and thus the UCCR cannot be considered a reliable indicator of treatment control. Similarly, UCCRs also cannot be used as an alternative to the ACTH stimulation test in dogs in which mitotane is used to partially destroy the adrenal cortex (Randolph et al. 1998, Angles et al. 1997). In contrast, the UCCR is a reliable indicator of persisting cortisol secretion after hypophysectomy or nonselective destruction of the adrenal cortex by mitotane therapy (Meij et al. 2002, Den Hertog et al. 1999).

In the measurement of urinary corticoids in unextracted urine by radioimmunoassay, not only free cortisol but also substances with a similar polarity are detected (Stolp et al. 1983). Several of these are precursors and metabolites of cortisol. This lack of specificity does not constitute a problem in the use of the UCCR for the diagnosis of hypercortisolism or for monitoring treatment after hypophysectomy or nonselective destruction of the adrenal cortex by mitotane (Meij et al. 2002, Den Hertog et al. 1999). It may, however, be important in evaluating the effect of trilostane therapy. When trilostane inhibits steroidogenesis by blocking HSD3B, the steroid precursors prior to the site of the blockade accumulate (Sieber-Ruckstuhl et al. 2006, Komanicky et al. 1978). Studies in dogs with PDH treated with trilostane have demonstrated significant increases in plasma 17α-OH-pregnenolone and dehydroepiandrostenedione, consistent with the inhibition of HSD3B. Trilostane also may inhibit other enzymes of the steroid hormone cascade, such as 11β-hydroxysteroid dehydrogenase type 1 (CYP11B1) (Sieber-Ruckstuhl et al. 2006). 17α-OH-pregnenolone and dehydro-epiandrostenedione also may have been measured by the urinary corticoid assay in the present study, which could explain why the UCCR did not decrease in association with the low plasma cortisol concentrations. Quantitative determination of urinary steroids conceivably could provide the answer, but the urinary steroid profile has not yet been characterized in dogs. It is a useful tool in humans in the diagnosis of adrenocortical carcinoma and some congenital deficiencies of enzymes involved in steroidogenesis (Gröndal et al. 1990, Kikuchi et al. 2000).

Some authors have speculated that steroid precursors accumulating as a result of enzyme inhibition by trilostane might exert some glucocorticoid effect (Wenger et al. 2003). This could explain why quite low post-ACTH cortisol concentrations appear to be necessary in some cases to avoid clinical signs of hypercortisolism. However, if the increased UCCRs in trilostane-treated dogs that are well-controlled reflect accumulated steroid precursors, it is unlikely that they have glucocorticoid effects. A better

explanation for the large variation in the post-ACTH plasma cortisol concentration in well-controlled dogs might be inter-individual differences in cortisol sensitivity. Although this effect has only been documented in humans (Russcher et al. 2006, Vermeer et al. 2004), differences in cortisol sensitivity also may exist in dogs.

Decrease in the UCCR below the upper limit of the reference range by trilostane sometimes led to signs of hypocortisolism. In some cases, the ACTH stimulation test also demonstrated too little adrenocortical reserve, and the signs were reversed by stopping trilostane treatment. That the UCCRs were not as low as may be expected in dogs with hypocortisolism, might be ascribed to detection of corticoids other than cortisol in the corticoid assay, but the simultaneous occurrence of clinical signs of hypocortisolism suggests that these corticoids have no clinically relevant intrinsic glucocorticoid effect.

Trilostane-induced hypocortisolism has been documented previously (Chapman et al. 2004, Sieber-Ruckstuhl et al. 2006, Ramsey et al. 2008, Galac et al. 2009). Because the action of the drug is reversible, temporarily stopping trilostane is sufficient in most cases. Because adrenocortical insufficiency can be fatal if not treated promptly, some warning of this risk during trilostane therapy could be of great value. Lowering of the UCCR below the upper limit of the reference range might serve this purpose. Recently, necrosis and apoptosis of the adrenal glands have been reported to occur in dogs treated with trilostane (Reusch et al. 2007). The severity appeared to be related to the dose and duration of trilostane administration as well as to individual sensitivity to the drug. In the present study, clinical signs of hypocortisolism occurred in few dogs several months after the dose of trilostane appeared to be optimal. In all of these, the UCCR decreased below the upper limit of the reference range before signs of hypocortisolism developed.

UCCR was measured both before and 6h after trilostane administration in order to determine which result would be more useful to evaluate treatment. In the majority of the dogs in which the dose was judged to be optimal, both of these UCCR values exceeded the upper limit of the reference range within 2 months, whereas in those dogs that developed hypocortisolism both results were below the upper limit of the reference range. Follow-up examinations indicated that the combination of a slightly increased basal UCCR and a 6h post-trilostane UCCR close to the upper limit of the reference range was associated with good control. This pattern was observed only after several months on the optimal dose of trilostane. Whether differences in clearance of various metabolites play a role (Stolp et al. 1983) needs to be examined before conclusions can be drawn.

It would be useful to have a protocol to determine the optimal dose of trilostane on the basis of resolution of the clinical signs of hypercortisolism and an ACTH stimulation test, together with use of the basal UCCR, to monitor treatment after clinical signs of hypercortisolism are satisfactory controlled. One benefit would be that measuring the UCCR is less time consuming, less invasive, and less expensive than performing ACTH

stimulation tests. Perhaps even more important, patients at risk of developing hypocortisolism could be identified and given appropriate attention.
In conclusion, the results of this study demonstrate that the UCCR cannot be used to determine the optimal dose of trilostane, but could be helpful in detecting dogs that are at risk of developing hypocortisolism during trilostane therapy.

Acknowledgments

The study was supported by Dechra Veterinary Products, United Kingdom.

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Chapter 8

Hypercortisolism in a dog due to ectopic secretion of adrenocorticotropic hormone

S. Galac[a], H.S. Kooistra[a], G. Voorhout[b],
T.S.G.A.M. van den Ingh[c], J.A. Mol[a], G. van den Berg[d], B.P. Meij[a]

Domest Anim Endocrinol 2005;28:338-348.

[a]*Department of Clinical Sciences of Companion Animals,*
[b]*Division of Diagnostic Imaging,*
[c]*Department of Pathology,*
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
[d]*Department of Endocrinology, University Hospital, Groningen, The Netherlands*

Abstract

Spontaneous hypercortisolism in dogs is known to be the result of excessive secretion of adrenocorticotropic hormone (ACTH) by the pituitary gland or excessive autonomous glucocorticoid secretion by an adrenocortical tumor. Here we report on an 8-year-old German shepherd dog in which ACTH-dependent hypercortisolism was a result of ectopic ACTH secretion and could be related to an abdominal neuroendocrine tumor. Hypercortisolism was diagnosed in on the basis of the history, clinical signs, and elevated urinary corticoid:creatinine ratios (UCCRs; 236 and 350 $\times 10^{-6}$; reference range $<10 \times 10^{-6}$). The UCCR remained elevated (226×10^{-6}) after three oral doses of dexamethasone (0.1 mg/kg body weight) at 8-h intervals. Ultrasonography revealed two equivalently enlarged adrenal glands, consistent with adrenocortical hyperplasia. Plasma ACTH concentration was clearly elevated (159 and 188 ng/L; reference range 5-85 ng/L) Computed tomography (CT) revealed that the pituitary was not enlarged. These findings were interpreted as indicating dexamethasone-resistant pituitary-dependent hypercortisolism. Transsphenoidal hypophysectomy was performed but within 2 weeks after surgery there was exacerbation of the clinical signs of hypercortisolism. Plasma ACTH concentration (281 ng/L) and UCCRs (1518 and 2176×10^{-6}) were even higher than before surgery. Histological examination of the pituitary gland revealed no neoplasia. Stimulation of the pituitary with corticotropin-releasing hormone did not affect plasma ACTH and cortisol concentrations. Treatment with trilostane was started and restored normocorticism. CT of the pituitary fossa 10 months after hypophysectomy revealed an empty sella. Hence it was presumed that there was ectopic secretion of ACTH. CT of the abdomen revealed a mass in the region of the pancreas and a few nodules in the liver. Partial pancreatectomy with adjacent lymph node extirpation was performed and the liver nodules were biopsied. Histological examination revealed a metastasized neuroendocrine tumor. Abdominal surgery was not curative and medical treatment with trilostane was continued. At 18 months after the abdominal surgery, the dog is still in good condition. In conclusion, the combination of 1) severe dexamethasone-resistant hypercortisolism with elevated

circulating ACTH levels, 2) definitive demonstration of the absence of pituitary neoplasia, and 3) an abdominal neuroendocrine tumor allowed the diagnosis of ectopic ACTH secretion.

Introduction

Hypercortisolism or Cushing's syndrome is the clinical state of persistent glucocorticoid excess. In dogs, spontaneous hypercortisolism may be caused by excessive secretion of adrenocorticotropic hormone (ACTH) by the pituitary or excessive autonomous glucocorticoid secretion by an adrenocortical tumor (Rijnberk 1996). In humans, hypercortisolism may also be the result of ectopic secretion of ACTH or corticotropin-releasing hormone (Meador et al. 1962, Strewler 2003).

The majority of the tumors that cause ectopic ACTH secretion in humans are primarily malignant tumors of neuroendocrine cell origin, such as small-cell lung carcinomas (Terzolo et al. 2001). The ectopic ACTH syndrome may also be due to bronchial and thymic carcinoids, pancreatic islet cell tumors, medullary carcinomas of the thyroid gland, or pheochromocytomas (3). In cases of ectopic ACTH secretion the rapidly developing physical changes are usually associated with extremely high plasma levels of ACTH and cortisol, together with marked hypokalemia.

Hypercortisolism due to ectopic ACTH secretion is often difficult to distinguish from pituitary-dependent hypercortisolism (PDH). Tumors causing the ectopic ACTH syndrome are frequently small and difficult to visualize (Trainer and Grossman 1991, Newell-Price et al. 1998). In addition, up to 10% of the human population has incidental nonsecreting pituitary tumors and pituitary imaging may therefore provide a false positive finding. On the other hand, pituitary imaging may fail to demonstrate a microadenoma causing PDH (Aron and Howlet 2000). Consequently, the differentiation between PDH and hypercortisolism due to ectopic ACTH secretion relies heavily on biochemical testing.

Circulating ACTH levels tend to be higher in the ectopic ACTH syndrome than in PDH, but there is a large overlap (Terzolo et al. 2001, Howlet et al. 1986). Dynamic noninvasive testing of the pituitary-adrenocortical axis is more reliable in differentiating the causes of ACTH-dependent hypercortisolism. In the classical form of the ectopic ACTH syndrome, the secretion is not suppressed by large doses of dexamethasone (Strewler 2003). Intravenous administration of corticotropin-releasing hormone (CRH) causes a definite increase in plasma ACTH and cortisol concentrations in the majority of patients with PDH, but rarely does so in those with the ectopic ACTH syndrome (Nieman et al. 1986, Malchoff et al 1988, Newell-Price et al. 2002). In humans, simultaneous bilateral inferior petrosal sinus sampling (IPSS) for ACTH has

been used successfully to distinguish a pituitary from an ectopic ACTH source (Manni et al. 1983, McCance et al. 1989). When pituitary and peripheral ACTH concentrations are compared, a gradient to the periphery is indicative for the ectopic ACTH secretion. To avoid the problem of intermittent ACTH secretion, the IPSS sampling can be combined with a CRH stimulation test (Oldfield et al. 1991).
We report here our findings in a dog, in which initially PDH was diagnosed. After hypophysectomy hypercortisolism persisted. Further studies revealed a metastatic neuroendocrine tumor as the most likely source of the ACTH excess.

Materials and methods

Endocrine tests and sample collection

Urine samples for measurement of urinary corticoids were collected by the owner at home on three consecutive mornings. After collection of the second urine sample, three doses of dexamethasone (0.1 mg/kg body weight) were given orally at 8-h intervals and on the following morning the third urine sample was collected.
Blood samples for hormone measurements were collected from the jugular vein and transferred to ice-chilled EDTA-coated tubes. Plasma was separated by centrifugation at 4 °C for 10 minutes and was then stored at –25 °C until assayed.
A CRH-stimulation test was performed according to methods described previously (16). Blood samples were collected for measurement of ACTH and cortisol at –15, 0, 5, 10, 20, 30, and 45 minutes after the intravenous administration of 1 µg ovine CRH (Peninsula Laboratories Inc.) per kg body weight.

Hormone determination

Urinary corticoid concentrations were measured by radioimmunoassay (RIA) as described previously (Rijnberk et al. 1988). The intra- and inter-assay 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 by calculating the urinary corticoid:creatinine ratio (UCCR).
Plasma ACTH concentration was measured using an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The antiserum is highly specific for ACTH (1-39). The intra- and inter-assay coefficients of variation were 3.2 and 7.8 %, respectively, and the sensitivity was 1 ng/L.

Plasma cortisol concentration was measured by RIA (Coat-A-Count, Cortisol, Diagnostic Product Corporation, Los Angeles, USA). The sensitivity was 1 nmol/L and the inter-assay coefficient of variation was 4-6.4 %.

Diagnostic imaging

Ultrasonography was performed with a high-definition ultrasound system (HDI 3000, Advanced Technology Laboratories/Philips, Eindhoven, The Netherlands), equipped with an 8-5 MHz broadband curved-array transducer. The abdomen was examined via the ventral and/or left and right lateral approach with the dog in dorsal or lateral recumbency, respectively.

Computed tomography (CT) was performed in the anaesthetized dog with a third-generation CT scanner (Tomoscan CX/S, Philips NV, Eindhoven, The Netherlands). Ventilation of the lungs was controlled manually and scans of the thorax and abdomen were made at the end of expiration.

During the initial examination of the pituitary gland, both a dynamic and a spatial series of scans were made (Van der Vlught-Meijer et al. 2003). Ten months after hypophysectomy, CT was performed to examine the pituitary fossa, the thorax, and the abdomen. With the dog in sternal recumbency, transverse scans of the base of the skull were made using 120 kV, 220 mA, 9 seconds scanning time, and 2-mm-thick contiguous slices from the rostral clinoid processes to the dorsum sellae, both before and following the intravenous administration of 60 ml of contrast medium (Telebrix 350, sodium and meglumine joxitalamate, containing 350 mg iodine/ml, Guerbet Nederland BV, Gorinchem, The Netherlands). With the dog remaining in sternal recumbency, CT of the thorax was performed using 120 kV, 220 mA, 4.5 seconds scanning time, and 10-mm-thick contiguous slices, without additional contrast medium.

With the dog in dorsal recumbency, CT of the abdomen was performed using 120 kV, 220 mA, 4.5 seconds scanning time, and 10-mm-thick contiguous slices, immediately following the additional administration of 60 ml of contrast medium.

Histological examination

For histological examination, the pituitary tissue, resected pancreatic nodule, pancreaticoduodenal lymph node, and liver biopsies, were fixed in 10% neutral buffered formalin and processed for embedding in paraffin. Sections of 3 µm were stained with hematoxylin and eosin (HE). Immunohistochemical staining was performed by the avidin-biotin complex (ABC) technique using polyclonal rabbit antibodies to synthetic $ACTH_{1\text{-}24}$ (Middleton et al. 1987), polyclonal rabbit antibodies to synthetic

α-melanocyte stimulatory hormone (α-MSH) (PU060-UP, Biogenex laboratories, San Ramon CA), and rabbit antibodies to porcine growth hormone (Spencer et al. 1983).

Case report

History and initial presentation

An 8-year-old intact male German shepherd dog was referred to the Department of Clinical Sciences of Companion Animals of Utrecht University because of polyuria, polydipsia, and polyphagia of a few weeks duration, and frequent panting without exertion. The hair coat was unchanged. The dog was lively and appeared to be in good physical condition; the only abnormality revealed by physical examination was hepatomegaly. Laboratory findings included an increase in plasma alkaline phosphatase (111 U/L; reference range 15-69 U/L) and a low plasma potassium concentration (3.4 mmol/L; reference range 3.6-4.8 mmol/L). Plasma urea, creatinine, glucose, sodium, calcium, and inorganic phosphorous concentrations were within reference ranges. Urine specific gravity was low (1.008). Urinalysis revealed no other abnormalities.

The history, clinical signs, and laboratory findings suggested hypercortisolism. The basal UCCRs on two consecutive days were 236 and 350×10^{-6} (reference range $<10 \times 10^{-6}$). After three oral doses of 0.1 mg dexamethasone per kg body weight, the UCCR was 226×10^{-6}. Basal plasma ACTH concentrations, collected with a 15-minute interval, were 159 and 188 ng/L (reference range 5-85 ng/L). In agreement with the evidence of dexamethasone-resistant PDH, ultrasonography revealed equal enlargement of both adrenal glands. On the dynamic CT scan the pituitary gland had a normal enhancement pattern. The pituitary was not enlarged (height 3.8 mm, pituitary height/brain area (P/B) value 0.23) (Figure 1A). A microadenoma of the pituitary was suspected and transsphenoidal hypophysectomy was performed, as described previously (Meij et al. 1998). After successful surgery and uneventful recovery, the dog was released from the hospital with hormone replacement therapy consisting of desmopressin for 14 days, cortisone acetate, and thyroxine, as described previously (Meij et al. 1998).

Histological examination of the pituitary revealed a normal structure and serial sections at 36 µm intervals revealed no tumor tissue. The pattern of immunohistochemical staining for GH and µ–MSH was normal. Immunohistochemical staining of the pars distalis for ACTH was negative, i.e., only sporadically cells were found that immunostained for ACTH.

Two weeks after the hypophysectomy, the dog was presented again because of extreme polyuria, polydipsia, polyphagia, and panting. The dog was in poor physical condition.

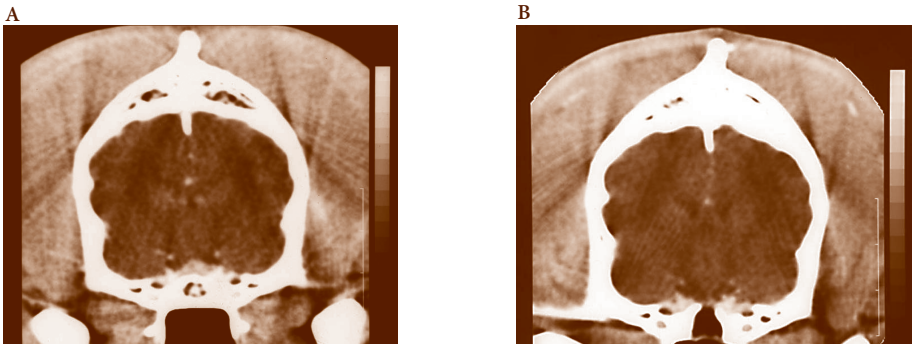

Figure 1
Contrast-enhanced transverse computed tomography (CT) image through the pituitary gland in an 8-year-old intact male German shepherd dog with ectopic adrenocorticotropin (ACTH) secretion;
A. At the time of the initial diagnosis of pituitary-dependent hypercortisolism (PDH). Pituitary gland height is 3.8 mm and the pituitary height/brain area value (P/B) is 0.23.
B. Contrast enhanced CT image of the pituitary fossa at 10 months after transsphenoidal hypophysectomy. The burr hole in the sphenoid bone is bordered by the contrast-enhanced cavernous sinuses. There is no evidence of pituitary remnants.

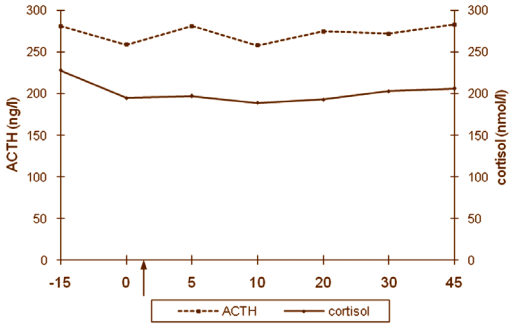

Figure 2
Plasma adrenocorticotropin (ACTH) and cortisol concentrations after intravenous injection (arrow) of 1 µg ovine corticotropin releasing hormone (oCRH) per kg body weight in an 8-year-old intact male German shepherd dog with ectopic ACTH secretion after complete hypophysectomy for initially presumed pituitary-dependent hyperadrenocorticism (PDH).

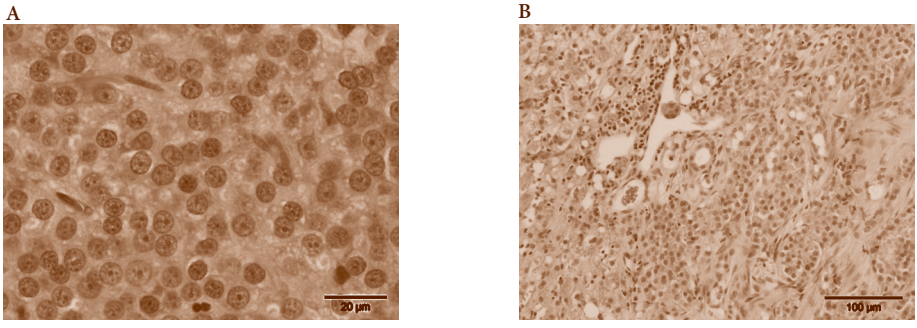

Figure 3
Histology of the pancreatic lymph node and liver tissue in an 8-year-old intact male German shepherd dog with ectopic adrenocorticotropin (ACTH) secretion. In the pancreatic lymph node (A) neuroendocrine cells predominate. In the liver (B) there is infiltration of neuroendocrine tumor cells.

The temporal muscles and muscles of the rear legs were atrophied and there was no regrowth of hair that had been clipped for surgery. The hypokalemia had worsened (2.2 mmol/L). Plasma alkaline phosphatase concentration had increased further (1129 U/L), most being the heat-stable isoenzyme (632 U/L), known to be induced by corticosteroid excess (Teske et al. 1989). Urine specific gravity remained low (1.004) in spite of the administration of desmopressin. The UCCRs, 24 hours after cortisone medication, were extremely high (1518 and 2176 × 10^{-6}). Basal plasma ACTH concentration was elevated (281 ng/L). CRH stimulation did not change plasma concentrations of ACTH and cortisol (Figure 2). Contrast-enhanced CT of the pituitary fossa revealed no remnants of pituitary tissue (Figure 1B).

On the basis of these findings, ectopic ACTH secretion was suspected. Total body CT revealed a rounded mass (2.6 × 3.3 cm in cross-section) in the region of the left lobe of the pancreas. The liver contained one large (3.6 cm) and two small (0.5 cm) hypodense lesions. During laparatomy, a 5-mm nodule was palpated in the left lobe of the pancreas, a lymph node 3 cm in diameter was found adjacent to the pancreas, and several nodules were observed in the liver. Partial pancreatectomy with extirpation of the adjacent lymph node was performed and crush biopsies of the liver nodules were collected. Histological examination revealed a metastasized neuroendocrine tumor (Figure 3). There was no immunostaining for ACTH in the neuroendocrine tissue.

Plasma ACTH and cortisol concentrations did not decrease following surgery and treatment with trilostane (Vetoryl, Arnolds, UK), a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (HSD3B), was started in a dose of 60 mg once daily. This resulted in reversal of the clinical signs and lowering of the UCCR to normal values within 2 months. Interrupting the treatment resulted in recurrence of signs of hypercortisolism and elevation of the UCCR within a few days (Table 1).

Eighteen months after abdominal surgery the dog was in good physical condition. There were no signs of hypercortisolism as long as trilostane was given. Blood examination and thoracic radiographs revealed no abnormalities. Ultrasonography revealed the continuing presence of the nodules in the liver.

Time	UCCR 1 (× 10^{-6})	UCCR 2 (× 10^{-6})
At initial diagnosis of hyperadrenocorticism	236	350
2 weeks after hypophysectomy	1518	2176
2 weeks trilostane	194	257
2 months trilostane	5.2	5.4
9 months trilostane, withdrawal for 2 days	72	74
11 months trilostane	3	3.5

Table 1
Basal urinary corticoid:creatinine ratio (UCCR) on two consecutive days at initial diagnosis of hypercortisolism, after hypophysectomy and during treatment with trilostane in an 8-year-old male German shepherd dog with ectopic adrenocorticotropin (ACTH) secretion. Reference range is < 10 × 10^{-6}.

Discussion

This case report documents the occurrence of hypercortisolism due to ectopic ACTH secretion associated with the presence of a neuroendocrine tumor. There has been a previous case report published on a dog with dexamethasone-resistant hypercortisolism, presumably caused by ACTH release from a primary hepatic carcinoid (Churcher 1999) However, the final proof of ectopic ACTH secretion was lacking in that case, because ACTH immunostaining of the carcinoid tissue was negative and the pituitary function and morphology were not evaluated. In our patient, hypercortisolism was initially thought be of pituitary origin. Plasma ACTH concentration was elevated and ultrasonography revealed two equivalently enlarged adrenal glands. The finding that the pituitary was of normal size with a normal enhancement pattern on dynamic CT could not exclude a pituitary microadenoma (Van der Vlugt-Meijer et al. 2003). However, neither the signs of hypercortisolism nor the elevation of the UCCR and of plasma ACTH concentration were reversed by complete hypophysectomy. Although an empty sella on postoperative CT images does not completely exclude the possibility of pituitary remnants (Meij et al. 1998), the absence of any neoplastic tissue in the excised pituitary made remnants or regrowth very unlikely. The negative immunohistochemical staining for ACTH in the normal adenohypophyseal tissue was consistent with feedback suppression by chronic cortisol excess due to ectopic ACTH secretion. In case of ectopic CRH secretion, positive immunostaining for ACTH and hyperplasia of normal adenohypophyseal corticotropes should have occurred (Strewler 2003).

Hypokalemia was an important biochemical feature of the disease in this dog. Among humans with hypercortisolism, hypokalemia is mainly present in those with ectopic ACTH syndrome, versus less than 10% of those with Cushing's disease (Howlett et al. 1986). Humans with ectopic ACTH syndrome usually have higher circulating levels of cortisol than do those with other forms of hypercortisolism. A high cortisol concentration saturates the renal protective tubular enzyme 11β-hydroxysteroid dehydrogenase (CYP11B2). This enables cortisol to access the type I mineralocorticoid receptor, resulting in a cortisol-induced mineralocorticoid effect (Stewart et al. 1995). In the dog described here, the very excessive cortisol secretion, reflected in the extremely elevated UCCR, may have resulted in hypokalemia via the same mechanism.

Administration of CRH causes a definite increase in plasma ACTH and cortisol concentrations in the majority of humans and dogs with Cushing's disease (Nieman et al. 1986, Trainer et al. 1995, Meij et al. 1997). In human patients with the ectopic ACTH syndrome, CRH rarely causes an increase in plasma ACTH concentration (Malchoff et al. 1988, Newell-Price et al. 2002). Consequently, the absence of a response to CRH in this dog after hypophysectomy is consistent with ectopic ACTH secretion.

Metastasized neuroendocrine tissue was found in the pancreatic lymph node and liver nodules. Immunohistochemical staining for ACTH was negative, just as in the case

report of Churcher (1999). The failure of ACTH immunohistochemistry in staining ectopic ACTH-secreting tumor tissue is well known in humans. Even in a patient in whom tumor resection had been curative, ACTH immunohistochemistry was negative (Aniszewski et al. 2001). Furthermore, in an immunohistochemical study of Coates and coworkers (1986), 6 of 18 tumors causing ectopic Cushing's syndrome were immunonegative for ACTH, as well as other proopiomelanocortin (POMC)-derived peptides and peptides with ACTH-releasing properties. The explanation may be that all peptide hormones produced by these tumors are secreted immediately. Consequently, the amounts of peptide hormones remaining in the cells are too small to be visualized by immunohistochemistry (Coates et al. 1986, Heintz et al. 1981). Moreover, the absence of immunohistochemical staining for ACTH in metastatic tissue does not disprove ectopic ACTH secretion, for only a subpopulation of the cells may secrete ACTH (Aniszewski et al. 2001).

Demonstrating POMC by immunohistochemistry and/or searching for the presence of POMC-mRNA by PCR may also not provide conclusive evidence, because the results of both can be positive in virtually all normal tissues, as well as in various tumors not associated with the ectopic ACTH syndrome (Clark 1988, DeBold et al. 1988, White et al. 1990). Odel (1991) even suggested that the term ectopic ACTH syndrome is a misnomer, since ectopic ACTH secretion represents a cancer-induced amplification of a biologic feature that normally exists in the cells from which the cancer originates. It has been reported that the ectopic ACTH syndrome most likely occurs when a highly tissue-specific promoter of the POMC gene, called CpG island promoter, is activated (Newell-Price et al. 2001).

The pancreas is a frequent site of ectopic ACTH-secreting tumors in humans (Orth 1987, Clark and Carney 1987). The clinical course of ACTH-secreting pancreatic islet cell tumors is often severe and rapidly progressive, and most patients already have hepatic metastases at the time of diagnosis (Doppman et al. 1994, Amikura et al. 1995). The optimal treatment for an ACTH-secreting neoplasm is complete surgical resection, a goal that—in patients with pancreatic islet cell tumors—often cannot be achieved (Aniszewski et al. 2001). In none of the patients with ACTH-secreting islet cell tumor studied by Amikura and coworkers (1995) was a biochemical cure achieved by aggressive surgical resection of the primary tumor and metastases. In the dog described here, removal of the pancreatic nodule and adjacent lymph node did not change the course of the disease. Apparently, the primary tumor was not found or liver metastases continued to secrete ACTH.

Treatment targeting the adrenal glands, such as bilateral adrenalectomy, chemical adrenocorticolysis, or inhibition of adrenal steroid secretion, is necessary when the source of ACTH secretion cannot be found or removed (Jex et al. 1985, Miller and Crapo 1993). Canine ACTH-dependent hypercortisolism is most often treated by selective or complete adrenocorticolysis by the administration of o,p'-DDD (mitotane)

(Peterson and Kintzer 1994, Den Hertog et al. 1999). The hypercortisolism in this dog was controlled successfully with trilostane, a competitive inhibitor of HSD3B. Since medicinal reduction or elimination of the glucocorticoid excess will reduce the clinical signs of hypercortisolism, whether due to PDH or to ectopic ACTH secretion, it is possible that until now the ectopic ACTH syndrome has been overlooked in the dog.
In conclusion, the diagnosis of ectopic ACTH secretion causing hypercortisolism in the dog described was made based on strong circumstantial evidence. The persistence of hypercortisolism and hyperadrenocorticotropism after complete hypophysectomy, no plasma ACTH and cortisol response after CRH administration following hypophysectomy, the failure to identify a pituitary adenoma in the pathology specimen, the finding of sporadic staining for ACTH in the normal adenohypophyseal tissue, and the demonstration of a metastatic neuroendocrine tumor are arguments for ectopic ACTH secretion. This case report indicates that the ectopic ACTH syndrome should be included in the differential diagnosis of hypercortisolism in dogs with elevated, nonsuppressible UCCR levels, high plasma ACTH concentrations, and normal findings on pituitary imaging.

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Chapter 9

Adrenocorticotropic hormone-independent hypercortisolism due to food-induced hypercortisolemia in a dog

S. Galac[a], V.J. Kars[a], G. Voorhout[b], J.A. Mol[a], H.S. Kooistra[a]

Vet J 2008;117:141-143.

[a] *Department of Clinical Sciences of Companion Animals,*
[b] *Division of Diagnostic Imaging,*
Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

Abstract

In addition to adrenocortical tumors, ectopic or aberrant expression of functional hormone receptors in the adrenal cortex may also cause adrenocorticotropic hormone (ACTH)-independent hypercortisolism. Here we report on a 6-year-old Vizsla dog in which ACTH-independent hypercortisolism was associated with meal-induced hypercortisolemia. Diagnosis was based on the history, physical findings, biochemical changes, and elevation of the urinary corticoid:creatinine ratio on two consecutive days (UCCR, 11 and 13 × 10^{-6}, reference range <8.3 × 10^{-6}). Basal plasma ACTH concentration was found by repeated measurements to be suppressed (<1 ng/L, reference range 5-85 ng/L) and administration of corticotropin releasing hormone (CRH) resulted in a minor increase (to 6 ng/L), consistent with ACTH-independent hypercortisolism. Ultrasonography and computed tomography (CT) revealed two uniformly enlarged adrenal glands. Magnetic resonance imaging (MRI) of the pituitary area showed a nonenlarged, normally enhancing pituitary gland. Based on these results, expression of functional aberrant adrenocortical receptors was suspected and the possibility of food-dependent hypercortisolism (FDH) was explored.

The UCCR on two separate occasions rose from 11 and 8 × 10^{-6} before a meal to 25 and 23 × 10^{-6} at 3 hours after ingestion of a meal. There was a corresponding increase in plasma cortisol concentration (from 90 to 150 nmol/L), while plasma ACTH concentration remained low or undetectable. Consistent with the diagnostic criteria for FDH in humans, administration of octreotide completely prevented meal-induced hypercortisolemia in this dog. The dog was treated successfully with the cortisol-synthesis-inhibitor trilostane (2 hours before meals) and at 26 months after the final diagnosis the dog was still in good condition.

The combination of 1) low plasma ACTH concentration in the absence of an adrenocortical tumor, 2) a more than 100% increase in the UCCR after ingestion of a meal, 3) prevention of the meal-induced increase in plasma cortisol concentration by octreotide, and 4) reversal of signs of hypercortisolism by administration of trilostane a few hours before meals led to the diagnosis of FDH due to aberrant expression of functional gastric-inhibitory polypeptide receptors in this dog.

Introduction

Adrenocorticotropic hormone (ACTH)-independent hypercortisolism results from excessive secretion of glucocorticoids by benign or malignant adrenocortical tumors (ATs) (Rijnberk 1996). However, ACTH-independent hypercortisolism may also be due to ectopic expression or hyperactive eutopic hormone receptors. In humans, various adrenocortical membrane-bound receptors functionally coupled to steroidogenesis have been reported, including gastric-inhibitory polypeptide (GIP), catecholamine, vasopressin, serotonin, and luteinizing hormone (LH) receptors (Lacroix et al. 2001, Christopoulos et al. 2005). The ectopic expression of GIP receptors underlies meal-induced or food-dependent hypercortisolism (FDH) (De Herder et al. 1996, N`Diaye et al. 1998, Lebrethon et al. 1998). Due to expression of functional adrenocortical GIP receptors, cortisol secretion is stimulated by food intake, leading to the development of Cushing's syndrome. FDH has been described in humans with bilateral adrenocortical hyperplasia (Lacroix et al. 1992, Reznik et al. 1992) as well as with adrenocortical neoplasia (De Herder et al. 1996, Chabre et al. 1998, Dall`Asta et al. 2004).

The diagnosis of FDH in humans is based on low fasting plasma cortisol levels and a postprandial increase in cortisol secretion. Pretreatment with somatostatin (octreotide) blocks the meal-induced release of GIP and consequently the GIP-induced rise in cortisol secretion (De Herder et al. 1996, Croughs et al. 2000). The response of plasma ACTH and cortisol concentrations to corticotropin-releasing hormone (CRH) stimulation is usually blunted in FDH, but in some cases the response is preserved (Croughs et al. 2000, Tsagarakis et al. 2001). The abnormal adrenocortical cells retain sensitivity to ACTH and the administration of synthetic ACTH leads to a rise in plasma cortisol concentration (De Herder et al. 1996, N`Diaye et al. 1998).

We report here our findings in dog with physical and biochemical changes consistent with hypercortisolism in which the combination of suppressed plasma ACTH levels and bilateral adrenal enlargement raised suspicion of ACTH-independent hypercortisolism due to aberrant expression of functional hormone receptors in the adrenal cortex.

Materials and methods

Endocrine tests and sample collection

For the diagnosis of hypercortisolism, urine samples for measurement of urinary corticoid concentration were collected by the owner at home on three consecutive mornings. After collection of the second basal urine sample, three doses of dexamethasone

(0.1 mg/kg body weight) were given orally at 8-h intervals and on the following morning the third urine sample was collected (Rijnberk et al. 1988).
The influence of food intake on urinary corticoid excretion was studied by collecting 1 urine sample before and 2 urine samples at 3-h and 6-h after a meal.
A CRH stimulation test was performed by collecting blood samples for measurement of ACTH and cortisol concentrations immediately before and 30 minutes after the i.v. administration of 1 µg ovine CRH (Peninsula Laboratories Inc.) per kg body weight. The same procedure was followed in a control group of 6 healthy beagle dogs.
The influence of food intake on plasma cortisol and ACTH concentrations was investigated on two consecutive days by collecting blood samples 30 minutes before and 30, 90, 150, and 210 minutes after intake of a protein-rich meal (meal test). On the second day, immediately after the collection of the first blood sample, octreotide (Sandostatin, Sandoz Pharmaceutical, Basel, Switzerland) was administered subcutaneously in a dose of 3 µg/kg body weight. The same procedure was followed in a control group of 6 healthy beagle dogs.
Blood samples for hormone measurements were collected from the jugular vein and transferred to ice-chilled EDTA-coated tubes. Plasma was separated by centrifugation at 4 °C for 10 minutes and stored at –25 °C until assayed.

Hormone determinations

Urinary corticoid concentrations were measured by radioimmunoassay (RIA) as described previously (Rijnberk et al. 1988). The intra- and inter-assay 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 by calculating the urinary corticoid:creatinine ratio (UCCR).
Plasma ACTH concentration was measured using an immunoradiometric assay (Nichols Institute, Wijchen, The Netherlands). The antiserum is highly specific for ACTH (1-39). The intra- and inter-assay coefficients of variation were 3.2 and 7.8 %, respectively, and the sensitivity was 1 ng/L.
Plasma cortisol concentration was measured by RIA (Coat-A-Count Cortisol, Diagnostic Product Corporation, Los Angeles, USA). The sensitivity was 1 nmol/L and the inter-assay coefficient of variation was 4.0 to 6.4 %.

Diagnostic imaging

Ultrasonography was performed with a high-definition ultrasound system (HDI 5000, Advanced Technology Laboratories/Philips, Eindhoven, The Netherlands), equipped

with an 8-5 MHz broadband curved-array transducer. The abdomen was examined via a ventral and ventrolateral approach with the dog in dorsal recumbency.
Thin-sliced 3D gradient-echo magnetic resonance imaging (MRI) of the pituitary gland was performed in the anesthetized dog with a 0.2 T open field MRI-system (Magnetom Open Viva, Siemens AG, München, Germany) as described previously (Van der Vlugt-Meijer et al, 2006). The height and width of the pituitary were measured on transverse images and the length was measured on sagittal reconstructions of the transverse images. The ratio between height of the pituitary gland and the area of the brain (P/B value), was calculated as described previously (Kooistra et al. 1997).
Computed tomography (CT) of the adrenal glands was performed in the anaesthetized dog with a single slice helical CT scanner (Philips Secura, Philips NV, Eindhoven, The Netherlands). With the dog in dorsal recumbency CT of the abdomen was performed using 120 kV, 280 mA, 1.4 seconds scanning time, and 5-mm-thick contiguous slices, from a plane cranial to the right kidney to the hilus of the left kidney.

Statistical analysis

The Kolmogorov-Smirnov test indicated that the plasma concentrations of ACTH and cortisol in the control dogs were not normally distributed. Consequently, differences in plasma hormone concentrations in the 6 control dogs were assessed using the non-parametric Friedman test. Dunnett's test was used as post-hoc test. Values are expressed as mean ± SEM and $P<0.05$ was considered significant.

Case report

History and initial presentation

A 6-year-old intact male Vizsla dog was referred to the Department of Clinical Sciences of Companion Animals of Utrecht University because of polyuria, polydipsia, polyphagia, and mild exercise intolerance. The hair coat was dull and there was non-pruritic symmetrical alopecia. These changes had been present with variable intensity for 2 years.
Physical examination revealed no abnormalities except a slightly pendulous abdomen and symmetrical alopecia. Laboratory findings included an elevated plasma alkaline phosphatase concentration (391 U/L; reference range <73 U/L), mainly due to the heat-stable isoenzyme (290 U/L) (Teske et al. 1989). Plasma urea, creatinine, glucose, sodium, potassium, calcium, and inorganic phosphate concentrations were within

reference ranges. Urinalysis revealed no abnormalities except a low specific gravity (1.010).

Because the history, physical findings, and laboratory findings all suggested hypercortisolism, the UCCR was measured. The basal UCCR on two consecutive days was 11.0 and 8.3×10^{-6} (reference range $<8.3 \times 10^{-6}$; Van Vonderen et al. 1998). After three high doses of dexamethasone orally, the UCCR was 0.9×10^{-6}, consistent with pituitary-dependent hypercortisolism (PDH).

The MRI of the pituitary gland revealed that the pituitary had a normal enhancement pattern and was not enlarged (P/B value 0.20, reference range <0.31; Kooistra et al. 1997). Basal plasma ACTH concentration in each of two samples collected with a 15-minute interval was <1 ng/L (reference range 5-85 ng/L; Javadi et al. 2006), indicating ACTH-independent hypercortisolism. Ultrasonography and CT scanning of the abdomen revealed uniform enlargement of both adrenal glands.

In the absence of evidence of an AT, expression of functional aberrant adrenocortical receptors was suspected and the possibility of FDH was explored. On two separate occasions, the UCCR 3 hours after ingestion of a protein-rich meal was 25 and 23×10^{-6}, which was more than double the basal UCCR (11 and 8.4×10^{-6}) (Table 1). Plasma cortisol concentration increased after ingestion of a protein-rich meal, from 90 to 150 nmol/L in 2.5 hours (Figure 1a). On the second day, the meal-induced rise in plasma cortisol concentration was completely prevented by administration of octreotide (Figure 1b). Moreover, octreotide administration resulted in a considerable decrease in plasma cortisol concentration (from 58 to 8 nmol/L). The plasma ACTH concentration was low or undetectable throughout the meal test (Figures 1c and 1d).

In the healthy control dogs, food intake also resulted in a slight increase in plasma cortisol concentration (from 40 ± 3 nmol/L at t=0 to 72 ± 6 nmol/L at t=120 min, P=0.03) (Figure 1a). This increase was associated with a significant increase in plasma ACTH concentration after a meal (P<0.05 at t=120 and t=180) (Figure 1c). Octreotide administration to the healthy control dogs prior to a meal resulted in a slight decrease in plasma cortisol concentration (from 58 ± 3 nmol/L at t=0 to 29 ± 3 nmol/L at t=60, P=0.01) (Figure 1b). In addition, plasma ACTH concentration decreased significantly (at t=60, P=0.003) when octreotide was administered before the meal (Figure 1d).

Administration of 0.25 mg synthetic ACTH to the Vizsla dog resulted in a rise in plasma cortisol concentration from a basal level of 63 nmol/L to 683 nmol/L at 90 minutes. Administration of CRH resulted in an increase in plasma cortisol concentration from 78 nmol/L to 138 nmol/L and in ACTH concentration from 3 ng/L to 6 ng/L. In the healthy control dogs CRH administration caused an increase in plasma cortisol from 32 ± 6 nmol/L to 211 ± 20 nmol/L and an increase in ACTH from 42 ± 7 ng/L to 385 ± 46 ng/L (for both P<0.001).

Time of day	UCCR day 1 ($\times 10^{-6}$)	UCCR day 2 ($\times 10^{-6}$)
08.00	12	9.5
11.00	11	8.7
14.00	13	6.4
16.00	11	8.4
19.00	25	23
22.00	16	10

Table 1
Urinary corticoid:creatinine ratio (UCCR) immediately before and 3 and 6 hours after ingestion of a meal on two consecutive days in a 6-year-old male Vizsla dog with adrenocorticotropin (ACTH)-independent hypercortisolism. At 8.00 only bread was given, while at 16.00 a protein-rich meal was given.

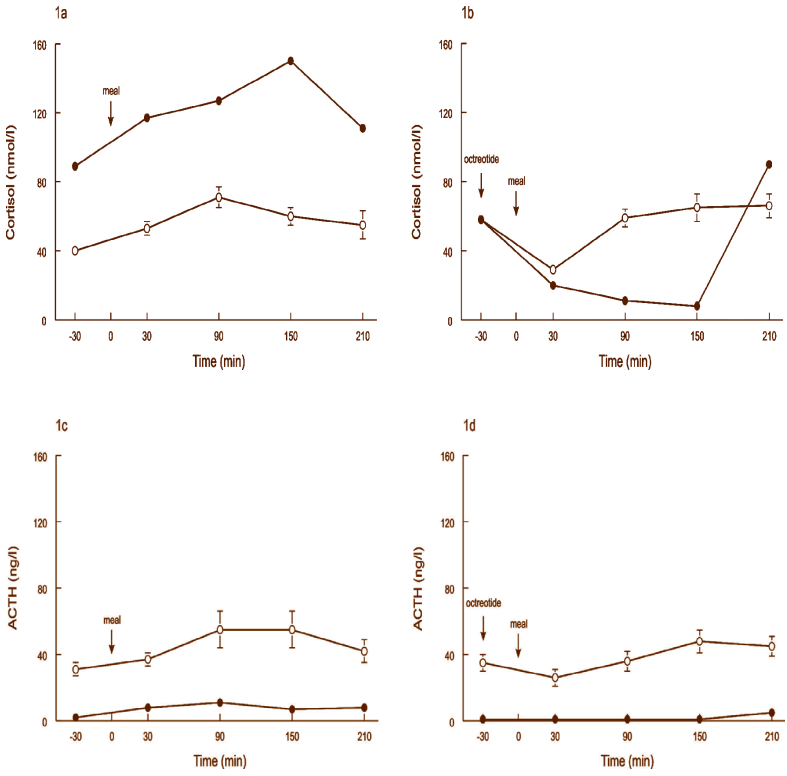

Figure 1
Plasma concentrations of cortisol (panels 1a and 1 b) and adrenocorticotropin (ACTH) (panels 1c and 1d) before and after ingestion of a protein-rich meal (at time 30 min) in a 6-year-old intact Vizsla dog with adrenocorticotropin (ACTH)-independent hypercortisolism and in 6 healthy beagle dogs, without (left panels) and with (right panels) pretreatment with octreotide in a subcutaneous dose of 3 µg/kg body weight (at time 0 min). Values in control dogs are expressed as mean ± SEM.

Treatment with trilostane (Vetoryl, Dechra), a competitive inhibitor of 3β-hydroxysteroid dehydrogenase (HSD3B) , was started in a dose of 2 mg/kg body weight once daily. Initially, trilostane was administered in the morning, when the dog was fed only bread, but there was no improvement until administration was changed to 2 hours before the main meal in the afternoon. After 26 months of treatment with trilostane the dog is still in good physical condition and without signs of hypercortisolism. Regular blood examinations included measurements of plasma urea, creatinine, glucose, sodium, potassium and alkaline phosphatase concentrations. All values were within the reference ranges. The ACTH stimulation test to control the dosage of trilostane, performed 3 hours after trilostane administration, revealed a basal plasma cortisol concentration of 28 nmol/L and a post-ACTH plasma cortisol concentration of 35 nmol/L.

Discussion

This case report documents the occurrence of ACTH-independent hypercortisolism in a dog due to meal-induced hypercortisolemia. The hypercortisolism was initially thought to be of pituitary origin, because the elevated UCCR was suppressed by dexamethasone (Galac et al. 1997) and bilateral adrenal gland enlargement was found by diagnostic imaging. However, plasma ACTH concentration was repeatedly found to be suppressed and in the absence of an AT to explain this, aberrant expression of adrenocortical receptors was considered and the possibility of meal-induced or FDH was explored.

In humans, FDH is usually associated with rather subtle signs and symptoms of Cushing's syndrome that have been present for several years (Swain et al. 1998). In the dog described here, clinical signs of hypercortisolism were and the UCCR was only slightly elevated. For the diagnosis of hypercortisolism, the first morning urine is collected for measurement of the UCCR and this will usually be before or very shortly after the first food intake. In subjects with FDH the UCCR will not be expected to be elevated at this time. Similarly, in humans with FDH low fasting plasma cortisol levels have been reported (De Herder et al. 1996). This Vizsla dog received its main meal in the afternoon and the UCCR was found to be clearly elevated only when determined following the meal. Based on criteria used in humans (Lacroix et al. 1999), the more than 50% elevation of the UCCR following the meal pointed to FDH.

The meal also resulted in a clear increase in plasma cortisol concentration, while plasma ACTH concentration remained low or undetectable. There was also a significant postprandial rise in plasma cortisol concentration in the control dogs, but it was associated with a significant increase in plasma ACTH concentration, which was presumably its cause. If it had been mediated by aberrant functional adrenocortical recep-

tors, the negative feedback of cortisol at the hypothalamic-pituitary level would presumably have resulted in a decrease in plasma ACTH concentration.
The fact that the meal-induced cortisol release was completely prevented by administration of octreotide also provides evidence for FDH. Octreotide blocks the meal-induced release of GIP and consequently the GIP-induced increase in cortisol secretion (De Herder et al. 1996, Croughs et al. 2000). Blocking GIP release by octreotide eliminated the main stimulus for adrenocortical cortisol secretion in this dog, resulting in a decrease in plasma cortisol concentration. ACTH release apparently remained suppressed to the extent that the negative feedback failed to raise the plasma ACTH level. However, octreotide administration also caused a slight but significant decrease in plasma cortisol concentration in the control dogs. This was associated with a significant decrease in the plasma ACTH concentration, suggesting that the cortisol decrease occurred secondary to the ACTH decline. Corticotrope cells have been shown to express somatostatin receptors (Hofland et al. 2005) and activation of these receptors results in suppression of ACTH release.

Administration of synthetic ACTH resulted in a considerable increase in the plasma cortisol concentration in the Vizsla, indicating that the adrenocortical cells had retained their sensitivity to ACTH, as has been reported in humans with FDH (De Herder et al. 1996, N`Diaye et al. 1998). However, the food-induced hypercortisolemia resulted in suppression at the hypothalamic-pituitary level, as shown by the low or undetectable plasma ACTH concentration. This suppression was incomplete, possibly as a result of the intermittent food-dependent nature of the hypercortisolemia (Croughs et al. 2000), as CRH administration still resulted in a minor increase in plasma ACTH concentration. A CRH-induced increase in plasma ACTH concentration has also been described in humans with FDH (Doppman et al. 2000, Croughs et al. 2000, Tsagarakis et al. 2001). Incomplete suppression at the hypothalamic-pituitary level may also explain the suppression of the UCCR in the Vizsla after administration of high doses of dexamethasone.
In the present case, diagnostic imaging revealed bilateral adrenal enlargement, but failed to demonstrate noduli. In most humans with FDH there are nodular changes in the adrenals (Doppman et al. 2000, Rockall et al. 2004), but diffuse enlargement without distinct nodules has also been reported (Doppman et al. 2000).
FDH can be diagnosed definitively by histological examination of adrenocortical tissue, by demonstration of abundant adrenocortical GIPR expression and by stimulation of cortisol production by GIP in a primary culture of adrenal cells (Groussin et al. 2002, Bertherat et al. 2005). Since the hypercortisolism in the Vizsla was controlled successfully by trilostane, adrenal tissue was not obtained for analysis.
The effect of trilostane, a competitive inhibitor of HSD3B, on cortisol metabolism begins shortly after administration and lasts for only a few hours (Neiger et al. 2002). Its short duration of activity explains why trilostane was ineffective when administered

in the morning but was effective when administered shortly before the main meal. In humans, bilateral adrenalectomy is the preferred long-term treatment of FDH (Swain et al. 1998). Pharmacological blockade of hypercortisolism with octreotide was attempted as an alternative to surgery (Reznik et al. 1992, De Herder et al. 1996, Croughs et al. 2000). It led to clinical and biological improvement initially but long-term treatment proved to be ineffective (Lacroix et al. 2000).
In conclusion, the distinct increase in UCCR and plasma cortisol concentration after ingestion of a meal, the low or undetectable plasma ACTH levels, the prevention of a meal-induced rise in plasma cortisol concentration by octreotide administration, and a good response to medical treatment with trilostane administered shortly before the main meal are strong arguments for the diagnosis of FDH in this dog.

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Chapter 10

Summarizing Discussion and Conclusions

Spontaneous hypercortisolism or Cushing's syndrome is characterized by physical and biochemical effects of chronic exposure to increased circulating levels of glucocorticoids. In the dog, hypercortisolism is usually due to hypersecretion of adrenocorticotropin (ACTH) by a pituitary corticotroph adenoma. ACTH is the major peptide regulating adrenocortical steroidogenesis, which is initiated by the binding of ACTH to its receptor (ACTH-R) (Mountjoy et al. 1992). Regulation of the ACTH-R gene is unique in that it is upregulated by its own ligand in a time- and dose-dependent manner (Mountjoy et al. 1994, Lebrethon et al. 1994). When hypercortisolism results from hypersecretion of cortisol by an adrenocortical tumor (AT), the plasma ACTH concentration is suppressed (Van Sluijs et al. 1995) and the ACTH/ACTH-R mechanism controlling steroidogenesis is inoperative. In the absence of an explanation for the ACTH-independent hypersecretion of cortisol by an AT, it is simply described as being autonomous. However, recent studies in humans have revealed that steroidogenesis in some cases of cortisol-producing bilateral macronodular adrenal hyperplasia and several unilateral adenomas is stimulated by either ectopic or overexpressed eutopic adrenocortical hormone receptors (Lacroix et al. 2001, Lacroix 2009). The aberrant expression of these receptors may also be responsible for hyperplasia, proliferative advantage, and tumor formation (Bordeaux et al. 2001, Li et al. 2005).

Chapter 3 reports the results of screening 23 surgically-removed canine cortisol-secreting ATs for the expression of luteinizing hormone receptor (LHR), gastric inhibitory polypeptide receptor (GIPR), and vasopressin receptors ($V_{1a}R$, $V_{1b}R$, and V_2R). Normal adrenal glands served as control tissues. Abundant mRNA for these receptors was quantified by means of QPCR, while the presence and localization of the receptors was determined by immunohistochemistry (IHC).

QPCR analysis revealed that the hypersecretion of cortisol by canine ATs was not associated with overexpression of any of the receptors studied. Nevertheless, there were strong indications of ectopic expression of GIPR and V_2R in the ATs, as IHC demonstrated several GIPR- and V_2R-immunopositive cells in tumorous zona fasciculata (ZF) tissue, while in normal adrenal glands the ZF was immunonegative for GIPR

and V_2R. It may be hypothesized that this ectopic expression of GIPR and V_2R protein plays a role in the pathogenesis of canine AT. *In vitro* tests with genetically engineered primary bovine adrenocortical cells demonstrated that ectopic expression of GIPR and V2R is a sufficient genetic event to trigger adrenocortical tumorigenesis (Mazzuco et al. 2006a, 2007).
Although the ZF of both normal adrenals and ATs was immunopositive for LHR, and QPCR did not reveal overexpression of LHR in ATs when compared with normal adrenals, a role for aberrant eutopic expression of LHR in adrenocortical tumorigenesis can still not be excluded. The mRNA expression in ATs, consisting almost entirely of tumorous ZF tissue, was compared with that in normal adrenals, including the strongly immunopositive zona glomerulosa (ZG) cells. The staining pattern in normal adrenals and ATs differed.. In normal adrenals, immunopositivity for LHR in ZF tissue was strongest just beneath the ZG, whereas the tumorous ZF tissue of the ATs was more uniformly immunopositive. In addition, immunostaining of LHR in some of the canine ATs was heterogeneous. This heterogeneous pattern, characterized by clusters of immunopositive areas close to areas of no staining, was observed mainly in carcinomas. The functional link between LHR expression and AT development has been studied by a design similar to that for the GIPR (Pabon et al. 1996, Mazzuco et al. 2006b). The enforced expression of LHR in genetically engineered adrenocortical cells transplanted under the renal capsule of adrenalectomized immunodeficient mice resulted in the development of hypercortisolemia, adrenal hyperplasia, and benign transformation, indicating that aberrant LHR expression is also sufficient to initiate the complete phenotypic alterations that ultimately lead to formation of a cortisol-secreting AT.

ACTH-independent steroidogenesis in ATs may be explained by upregulation of genes encoding steroidogenic enzymes of cortisol biosynthesis. Human cortisol-secreting adenomas do indeed overexpress mRNA-encoding cholesterol side-chain cleavage enzyme (CYP11A), 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase /17,20-lyase (CYP17), and 11β-hydroxylase type 1 (CYP11B1), when compared with normal adrenals (Bassett et al. 2005). In a screening of 38 surgically removed ATs in dogs with hypercortisolism (**Chapter 4**), the mRNA expression of steroidogenic acute regulatory protein (StAR), CYP11A, HSD3B, CYP17, 21-hydroxylase (CYP21), and CYP11B1 did not differ significantly between normal adrenal glands and cortisol-secreting adenomas and carcinomas. Consequently, hypercortisolemia in dogs with ATs cannot be ascribed to upregulation of the genes encoding for steroidogenic enzymes.
A relatively great abundance of mRNA encoding HSD3B was found in tumorous ZF tissue. This finding justifies medical treatment with trilostane, a competitive inhibitor of HSD3B, to control cortisol secretion and consequently clinical signs due to hypercortisolism in dogs with an (inoperable) ATs (Eastwood et al. 2003, Benchekroun et al. 2008).

QPCR analysis revealed significant downregulation of the gene encoding ACTH-R in canine cortisol-secreting adrenocortical carcinomas, which was substantiated at the transcriptional level by Western blot analysis. The presence of the ACTH/ACTH-R signaling cascade has been found to be essential to maintain a highly differentiated adrenocortical cellular phenotype (Reincke et al. 1997). Low expression of the ACTH-R gene and loss of heterozygosity (LOH) have been described in highly malignant and nonfunctional human ATs (Reincke et al. 1997, 1998). It may be hypothesized that loss of the ACTH-R signal transduction cascade in canine adrenocortical carcinomas leads to loss of differentiation and enhanced clonal expansion of AT cells, similar to that described in humans (Reincke et al. 1997, 1998, Beuschlein et al. 2001).

ACTH-R expression in canine adrenocortical adenomas did not differ from that in normal adrenal glands. Apparently, the suppressed plasma ACTH concentration in these patients did not affect the expression of its receptor, as would be expected from the regulation mechanism of the ACTH-R gene demonstrated in cultured human and bovine adrenocortical cells (Lebrethon et al. 1994, Penhoat et al. 1995).

Administration of ACTH to human patients with a cortisol-secreting adenoma leads to a further increase in the production of cortisol, indicating that the ACTH-R is functional (Imai et al. 2001, Bertagna and Orth 1981). Efforts were made to differentiate between adrenocortical adenomas and carcinomas in dogs on the basis of the post-ACTH plasma cortisol concentration, but the results were inconclusive (Peterson et al. 1982, Feldman 1985). One of the major problems in interpreting the results of the ACTH-stimulation test in dogs with a cortisol-secreting AT is that the differentiation between adrenocortical adenoma and carcinoma by conventional histopathology is very difficult (Labelle et al. 2004). The results of this study suggest that determination of ACTH-R expression, and possibly also the outcome of an ACTH-stimulation test, may provide the pathologist with additional information allowing a better differentiation between adrenocortical carcinomas and adenomas. More reliable identification of adrenocortical carcinoma would enable postoperative adjuvant therapy to be initiated only in those patients that may really benefit from it. In order to make the pathological diagnosis as reliable as possible, all of the ATs described in this thesis were evaluated by one pathologist using the same criteria. Despite this approach, misdiagnoses are possible. A scoring system that takes into account not only the histopathology, but also the clinical picture and follow-up, may be more reliable. Such a classification is used to judge the nature of ATs in humans (Weiss et al. 1989) and may have to be introduced in veterinary medicine as well.

When the diagnosis of hypercortisolism has been confirmed, it is necessary to differentiate between pituitary-dependent hypercortisolism (PDH) and hypercortisolism due to AT, because the respective treatments are usually different. The study reported

in **Chapter 5** revealed that basal urinary corticoid:creatinine ratios (UCCRs) of dogs with PDH and AT did not differ significantly, although a UCCR exceeding 100 x 10^{-6} was almost exclusively associated with PDH. This indicates that the hyperplastic adrenal cortices in dogs with PDH have a greater capacity to produce cortisol than does neoplastic adrenocortical tissue.
Using the UCCR to measure the response in a high-dose dexamethasone suppression test (HDDST), the maximum decrease in the UCCR in dogs with AT was 43.7%. A decrease in the UCCR of more than 50% was thus taken to be indicative of PDH, consistent with the generally accepted criterion of 50% suppression for the HDDST using the measurement of plasma cortisol concentration (Feldman et al. 1996) In the majority (34 of 49) dogs with an AT, the UCCR *increased* after dexamethasone administration. A similar effect has been described in humans with functional AT (Flack et al. 1992, Mantero et al. 2004). The reason for this paradoxical increase remains unclear, but it suggests that dexamethasone has a direct effect on the AT.

The goal of trilostane treatment is to suppress plasma cortisol concentration to such an extent that the signs of hypercortisolism disappear. The optimal dose is determined by resolution of clinical signs and the results of an ACTH-stimulation test (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). In a retrospective study of 63 dogs with PDH (**Chapter 6**), the dose of trilostane was considered to be optimal when the post-ACTH plasma cortisol concentration was <190 nmol/L and the owner reported resolution of the clinical signs of hypercortisolism, such as polyuria, polydipsia, and polyphagia. Basal plasma cortisol concentrations were significantly lower after the optimal trilostane dose was achieved than before treatment, whereas basal plasma ACTH concentrations were significantly higher. The increase in plasma ACTH concentration during trilostane therapy is the result of negative feedback initiated by the low circulating cortisol concentration (Witt and Neiger 2004, Sieber-Ruckstuhl et al. 2006). Accordingly, a markedly increased plasma ACTH concentration is expected during chronic hyposecretion of cortisol, as occurs in primary hypoadrenocorticism (Peterson et al. 1996, Javadi et al. 2003). Indeed, in 5 dogs with trilostane-induced hypocortisolism, plasma ACTH concentrations were very high. Moreover, there was no overlap in plasma ACTH concentrations between dogs receiving the optimal dose of trilostane and those with trilostane-induced hypocortisolism. It can be concluded that trilostane therapy affects the pituitary-adrenocortical axis and that measurement of plasma ACTH concentration will be found useful to detect trilostane overdosage.
The results of the study described in **Chapter 6** demonstrate that trilostane not only affects the pituitary-adrenocortical axis but also influences the renin-angiotensin-aldosterone (ALD) system. Although trilostane administration was not associated with a significant decrease in plasma ALD concentration, plasma renin activity was

significantly higher when trilostane dosage was considered to be optimal than it was before trilostane treatment. Elevated plasma renin activity during trilostane therapy has also been described in humans (Komanicky et al. 1978). Higher plasma renin activity during treatment can be explained by a trilostane-induced decrease in ALD secretion. Subsequently, the decline in plasma ALD concentration results in a lower circulating volume due to a decrease in ALD-mediated renal reabsorption of sodium. The decrease in circulating volume stimulates renin secretion, and the activation of the renin-angiotensin system stimulates ALD synthesis and secretion. This sequence of events indicates that plasma renin activity is a better guide to mineralocorticoid deficiency than is plasma ALD concentration. To detect abnormalities in the renin-ALD system, the ratio of plasma ALD to renin activity has been identified as a sensitive screening test in humans (Lim et al. 1999), cats (Javadi et al. 2005), and dogs (Javadi et al. 2006). Indeed, the ALD:renin ratio was significantly lower during trilostane treatment than before treatment.

The UCCR has proved to be a reliable parameter in the initial diagnosis of hypercortisolism and trustworthy in evaluating cortisol secretion after hypophysectomy or non-selective destruction of the adrenal cortex by mitotane therapy (Meij et al. 2002, Den Hertog et al. 1996). The results of the study presented in **Chapter 7** demonstrate, however, that the UCCR cannot be used as an alternative to the ACTH-stimulation test to determine the optimal dose of trilostane. In the majority of dogs studied the UCCR did not decline below the upper limit of the reference range for at least 2 months after the optimal dose of trilostane was reached. This might be a combined effect of the mechanism of action of trilostane and the method of measuring urinary corticoids. Trilostane is a competitive inhibitor of HSD3B (Pots et al. 1978) and it may influence additional enzymes of the steroid hormone cascade, such as CYP11B1 (Sieber-Ruckstuhl et al. 2006). In keeping with its inhibitory effects on HSD3B, it caused a significant increase in plasma concentrations of 17α-OH-pregnenolone and dehydroepiandrostenedione in human patients being treated with it for hypercortisolism (Komanicky et al. 1978, Sieber-Ruckstuhl et al. 2006). In the measurement of urinary corticoids in unextracted urine by radioimmunoassay, not only cortisol but also some substances with a similar polarity are included (Stolp et al. 1983). It is possible that the above mentioned precursors were included in the measurement of urinary corticoids and that this is why the UCCR did not decline to within the reference range despite low plasma cortisol concentrations and good clinical control of hypercortisolism.
In a few dogs the UCCR did decline below the upper level of the reference range during trilostane therapy. In the long-term follow-up, these dogs developed clinical signs of hypocortisolism and/or the ACTH-stimulation test demonstrated too little adrenocortical reserve. Trilostane-induced hypocortisolism has been documented previously

(Chapman et al. 2004, Sieber-Ruckstuhl et al. 2006, Ramsey et al. 2008). Apparently, dogs at risk for developing hypocortisolism during trilostane therapy can be recognized by the finding of a UCCR within the reference range. Because adrenocortical insufficiency can be fatal if not treated promptly, a warning that the dog is at risk during trilostane therapy is of great value. This seems especially important now that it is clear that necrosis and apoptosis of the adrenal glands can occur in dogs treated with trilostane (Reusch et al. 2007). The degree of its severity seems to be related not only to the dose of trilostane and individual sensitivity to the drug, but also to the duration of the trilostane treatment.

The ectopic ACTH secretion syndrome is an important differential diagnosis in ACTH-dependent hypercortisolism in humans. Various types of tumors, from well-differentiated and indolent carcinoids to highly aggressive small cell carcinomas, can secrete ACTH (Newell-Price et al. 1998, Raffin-Sanson et al. 2000). They are usually small and difficult to visualize even by cross-sectional diagnostic imaging techniques. **Chapter 8** is a case report of a dog in which PDH due to a microadenoma was diagnosed and hypophysectomy was performed. When despite complete removal of the pituitary gland the clinical signs of hypercortisolism persisted, the UCCR and plasma ACTH concentration remained elevated, and histological examination of the excised pituitary revealed no neoplasia, ectopic secretion of ACTH was suspected. Computed tomography of the abdomen located a tumor in the region of the pancreas. Laparotomy revealed the tumor nodule in the pancreas as well as metastases in an adjacent lymph node and the liver. Partial pancreatectomy and excision of the lymph node were performed, and a neuroendocrine tumor with metastasis in the lymph node was diagnosed by histological examination. Immunohistochemical staining for ACTH was negative in the tumor and its metastases, but this does not disprove ectopic ACTH secretion, for failure of positive immunohistochemical staining for ACTH in ectopic ACTH-secreting tissue has been reported in humans (Coates et al. 1986, Aniszewski et al. 2001). The explanation may be that the peptide hormones produced by these tumors are secreted immediately, so that the amounts remaining in the cells are too small to be visualized by IHC (Heitz et al. 1981).
Based on this case report, ectopic ACTH secretion should be considered in dogs with severe hypercortisolism in which plasma ACTH concentration is very high and is not suppressed by high doses of dexamethasone, and in which diagnostic imaging does not reveal a pituitary tumor.

Chapter 9 presents a case report of a dog with mild clinical signs of hypercortisolism, slightly elevated UCCRs, and suppressed plasma ACTH concentration. This combina-

tion is consistent with primary adrenocortical hyperfunction, which in dogs is synonymous with AT. However, diagnostic imaging in this dog revealed bilaterally enlarged, nontumorous adrenal glands, raising suspicion of ACTH-independent bilateral adrenal hyperplasia due to expression of aberrant hormone receptors. The UCCR was increased by >50% by ingestion of a meal. Based on the criteria used in humans (Lacroix et al. 1999), this suggests food-dependent hypercortisolism (FDH). Moreover, the ingestion of a meal also caused a significant increase in plasma cortisol concentration, while plasma ACTH concentration remained undetectable. The meal-induced cortisol release was completely inhibited by administration of octreotide. These observations strongly suggest ectopic expression of gastric inhibitory polypeptide receptor (GIPR) in the adrenal cortex of this dog, because octreotide blocks the meal-induced release of gastric inhibitory polypeptide (GIP) and consequently the GIP-induced increase in cortisol secretion (De Herder et al. 1996, Croughs et al. 2000). In addition, medical treatment with trilostane was only effective when administered shortly before the main meal, which is another strong argument for the diagnosis of FDH in this dog.

The ultimate confirmation of FDH would require (1) stimulation of adrenal cortisol production *in vivo* by infusion of physiological concentrations of GIP (Lacroix et al. 1999) and (2) detection of mRNA encoding GIPR in the ZF and confirmation of its functionality by *in vitro* testing (De Herder et al. 1996). Unfortunately, GIP for infusion was not available at the time this dog was being examined and adrenal tissue could not be obtained for *in vitro* testing because the dog responded very well to treatment with trilostane.

When the UCCR is used for the diagnosis of hypercortisolism, the first morning urine is usually collected by the owner at home and thus usually after overnight fasting. Since in FDH the release of cortisol is only stimulated by meal-induced GIP secretion, the diagnosis of FDH can easily be missed. Therefore in dogs with mild signs of hypercortisolism, suppressed plasma ACTH concentrations, and enlarged adrenal glands, measuring the UCCR after a meal should be the first step in further diagnostics of ACTH-independent hypercortisolism.

Recognition of the ectopic ACTH secretion syndrome and FDH in dogs has reshaped the differential diagnosis of hypercortisolism in this species, and makes the process of identifying the cause even more interesting than it already was.

Conclusions

- Hypersecretion of cortisol by canine ATs is not associated with overexpression of the genes encoding LHR, GIPR, $V_{1a}R$, $V_{1b}R$, or V_2R.
- Ectopic expression of GIPR and V_2R protein, and eutopic expression of LHR protein in tumorous ZF tissue, may play a role in the pathogenesis of canine cortisol-secreting ATs.
- Hypercortisolemia in dogs with cortisol-secreting ATs cannot be ascribed to upregulation of the genes encoding for steroidogenic enzymes.
- Significant downregulation of ACTH-R may contribute to the malignant character of cortisol-secreting carcinomas.
- The generally-accepted criterion of 50% suppression of plasma cortisol concentration by a high dose of dexamethasone in the differentiation between PDH and AT is also applicable to the UCCR.
- Trilostane treatment affects both the pituitary-adrenocortical axis and the renin-angiotensin-ALD system.
- The UCCR cannot be used as an alternative to the ACTH-stimulation test to determine the optimal dose of trilostane.
- The basal plasma ACTH concentration and the UCCR may both be used to detect dogs at risk for developing hypocortisolism during trilostane therapy.
- The ectopic ACTH secretion syndrome occurs in dogs and should be suspected in cases of severe and nonsuppressible hypercortisolemia.
- The differential diagnosis of canine ACTH-independent hypercortisolism should include FDH.

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Chapter 11

Samenvatting en conclusies

Het syndroom van Cushing (hypercortisolisme) wordt veroorzaakt door een chronische overmaat aan circulerende glucocorticoïden. Bij ongeveer 85% van de honden met spontaan hypercortisolisme is sprake van een hypofyse-adenoom. Dit hypofyse-adenoom geeft teveel bijnierschors-stimulerend hormoon oftewel adrenocorticotroop hormoon (ACTH) af, hetgeen leidt tot beiderzijdse bijnierschorshyperplasie en een verhoogde afgifte van cortisol. In de resterende 15% van de gevallen berust het hypercortisolisme op een glucocorticoïd-producerende bijnierschorstumor. Men onderscheidt dus twee vormen van spontaan hypercortisolisme: ACTH- of hypofyse-afhankelijk hypercortisolisme (HAH) of bijnier-afhankelijk hypercortisolisme.
Onder normale omstandigheden is activatie van de ACTH receptor (ACTH-R) door ACTH de belangrijkste stimulus voor de vorming en afgifte van glucocorticoïden. Bij bijnier-afhankelijk hypercortisolisme wordt de hypofysaire ACTH afgifte onderdrukt en is de plasma ACTH concentratie dus laag (Galac et al. 2010). Dientengevolge kan de steroïdogenese niet geïnduceerd worden via de ACTH-R. Omdat er voorlopig nog geen goede verklaring is voor ACTH-onafhankelijke steroïdogenese, wordt de hypersecretie van cortisol door een bijnierschorstumor aangeduid als autonoom. Onderzoeken bij de mens lieten zien dat bij eenzijdige adenomen en macronodulaire bijnierschorshyperplasieën de steroïdogenese kan worden gestimuleerd door ectopische of overactieve eutopische bijnierschorsreceptoren (Lacroix et al. 2001, Lacroix 2009). De aberrante expressie van deze hormoonreceptoren is ook verantwoordelijk voor hyperplasie en proliferatie van bijnierschorscellen en kan bovendien tot tumorvorming leiden (Bordeaux et al. 2001, Li et al. 2005).

In **Hoofdstuk 3** zijn de resultaten beschreven van een onderzoek naar de expressie van de receptoren voor luteïnizerend hormoon (LHR), gastic-inhibitory polypeptide (GIPR) en vasopressine ($V_{1a}R$, $V_{1b}R$, and V_2R) in 23 cortisol-producerende bijnierschorstumoren. Normaal bijnierweefsel van gezonde honden werd als controle gebruikt. De hoeveelheid mRNA werd gekwantificeerd met QPCR en de eiwitexpressie en de lokalisatie van de receptoren werden onderzocht met immunohistochemie (IHC). De QPCR analyse toonde geen verhoogde expressie van bovengenoemde re-

ceptoren aan. De verklaring voor hypercortisolisme ligt dus niet in een verhoogde expressie van deze hormoonreceptoren. Immunohistochemisch onderzoek liet echter zien dat deze receptoren wel degelijk een rol in de pathogenese van de bijnierschorstumoren kunnen spelen. Het tumoreuze zona fasciculata weefsel was namelijk immunopositief voor GIPR and V_2R, terwijl in normaal zona fasciculata weefsel deze receptoren niet aanwezig waren. Er is dus sprake van ectopische GIPR en V_2R expressie in bijnierschorstumorweefsel. De potentiële rol van deze receptoren in de pathogenese van bijnierschorstumoren wordt ondersteund door *in vitro* testen, waarbij is aangetoond dat ectopische expressie van GIPR en V_2R de groei van de bijnierschorscellen kan stimuleren en vervolgens tot het ontstaan van een bijnierschorstumor kan leiden (Mazzuco et al. 2006a, 2007).

Immunohistochemische kleuring van LHR toonde aan dat deze receptoren in de normale bijnier van de hond het meest intensief tot expressie komen in de zona glomerulosa. Ook de zona fasciculata van de normale bijnierschors was immunopositief voor LHR; met een duidelijk afnemende intensiteit in de richting van de zona reticularis. Het bijnierschorstumorweefsel was eveneens immunopositief voor LHR. Bij de meeste tumoren was het aankleuringspatroon homogeen, maar vooral bij carcinomen werd een heterogeen aankleuringspatroon gezien met groepjes cellen met sterke immunopositieve kleuring omringd door immunonegatieve cellen. Het mogelijke verband tussen de expressie van LHR en het ontstaan van bijnierschorstumoren is gebaseerd op *in vitro* onderzoeken vergelijkbaar met eerdergenoemde studies met GIPR en V_2R (Pabon et al. 1996, Mazzuco et al. 2006b). De resultaten van deze studies laten zien dat ook aberrante expressie van de LHR kan leiden tot het ontstaan van een bijnierschorstumor. Bij de hond wordt de LHR dus tot expressie gebracht in zowel normaal zona fasciculata weefsel als in tumoreus zona fasciculata weefsel. Aangezien het aankleuringspatroon in het tumorweefsel nogal verschilde van dat in het normale weefsel is een eutopische rol van de LHR in de pathogenese van de bijnierschorstumoren zeer goed denkbaar.

Een andere verklaring voor hypercortisolemie bij bijnierschorstumoren zou een verhoogde expressie van enzymen betrokken bij de steroïdogenese kunnen zijn. Bij de mens is een verhoogde expressie van cholesterol-side-chain enzyme (CYP11A), 3β-hydroxysteroid dehydrogenase (HSD3B), 17α-hydroxylase/17,20-lyase (CYP17) en 11β-hy-droxylase type 1 (CYP11B1) beschreven in bijnierschorsadenomen (Bassett et al. 2005). In **Hoofdstuk 4** zijn de resultaten van een QPCR analyse beschreven waarin de expressie van steroid acute regulatory protein (StAR), CYP11A, HSD3B, CYP17, 21-hydroxylase (CYP21) en CYP11B1 is bepaald in 38 bijnierschorstumoren en is vergeleken met de genexpressie van deze enzymen in normale bijnieren. mRNA expressies van de enzymen in bijnierschorstumoren en normale bijnieren waren niet verschillend. Geconcludeerd is dat de hypercortisolemie niet kan worden toegeschreven aan een verhoogde expressie van steroïdogenen enzymen. De resultaten van het

onderzoek lieten wel een relatief hoge expressie van HSD3B in bijnierschorsadenomen zien, hetgeen verklaart dat behandeling van honden met een functioneel bijnierschorsadenoom met trilostane, een competitieve remmer van HSD3B, succesvol is in het verlagen van de cortisolafgifte door de tumor (Eastwood et al. 2003, Benchekroun et al. 2008).

De resultaten van het onderzoek beschreven in **Hoofdstuk 4** geven ook aan dat in bijnierschorscarcinomen de expressie van de ACTH-R significant lager is dan die in adenomen en normale bijnieren. Om het hoog gedifferentieerde fenotype van de bijnierschorscellen te behouden wordt het functioneren van de ACTH/ACTH-R cascade van groot belang geacht (Reincke et al. 1997). Een lage expressie van de ACTH-R en verlies van heterozygotie (LOH) zijn beschreven bij maligne en hormonaal-inactieve tumoren van de bijnierschors bij de mens (Reincke et al. 1997, 1998). Het is aannemelijk dat ook bij de hond een verlaagde expressie van de ACTH-R tot een snelle groei van slecht gedifferentieerde bijnierschorscellen kan leiden.

De regulatie van het gen dat codeert voor de ACTH-R is uitzonderlijk, omdat aangetoond is dat de expressie van dit gen positief beïnvloed wordt door ACTH zelf. De relatie tussen ACTH en de ACTH-R is tijd- en de dosis-afhankelijk. Hoe hoger de ACTH concentratie is en hoe langer deze concentratie hoog is, hoe groter de expressie van de ACTH-R is en vice versa (Mountjoy et al. 1994, Lebrethon et al. 1994). Uit de resultaten van het onderzoek beschreven in **Hoofdstuk 4** blijkt echter dat het aantal ACTH-R in de functionele bijnierschorsadenomen niet verschilde met die in de normale bijnieren. Deze bevinding geeft aan dat de verlaagde plasma ACTH concentratie blijkbaar geen effect heeft gehad op de expressie van de ACTH-R, wat men wel zou verwachten gezien het veronderstelde verband tussen ACTH en de ACTH-R.

Onderzoek bij mensen met bijnierschorsadenomen heeft uitgewezen dat toediening van ACTH tot een verhoogde cortisolafgifte leidt (Imai et al. 2001, Bertagna and Orth 1981), hetgeen aantoont dat de ACTH-R functioneel is in de bijnierschorsadenomen. Vergelijkbare onderzoeken werden ook bij honden met bijnierschorstumoren uitgevoerd (Peterson et al. 1982, Feldman 1985), maar omdat de differentiatie tussen bijnierschorsadenomen en carcinomen uiterst lastig is kan niet teveel belang aan de uitkomsten van deze studies worden toegekend. Op basis van de bevindingen beschreven in **Hoofdstuk 4** kan gepostuleerd worden dat de plasma cortisol concentratie na ACTH toediening bij honden met een bijnierschorscarcinoom veel minder zou moeten stijgen dan bij honden met een bijnierschorsadenoom, maar dat zal in de praktijk nog bewezen moeten worden. Indien de hypothese echter klopt dan zouden de resultaten van een ACTH-stimulatietest aanvullende informatie kunnen opleveren over de aard van de bijnierschorstumor, hetgeen kan bijdragen aan een beter afgewogen therapiekeuze en een betrouwbaardere prognosestelling. Bij de mens wordt bij de differentiatie tussen bijnierschorsadenomen en bijnierschorcarcinomen al gebruik gemaakt van klinische gegevens (Weiss et al. 1989). Het zou wenselijk zijn als in de nabije toe-

komst ook bij honden met bijnierschorstumoren een beoordelingssysteem gemaakt wordt waarbij zowel histopathologische als klinische gegevens worden gebruikt.

Nadat de diagnose hypercortisolisme is gesteld is het noodzakelijk om te differentiëren tussen hypofyse- en bijnier-afhankelijk hypercortisolisme, omdat de behandeling van deze twee vormen verschillend is. Een van de doelstellingen van het onderzoek beschreven in **Hoofdstuk 5** was om na te gaan of de basale urine corticoïd/kreatinine (UCK) ratio ook zou kunnen worden gebruikt voor de differentiatie. De resultaten van dit onderzoek laten echter zien dat de basale UCK ratio's bij hypofyse- en bijnier-afhankelijk hypercortisolisme niet significant verschilden, maar waarden hoger dan 100 x 10^{-6} werden vrijwel alleen gevonden bij HAH. Deze bevindingen suggereren dat de hyperplastische bijnierschors bij honden met HAH een grotere capaciteit heeft voor cortisolproductie dan tumoreus bijnierschorsweefsel.

Als bepaling van de basale UCK ratio's gecombineerd werd met een hoge-dosis dexamethason suppressietest was de maximale daling van de UCK ratio bij honden met een bijnierschorstumor 43.7%. Een daling van de UCK ratio van meer dan 50% wijst dus op HAH. Dit komt overeen met het percentage dat ook wordt aangehouden als bij de hoge-dosis dexamethason suppressietest gebruik wordt gemaakt van de plasma cortisol concentratie (Feldman et al. 1996). Bij de meeste honden (34 van 49) met een bijnierschorstumor werd na de dexamethason toediening een hogere UCK ratio gemeten dan de basale waarden. De oorzaak van deze paradoxale stijging van de cortisolproductie na dexamethasontoediening, die ook bij mensen met een functionele bijnierschorstumor bekend is (Flack et al. 1992), is nog onduidelijk.

Trilostane is in de laatste jaren het meest gebruikte medicijn voor hypercortisolisme bij de hond geworden. Het doel van de behandeling met trilostane is om de plasma cortisol concentratie zo ver te onderdrukken dat de klinische verschijnselen van hypercortisolisme verdwijnen. De optimale trilostane dosis wordt bepaald op basis van de verbetering van de klinische verschijnselen en de resultaten van de ACTH-stimulatietest (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). In **Hoofdstuk 6** wordt een retrospectieve studie van 63 honden die vanwege HAH met trilostane worden behandeld beschreven. De trilostane dosering werd als optimaal bestempeld als de plasmaconcentratie van cortisol na ACTH toediening minder was dan 190 nmol/l en, volgens de eigenaar, de belangrijkste verschijnselen van hypercortisolisme zoals polyurie, polydipsie en polyfagie verdwenen waren. De resultaten van de studie laten zien dat de basale plasmaconcentratie van cortisol, op het moment dat de dosering optimaal was, significant lager was dan voor de behandeling, terwijl de plasmaconcentratie van ACTH juist significant steeg. De hoge plasmaconcentratie van ACTH is het gevolg van de afgenomen negatieve terugkoppeling van cortisol op de hypothalamus-hypofyse-as. Extreem hoge plasmaconcentraties van ACTH kunnen verwacht

worden bij een chronische hyposecretie van cortisol, zoals bij de ziekte van Addison (Peterson et al. 1996, Javadi et al. 2003). In overstemming hiermee werden bij 5 honden met trilostane-geïnduceerd hypocortisolisme extreem hoge plasma-ACTH-concentraties gemeten. Er was geen overlap tussen de basale plasma-ACTH-concentraties van goed gereguleerde honden en honden met trilostane-geïnduceerd hypocortisolisme. Op basis van de resultaten van deze retrospectieve studie is geconcludeerd dat de behandeling met trilostane een effect heeft op de hypofyse-bijnierschors-as en dat de plasma-ACTH-concentratie gebruikt kan worden om een overdosering met trilostane vast te stellen.

Het onderzoek dat beschreven is in **Hoofdstuk** 6 laat ook zien dat trilostane een effect heeft op het renine-angiotensine-aldosteron systeem. Ondanks het feit dat het gebruik van trilostane niet leidde tot een significante daling van plasmaconcentratie van aldosteron (ALD), was de plasma-renine-activiteit tijdens de behandeling significant hoger dan voor de behandeling met trilostane. Een verhoogde renine-activiteit tijdens behandeling met trilostane is ook bij de mens beschreven (Komanicky et al. 1978). Een hoge plasma-renine-activiteit tijdens behandeling met trilostane zou kunnen ontstaan door een trilostane-geïnduceerde afname in de productie en afgifte van ALD. De daling van de plasma-ALD-concentratie veroorzaakt vervolgens een verminderde renale reabsorptie van natrium en een lager circulerend volume, waardoor de afgifte van renine wordt gestimuleerd. De activatie van het renine-angiotensin systeem stimuleert vervolgens de ALD secretie, waardoor de plasma-ALD-concentratie vergelijkbaar wordt met die voor de behandeling met trilostane. Voor het detecteren van veranderingen in het renine-angiotensine-ALD systeem is het dus noodzakelijk om naast de plasma-ALD-concentratie ook de plasma-renine-activiteit te bepalen. Inderdaad is zowel bij mensen (Lim et al. 1999) als bij honden (Javadi et al. 2006) en katten (Javadi et al. 2005) de ratio tussen de plasma-ALD-concentratie en de plasma-renine-activiteit de meest informatieve parameter als het gaat om vaststellen van verstoringen van het renine-angiotensine-ALD systeem. De resultaten van het onderzoek beschreven in **Hoofdstuk 6** laten zien dat de ALD/renine ratio tijdens de behandeling met trilostane significant lager was dan voor de behandeling. Hiermee wordt het effect van trilostane op het renine-angiotensine-ALD systeem bewezen.

De UCK ratio wordt gebruikt om de diagnose hypercortisolisme vast te stellen en om de restfunctie van de bijnierschors te beoordelen na hypofysectomie of totale destructie van de bijnierschors met de mitotane (Den Hertog et al. 1996, Meij et al. 2002). De resultaten van het onderzoek beschreven in **Hoofdstuk 7** laten zien dat de bepaling van de UCK ratio niet als een alternatief voor de ACTH-stimulatietest gebruikt kan worden om de juiste dosering van trilostane vast te stellen. Bij de meeste honden daalde de UCK ratio, binnen 2 maanden nadat de juiste dosering van de trilostane was vastgesteld, niet tot een waarde in het referentiegebied. De verklaring hiervoor is hoogstwaarschijnlijk dat de gebruikte radioimmunoassay behalve cortisol ook een groot aan-

tal precursors meemeet. Trilostane is een competitieve remmer van 3HSDB (Pots et al. 1978) met mogelijk ook een effect op CYP11B1 (Sieber-Ruckstuhl et al. 2006). Bij mensen met hypercortisolisme behandeld met trilostane werden hoge concentraties van 17α-OH-pregnenolone en dehydroepiandrostenedione gemeten (Komanicky et al. 1978). Het is niet ondenkbaar, dat deze precursors bij de therapie met trilostane ook bij de hond vrijkomen en dat zij de reden zijn voor een verhoogde UCK ratio tijdens de behandeling, terwijl de plasmaconcentratie van cortisol onderdrukt was en het klinisch beeld van hypercortisolisme duidelijk verbeterde.
Bij enkele honden met hypercortisolisme daalde de UCK ratio wel binnen het referentiegebied binnen 2 maanden nadat de juiste dosering van trilostane gegeven werd. Al deze honden ontwikkelden in de loop van de tijd (klinische verschijnselen van) hypocortisolisme en/of er werd met een ACTH-stimulatietest vastgesteld dat de reservecapaciteit van de bijnierschors te gering was. Trilostane kan, vooral als een hoge dosering gegeven wordt, hypocortisolisme of zelfs de ziekte van Addison veroorzaken (Chapman et al. 2004, Sieber-Ruckstuhl et al. 2006, Ramsey et al. 2008). De oorzaak van het hypocortisolisme is trilostane-geïnduceerde necrose en apoptose van bijnierschorscellen (Reusch et al. 2007). Zoals uit de resultaten weergegeven in **Hoofdstuk 7** blijkt, kan het risico op hypocortisolisme bij honden die behandeld worden met trilostane tijdig worden onderkend door het vaststellen van een UCK ratio binnen de referentiewaarden. Deze bevinding heeft grote klinische betekenis, aangezien hypocortisolisme nogal eens fataal verloopt als de diagnose niet op tijd gesteld wordt.

Ectopische ACTH-secretie is bij diverse benigne en maligne tumoren beschreven, van goed gedifferentieerde carcinoïden tot zeer maligne carcinomen (Newell-Price et al. 1998, Raffin-Sanson et al. 2000). Deze tumoren hebben een gezamenlijk kenmerk: zij zijn klein en niet gemakkelijk te vinden, zelfs niet met de nieuwste beeldvormende technieken. In **Hoofdstuk 8** wordt een casus gepresenteerd van een hond met een initiële diagnose HAH door een microadenoom. Echter, na hypofysectomie verergerden de klinische verschijnselen van hypercortisolisme en in overeenstemming hiermee liet laboratoriumonderzoek zeer hoge basale UCK ratio's en plasma-ACTH-concentraties zien. Toen ook het histopathologisch onderzoek van de verwijderde hypofyse de aanwezigheid van een tumor niet kon bevestigen, werd er gedacht aan ectopische ACTH-secretie. Met een CRH-stimulatietest werd vastgesteld dat CRH toediening niet leidde tot een stijging van de plasmaconcentraties van ACTH en cortisol, passend bij ectopische ACTH-secretie. Een CT-scan van het abdomen liet een massa in de alvleesklier zien. Tijdens de laparotomie werden behalve deze massa ook enkele uitzaaiingen in de lever gevonden. Histopathologisch onderzoek toonde een neuroendocriene tumor aan. Dergelijke neuroendocriene tumoren zijn nogal eens de bron van ectopische ACTH-secretie bij de mens. Ondanks het feit dat immunohistochemisch onderzoek

op ACTH in de tumor en metastases van de patiënt negatief was is de diagnose ectopische ACTH secretie nog steeds zeer waarschijnlijk. Negatieve IHC wordt namelijk ook regelmatig gezien bij ectopische ACTH-secretie bij de mens (Heintz et al. 1981, Coates et al. 1986, Aniszewski et al. 2001). Het geproduceerde ACTH wordt waarschijnlijk direct uitgescheiden, waardoor de hoeveelheid ACTH in de tumorcellen zo minimaal is dat dit niet aantoonbaar is met IHC. Ectopische ACTH-secretie komt dus ook bij de hond voor. In de toekomst zal hieraan gedacht moeten worden bij honden met zeer hoge plasmaconcentraties van cortisol die niet te onderdrukken zijn met een hoge dosis dexamethason en waarbij in de hypofyse geen tumor zichtbaar gemaakt kan worden met diagnostische beeldvorming.

In **Hoofdstuk 9** wordt een casus beschreven van een hond met milde klinische verschijnselen van hypercortisolisme, licht verhoogde UCK ratio's en zeer lage plasmaconcentraties van ACTH. Deze combinatie wijst op een bijnier-afhankelijk hypercortisolisme. Met echografisch onderzoek werd echter geen bijnierschorstumor gevonden, maar beiderzijds vergrote bijnieren. Op basis hiervan werd gedacht aan ACTH-onafhankelijke hyperplasie door de aanwezigheid van functionele aberrante hormoonreceptoren. Bepaling van de UCK ratio's voor en na een maaltijd was de eerstvolgende stap in de diagnostiek. Een toename met meer dan 50% van de UCK ratio na de maaltijd bij deze hond was diagnostisch voor voedsel-afhankelijk hypercortisolisme, ervan uitgaande dat de criteria zoals die bij de mens worden toegepast ook voor de hond mogen worden gebruikt (Lacroix et al. 1999). Het verorberen van een maaltijd leidde ook tot een significante stijging van de plasmaconcentratie van cortisol, terwijl de plasma-ACTH-concentratie onderdrukt bleef. De voedsel-geïnduceerde cortisolafgifte kon onderdrukt worden met octreotide, zoals ook beschreven wordt bij de mens (De Herder et al. 1996, Croughs et al. 2000). Gezien het feit dat octreotide een sterk remmend effect op de afgifte van GIP (gastric-inhibitory polypeptide) heeft is het zeer waarschijnlijk dat het hypercortisolisme bij deze patiënt werd veroorzaakt door een aberrante expressie van GIPR.

De diagnose voedsel-afhankelijk hypercortisolisme bij deze hond zou definitief vastgesteld kunnen worden als (1) een infuus met GIP tot een verhoogde secretie van cortisol zou leiden (Lacroix et al. 1999) en als (2) expressie van GIPR in de zona fasciculata aangetoond zou worden en de functionaliteit van deze receptor met *in vitro* testen bevestigd zou worden (De Herder et al. 1996). Helaas waren beide, het GIP infuus en het bijnierschorsweefsel van deze hond, niet beschikbaar. De hond werd behandeld met trilostane en reageerde (pas) goed op de therapie toen trilostane enkele uren voor de maaltijd werd gegeven, hetgeen de diagnose voedsel-afhankelijk hypercortisolisme ondersteunt.

De diagnose voedsel-afhankelijk hypercortisolisme kan eenvoudig worden gemist, omdat voor de diagnostiek de UCK ratio in ochtendurine wordt bepaald en op dat tijdstip zijn de meeste honden nog nuchter. Honden met milde klinische verschijnselen

van hypercortisolisme, lage plasma-ACTH-concentraties en beiderzijds vergrote bijnieren moeten als verdacht van bilaterale hyperplasie door de expressie van aberrante hormoonreceptoren worden beschouwd. De eerste stap in de diagnostische procedure zou een UCK ratio bepaling voor en na een maaltijd kunnen zijn.
Ectopische ACTH-secretie en voedsel-afhankelijk hypercortisolisme zijn nieuwe entiteiten in de veterinaire endocrinologie. De differentiaal diagnose van hypercortisolisme wordt hiermee niet gemakkelijker, maar wel interessanter en uitdagender.

Conclusies

- Hypercortisolemie ten gevolge van functionele bijnierschorstumoren wordt bij de hond niet veroorzaakt door een verhoogde expressie van genen coderend voor LHR, GIPR, $V_{1a}R$, $V_{1b}R$, of V_2R.

- Ectopische expressie van GIPR en V_2R en eutopische expressie van LHR in bijnierschorstumorweefsel zouden wel eens een rol kunnen spelen in de pathogenese van cortisolproducerende bijnierschorstumoren bij de hond.

- Hypercortisolemie ten gevolge van functionele bijnierschorstumoren wordt bij de hond niet veroorzaakt door een verhoogde expressie van genen coderend voor enzymen van de steroïdogenese.

- Bijnierschorscarcinomen hebben een significant lagere expressie van de ACTH-R dan bijnierschorsadenomen en normaal bijnierweefsel.

- Het criterium dat een daling van meer dan 50% van de plasmaconcentratie van cortisol na toediening van dexamethason wijst op hypofyse-afhankelijk hypercortisolisme, kan ook worden gebruikt als in plaats van plasma-cortisol de urine corticoïd/kreatinine ratio wordt gebruikt.

- Behandeling met trilostane heeft een effect op zowel de hypofyse-bijnierschors-as als op het renine-angiotensine-aldosteron systeem.

- De urine corticoïd/kreatinine ratio kan niet gebruikt worden in plaats van de ACTH-stimulatietest om de optimale dosering van trilostane te vinden.

- De basale plasma-ACTH-concentratie en de urine corticoïd/kreatinine ratio kunnen worden gebruikt om trilostane-geïnduceerd hypocortisolisme op te sporen.

- Aan ectopische ACTH-secretie bij de hond moet vooral worden gedacht bij zeer hoge urine corticoïd/kreatinine ratio's die niet te remmen zijn met dexamethason.

- Differentiaal diagnostisch moet bij de ACTH-onafhankelijk hypercortisolisme ook gedacht worden aan voedsel-afhankelijk hypercortisolisme.

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Chapter 12

Izvleček in razprava

S spontanim Cushingovim sindromom opisujemo fizikalne in biokemijske spremembe v organizmu, ki nastanejo kot posledica kroničnega delovanja glukokortikoidov. Pri psih je hiperkortizolizem večinoma posledica povečanega izločanja hormona ACTH iz adenoma hipofize. Hormon ACTH je vodilni peptid za uravnavanje steroidogeneze. Njegovo delovanje je pogojeno z vezavo na ACTH receptor (ACTH-R) (Mountjoy et al. 1992). Razmerje med ACTH in ACTH-R je v biološkem smislu edinstveno, saj dolgotrajno povečana koncentracija ACTH privede do povečane izraženosti ACTH-R gena in obratno (Mountjoy et al. 1994, Lebrethon et al. 1994).
Hiperkortizolizem se lahko v manjšem odstotku primerov pojavi tudi kot posledica povečanega izločanja kortizola iz tumorja nadledvične žleze (NŽ). V tem primeru je koncentracija ACTH zmanjšana zaradi fiziološke negativne povratne zveze (Van Sluijs et al. 1995). Slednja naj bi, po zgoraj omenjenemu principu razmerja med ACTH in ACTH-R, privedla tudi do zmanjšane izraženosti ACTH-R gena. Povečano izločanje kortizola iz tumorjev NŽ zato ne more biti posledica fiziološkega mehanizma aktivacije steroidogeneze. Vzrok za hiperkortizolizem v primeru tumorja NŽ je še nepojasnjen in se običajno opisuje kot avtonomen. Raziskave pri ljudeh so pokazale, da je steroidogeneza v primerih makronodularne hiperplazije NŽ in unilateralnih adenomov lahko izzvana, če so v tkivu izraženi eutopičnimi ali ektopičnimi adrenokortikalnimi hormonskimi receptorji (Lacroix et al. 2001, Lacroix 2009). Izraženost teh receptorjev lahko privede tudi do hiperplazije, proliferativne rasti in nastanka tumorjev (Bordeaux et al. 2001, Li et al. 2005).

V **tretjem poglavju** doktorske dizertacije so predstavljeni rezultati študije o izraženosti receptorja luteinizirajočega hormona (LHR), receptorja gastričnega zaviralnega peptide (GIPR) in treh vazopresinskih receptorjev ($V_{1a}R$, $V_{1b}R$ in V_2R). Poskusi so bili opravljeni na tkivu tumorjev NŽ, ki so bili pridobljeni z adrenalektomijo pri 23 psih s hiperkortizolizmom. Tkivo normalne NŽ je bilo uporabljeno za kontrolo. Količina mRNA omenjenih receptorjev je bila kvantificirana s PCR, prisotnost in lokalizacija receptorjev pa z imunohistokemijo.
Kvantitativna PCR (QPCR) analiza je pokazala, da povečano izločanje kortizola iz tumorjev NŽ ni povezano s povečano izraženostjo proučevanih receptorjev. Imuno-

histokemijski rezultati so bili zato toliko bolj presenetljivi, saj so dokazali izraženost GIPR in V_2R v tumorjih NŽ, ki izvirajo iz snopičaste plasti (zone fasciculate), medtem ko v normalnem tkivu NŽ ta receptorja nista bila prisotna. Predpostavili smo, da ta ektopična izraženost GIPR in V_2R igra vlogo v patogenezi tumorjev NŽ pri psih. *In vitro* testiranja na primarni celični kulturi iz bovinih adrenokortikalnih celic so pokazala, da ektopična izraženost GIPR in V_2R lahko izzove rast adrenokortikalnih celic in privede do nastanka tumorja (Mazzuco et al. 2006a, 2007).

Imunohistokemijsko barvanje LHR je bilo pozitivno pri zdravih NŽ in tumorjih NŽ. Pri zdravih NŽ je bila najmočnejsa imunopozitivna reakcija na meji med klobčičasto in snopičasto plastjo, intenzivnost obarvanosti se je zmanjševala v smeri proti mrežasti plasti (zona reticularis). Večina tumorjev NŽ se je obarvala homogeno, pri karcinomih pa smo opazili tudi heterogen tip reakcije. Značilnost tega tipa reakcije so otočki tkiv, ki so se obarvali imunopozitivno. Vzročno povezavo med izraženostjo LHR in nastankom tumorjev so raziskovali *in vitro* (Pabon et al. 1996, Mazzuco et al. 2006b). Dokazali so, da eutopična ali ektopična izraženost LHR v celični kulturi lahko privede do hiperkortizolemije, hiperplazije in benigne transformacije celic NŽ. Eutopična vloga LHR v patogenezi tumorjev NŽ pri psih ni izključena.

Ena od možnih razlag za povečano izločanje hormona kortizola iz tumorjev NŽ je, da je vzrok tega povečana izraženost genov, ki kodirajo encime, vključene v steroidogenezo. V adenomih NŽ pri ljudeh so dokazali povečano izraženost mRNA, ki kodira encim za cepitev stranske verige holesterola (CYP11A), 3β-hydroxysteroid dehydrogenazo (HSD3B), 17α-hydroxylazo/17,20-lyazo (CYP17) in 11β-hydroxylazo, tip 1 (CYP11B1) (Bassett et al. 2005). V **četrtem poglavju** so predstavljeni rezultati študije, v kateri smo primerjali izraženost steroidnega akutnega regulatornega proteina (StAR), CYP11A, HSD3B, CYP17, 21-hydroxylaze (CYP21), in CYP11B1 v 38 tumorjih NŽ in te primerjali z izraženostjo encimov v normalnih NŽ. Rezultati kažejo, da izraženost mRNA, ki kodira steroidne encime, ni signifikantno različna med tumorji NŽ in normalnimi NŽ. Zaključimo lahko, da povečano izločanje hormona kortizola iz tumorjev NŽ ni posledica povečane izraženosti genov, ki kodirajo steroidogenetične encime.

Analiza QPCR je pokazala, da je bila v adenomih NŽ relativno velika izraženost mRNA, ki kodira HSD3B. Ta ugotovitev upravičuje uporabo trilostana, kompetitivnega inhibitorja HSD3B, za zdravljenje hiperkortizolizma pri psih z inoperabilnim adenomom NŽ (Eastwood et al. 2003, Benchekroun et al. 2008).

Pri karcinomih NŽ smo v naši raziskavi ugotovili signifikantno zmanjšano izraženost gena za ACTH-R. Prisotnost ACTH-R naj bi bila osnovnega pomena za vzdrževanje visoko diferenciranega fenotipa celic skorje NŽ (Reincke et al. 1997). Nizka izraženost gena za ACTH-R in izguba heterozigotnosti (LOH) sta opisani pri malignih in hormonsko neaktivnih tumorjih NŽ pri ljudeh (Reincke et al. 1997, 1998). Predpostavimo lahko, da tudi pri psih s karcinomom NŽ zmanjšano izraženje ACTH-R gena privede do izgube diferenciacije celic NŽ in do hitre rasti

slabo diferenciranih celic NŽ (Reincke et al. 1997, 1998, Beuschlein et al. 2001). Količini mRNA za ACTH-R v adenomih NŽ in normalnih NŽ se nista razlikovali. To pomeni, da zmanjšana koncentracija ACTH pri psih z adenomom NŽ ni imela učinka na izraženost ACTH-R, kot bi bilo pričakovati glede na fiziološki odnos med ACTH in ACTH-R.

V humani medicini so dokazali funkcionalnost ACTH-R v tumorjih NŽ, saj je dajanje sintetičnega ACTH privedlo do povečanja koncentracije kortizola (Imai et al. 2001, Bertagna in Orth 1981). Pri psih s tumorjem NŽ so v preteklosti s pomočjo ACTH stimulacijskega testa že poskusili ločiti med adenomomi in carcinomi NŽ, vendar rezultati niso potrdili učinkovitosti tega pristopa (Peterson et al. 1982, Feldman 1985). Naša študija kaže na zmanjšano izraženost ACTH-R v karcinomih NŽ in predpostavimo lahko, da v teh tumorjh ne bi prišlo do povečanja koncentracije kortizola po dajanju sintetičnega ACTH. V klinični praksi bi zatorej ACTH stimulacijski test morda lahko bil v pomoč pri ločevanju med adenomi in karcinomi NŽ. Za izboljšanje zanesljivosti diagnoze tumorjev NŽ bi bilo priporočljivo tudi v veterinarski medicini uvesti t.i. sistem točkovanja, pri katerem o naravi tumorja NŽ ne odloča samo patološka slika tumorja, temveč tudi klinični znaki, velikost tumorja, rezultati endokrinih testov in postoperativno obdobje. Tak sistem je že dolga leta v uporabi v humani medicini (Weiss et al. 1989).

Potem ko smo postavili diagnozo hiperkortizolizem, je treba ločiti med hipofizno in adrenalno obliko bolezni, saj se zdravljenji teh oblik razlikujeta. V **petem poglavju** opisujemo uporabo osnovnega razmerja med kortizolom in kreatininom v urinu (RKKU) v te namene. Velikost osnovnega RKKU pri psih s hipofizno obliko hiperkortizolizma se ne razlikuje od RKKU pri psih s tumorjem NŽ, vendar se visoke vrednosti (>100 x 10^{-6}) pojavljajo izključno pri psih s tumorjem hipofize. Pomeni, da ima hiperplastična skorja NŽ večjo sposobnost izločanja kortizola kot tumor NŽ. Razlikovanje med dvema oblikama hiperkortizolizma je možno tudi s testom z visokim odmerkom dexametazona (HDDST). Študije, narejene z meritvami kortizola v krvni plazmi, so pokazale, da se pri večini psov s hipofizno obliko hiperkortizolizma koncentracija kortizola zmanjša za več kot 50% (Feldman et al. 1996). Naša raziskava potrjuje, da se omenjeni kriterij za zmanjšanje koncentracije kortizola v plazmi lahko uporablja tudi pri testiranju z RKKU, saj je pri psih s tumorjem NŽ največje zmanjšanje RKKU po deksametazonu znašalo 43%. Pri večini psov (34 od 49) s tumorjem NŽ je prišlo po dajanju deksametazona do povečanja RKKU. Podobne učinke so opisali tudi pri ljudeh s hormonsko aktivnimi tumorji NŽ (Flack et al. 1992). Vzrok tega paradoksalnega povečanja koncentracije kortizola ostaja zaenkrat nepojasnjen, nakazuje pa na neposreden učinek deksametazona na celice tumorja NŽ.

Trilostan je v zadnjem času postal najpomembnejše zdravilo za zdravljenje hiperkorti-

zolizma pri psih. Trilostan je kompetitivni zaviralec HSD3B in zmanjša koncentracijo kortizola v krvni plazmi. Odmerek trilostana določimo na podlagi klinične slike in rezultatov ACTH stimulacijskega testa, s katerim določimo rezervo skorje NŽ v trenutku, ko je delovanje trilostana največje (2 do 3 ure po dajanju zdravila) (Neiger et al. 2002, Ruckstuhl et al. 2002, Wenger et al. 2004). V retrospektivni študiji, ki je vključevala 63 psov s hipofizno obliko hiperkortizolizma (**šesto poglavje**), smo odmerek razglasili kot učinkovit, ko je bila koncentracija kortizola < 190 nmol/L in ko so najpogostejši znaki hiperkortizolizma, kot so poliurija in polidipsija ter polifagija, izginili. Osnovna koncentracija kortizola je bila v času, ko so psi prejemali učinkovit odmerek trilostana, signifikantno nižja kot pred zdravljenjem. Nasprotno pa je bila osnovna koncentracija ACTH v tem času signifikantno višja kot pred zdravljenjem, kar je posledica vpliva negativne povratne zveze na zmanjšanje koncentracije kortizola (Witt and Neiger 2004, Sieber-Ruckstuhl et al. 2006). V primeru kroničnega zmanjšanja koncentracije kortizola, kot recimo, pri Addisonovi bolezni, se osnovna koncentracija ACTH še močneje zveča (Peterson et al. 1996, Javadi et al. 2003). V skladu s tem smo pri petih psih, pri katerih je uporaba trilostana izzvala hipokortizolizem, izmerili močno povečano koncentracijo ACTH, ki je bila veliko večja kot pri psih, ki so prejemali učinkovit odmerek trilostana. Iz tega smo sklepali, da ima zdravljenje s trilostanom učinek na hipofizno-nadledvično os in da je merjenje osnovne koncentracije ACTH smiselno predvsem za odkrivanje predoziranja trilostana.

V **šestem poglavju** opisujemo še učinke zdravljenja s trilostanom na os renin-angiotenzin-aldosteron. Čeprav dajanje učinkovitega odmerka trilostana ni izzvalo signifikantnega zmanjšanja koncentracije aldosterona, pa je bila plazemska aktivnost renina v času dajanja učinkovitega odmerka signifikantno večja kot pred zdravljenjem. Podoben učinek trilostana je opisan tudi pri ljudeh (Komanicky et al. 1978). Povečano koncentracijo plazemske aktivnosti renina lahko razložimo kot posledico zmanjšanja koncentracije aldosterona, ki jo je izzvalo zdravljenje s trilostanom. Nepravilnosti na osi renin-aldosteron najnatančneje ponazarja razmerje med koncentracijo aldosterona in plazemsko aktivnostjo renina (Lim et al. 1999, Javadi et al. 2005, 2006). V naši raziskavi je bilo to razmerje med zdravljenjem signifikantno nižje kot pred njim, kar kaže na učinek trilostana na os renin-aldosteron.

Z merjenjem RKKU lahko z veliko zanesljivostjo diagnosticiramo hiperkortizolizem in spremljamo stanje kortizola med zdravljenjem z mitotanom ali po hipofizektomiji (Meij et al. 2002, Den Hertog et al. 1996). Glede na to, da je merjenje RKKU enostavno, neinvazivno in relativno poceni, bi bilo morebiti uporabno tudi za določitev učinkovitega odmerka trilostana in bi lahko nadomestilo ACTH stimulacijski test. V **sedmem poglavju** navajamo rezultate prospektivne študije, v kateri smo merili RKKU pri psih s hipofizno obliko hiperkortizolizma najprej pred in potem vsakih 14 dni vsaj 2 meseca po dosegu učinkovitega odmerka trilostana. Ugotovili smo, da se pri večini

psov RKKU med zdravljenjem s trilostanom ni zmanjšalo pod zgornjo normalno vrednost vsaj 2 meseca po dosegu učinkovitega odmerka. Navidezno protislovnost teh rezultatov lahko razložimo z mehanizmom delovanja trilostana, ki lahko poleg HSD3B zavre tudi druge steroidne encime, kot je, recimo, CYP11B1 (Pots et al. 1978, Sieber-Ruckstuhl et al. 2006). Pri ljudeh s hiperkortizolizmom, ki so prejemali trilostan, so v krvi izmerili zvečani koncentraciji 17α-OH-pregnenolona in dehidroepiandrostenediona (Komanicky et al. 1978, Sieber-Ruckstuhl et al. 2006). Meritev kortikoidov v urinu zajema večinoma kortizol, vendar tudi nekatere substance s podobno polarnostjo, kot so recimo prekurzorji, ki se kopičijo pri delovanju trilostana (Stolp et al. 1983). Domnevamo, da je na velikost RKKU vplivala prisotnost prekurzorjev v urinu psov in da se zato RKKU ni zmanjšalo kljub očitnemu kliničnemu učinku zdravila.
Pri nekaj psih se je RKKU med zdravljenjem s trilostanom zmanjšalo pod zgornjo referenčno mejo. Pri teh psih so se nekaj let po začetku zdravljenja pojavili bodisi znaki hipokortizolizma bodisi je ACTH stimulacijski test pokazal premajhno rezervo NŽ. Očitno je, da zmanjšanje RKKU nakazuje možnost hipokortizolizma, kar je zelo pomembno odkritje. Hipokortizolizem je klinično stanje, ki zahteva hitro ukrepanje in je brez ustrezne terapije lahko življenjsko nevarno. Predvsem zdravljenje z velikimi odmerki trilostana lahko privede do hipokortizolizma (Chapman et al. 2004, Sieber-Ruckstuhl et al. 2006, Ramsey et al. 2008). Patohistološke preiskave NŽ psov, ki so dobivali trilostan, so pokazale nekrozo in apoptozo celic skorje NŽ, katerih stopnja je odvisna od individualne občutljivosti in od dolžine dajanja trilostana (Reusch et al. 2007).

Ektopični ACTH sindrom pri človeku se pojavlja kot posledica različnih tipov tumorjev, katerih skupna značilnost je, da so večinoma majhni in da jih je zelo težko diagnosticirati tudi z modernim diagnostičnimi tehnikami (Newell-Price et al. 1998, Raffin-Sanson et al. 2000). V **osmem poglavju** opisujemo primer psa s hipofizno obliko hiperkortizolizma. Po odstranitvi hipofize (hipofizektomiji) se klinični znaki hiperkortizolizma niso izboljšali, RKKU in osnovna koncentracija ACTH sta ostala povečana. Natančna patohistološka preiskava hipofize tumorja ni potrdila, kar je privedlo do suma ektopičnega ACTH sindroma. Računalniška tomografija (CT) abdomna je pokazala tumor na področju trebušne slinavke, ki smo ga kirurško odstranili. Med celiotomijo smo poleg tumorja v pankreasu odkrili tudi nodularne spremembe na jetrih in povečano prilegajočo se bezgavko. S patohistološko preiskavo tumorja trebušne slinavke smo diagnosticirali neuroendokrin tumor, ki je metastaziral v prilegajočo se bezgavko in v jetra. Immunohištolosko obarvanje z ACTH je bilo tako v tumorju kot tudi v metastazah negativno, vendar to ektopičnega ACTH sindroma ne izključuje (Coates et al. 1986, Aniszewski et al. 2001). Navedeno lahko razložimo s tem, da je ektopično izločanje ACTH iz tumorja trenutno in v celicah ni zalog ACTH, ki bi privedle do pozitivne imunohistološke reakcije (Heitz et al. 1981).

Torej bo morala diferencialna diagnostika od ACTH odvisnega hiperkortizolizma v bodoče vključevati tudi ektopični ACTH sindrom. Predvsem pri psih z močno izraženimi kliničnimi znaki hiperkortizolizma, močno povečano koncentracijo ACTH in kortizola, ki se se zmanjša po dajanju visokega odmerka deksametazona, ter kadar s CT hipofize tumorja ni možno dokazati.

V **devetem poglavju** opisujemo klinični primer psa z relativno blagimi znaki hiperkortizolizma, rahlo povečanim RKKU in zmanjšano koncentracijo ACTH, kar kaže na tumor NŽ. V nasprotju s pričakovanji je ultrazvočna preiskava abdomna pokazala bilateralno hiperplazijo NŽ, zato smo postavili sum primarne adrenokortikalne hiperplazije, ki nastane kot posledica nenormalne izraženosti hormonskih receptorjev. RKKU se je nekaj ur po zaužitju obroka povečalo za več kot 50%, kar pri ljudeh nakazuje na od hrane odvisen hiperkortizolizem (Lacroix et al. 1999). Po hranjenju se je povečala tudi osnovna koncentracija kortizola, čeprav je koncentracija ACTH ostala neizmerljivo nizka. Diagnostiko od hrane odvisnega hiperkortizolizma smo nadaljevali s testom z oktreotidom, ki zavre povečanje GIP po obroku (De Herder et al. 1996, Croughs et al. 2000). Dajanje oktreotida pri našem pacientu je povečanje koncentracije kortizola po obroku popolnoma zavrlo, s čimer smo posredno dokazali ektopično izraženost GIPR v skorji NŽ in od hrane odvisen hiperkortizolizem. Dodaten argument za pravilnost diagnoze pri tem psu predstavlja dejstvo, da je bilo zdravljenje s trilostanom uspešno šele takrat, ko je pacient zdravilo dobil nekaj ur pred hranjenjem in ne na tešče kot običajno.

Dokončen dokaz za od hrane odvisen hiperkortizolizem bi bilo zvečanje koncentracije kortizola po dajanju infuzije s fiziološkimi koncentracijami GIP, izraženost mRNA za GIPR v snopičasti plasti NŽ in *in vitro* dokaz funkcionalnosti GIPR (De Herder et al. 1996, Lacroix et al. 1999). Žal nam tako infuzija z GIP kakor tudi tkivo NŽ nista bila na voljo.

Za merjenje RKKU zbirajo lastniki po navodilih prvi jutranji urin. Običajno psi pred tem še niso zaužili obroka. Glede na to, da se pri od hrane odvisnemu hiperkortizolizmu koncentracija kortizola zveča izključno po zaužitju obroka, je z regularnim testiranjem na hiperkortizolizem to diagnozo težko postaviti. Pri psih s slabo izraženimi znaki hiperkortizolizma, obojestransko povečanimi NŽ in zmanjšano koncentracijo ACTH je priporočljivo testiranje z merjenji RKKU po obroku.

Zaključki

- Povečano izločanje kortizola iz tumorjev NŽ ni posledica povečane izraženosti genov za LHR, GIPR, $V_{1a}R$, $V_{1b}R$ ali V_2R.

- Ektopična izraženost GIPR in V_2R in eutopična izraženost LHR v tumorjih NŽ, ki izvira iz snopičaste plasti (zone fasciculate) lahko igra vlogo v patogenezi tumorjev NŽ.

- Povečano izločanje kortizola iz tumorjev NŽ ni posledica povečane izraženosti genov encimov steroidogeneze.

- Signifikantno zmanjšana izraženost ACTH-R lahko pripomore k malignemu karakterju tumorjev NŽ.

- Splošno uveljavljeno načelo, da zmanjšanje plazemske koncentracije kortizola za manj kot 50% nakazuje adrenalni hiperkortizolizem, se lahko prenese tudi v diagnostiko z meritvami RKKU.

- Zdravljenje s trilostanom ima učinek na hipofizno-adrenokortikalno os kot tudi na os renin-angiotenzin-aldosteron.

- Merjenje RKKU ni priporočljivo kot nadomestilo za ACTH stimulacijski test za določanje učinkovitega odmerka trilostana.

- Merjenje osnovne koncentracije ACTH in RKKU pomaga pri ugotavljanju tega, ali obstaja možnost hipokortizolizma med zdravljenjem s trilostanom.

- Ektopični ACTH sindrom se pojavlja tudi pri psih in nanj je treba pomisliti v primerih močno povečane koncentracije kortizola, ki se ne zmanjša po dajaju visokega odmerka deksametazona.

- Diferencialna diagnostika od ACTH neodvisnega hiperkortizolizma mora v bodoče upoštevati tudi od hrane odvisen hiperkortizolizem.

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Acknowledgments/Dankwoord/Zahvala

Mijn eerste bezoek aan het Departement Geneeskunde van Gezelschapsdieren van de Faculteit Diergeneeskunde in Utrecht was op maandag, 1 november 1994; als roulant voor 6 maanden. De herinnering aan deze dag is nog levendig aanwezig: polikliniek endocrinologie, veel patiënten, prettige sfeer en De Meester zelf: Ad Rijnberk.

Prof. dr. Ad Rijnberk
Beste Ad. Je bent de 'godfather' van de endocrinologie, binnen en buiten de grenzen van Nederland. Ik prijs me gelukkig dat ik met jou mocht samenwerken. Ik bewonder je wetenschappelijk denken en de manier waarop je werkt. De laatste twee artikelen in het proefschrift zijn het bewijs dat jouw scholing mij geleerd heeft te denken buiten de bestaande kaders.

Na dit eerste bezoek aan Utrecht heb ik veel heen en weer gereisd (en geleefd) tussen Utrecht en Ljubljana, totdat ik in 2001 mijn plekje had gevonden. Een nieuw levensverhaal is in het centrum van Utrecht begonnen en is uitgegroeid tot het allermooiste dat mij kon overkomen. Tevens werd mij het voorstel gedaan om een promotieonderzoek te gaan doen. Dat heb ik met twee handen aangepakt. Ik heb genoten van het onderzoek en ik moest er aan herinnerd worden dat het tijd is om het promotieonderzoek af te ronden.

Mijn promotor, Prof. dr. Jan Rothuizen
Beste Jan. De adviezen die je aan mij gaf tijdens mijn promotietraject deden mij goed, niet alleen vanwege hun inhoudelijke waarde maar ook omdat jij mij het gevoel gaf vertrouwen in mij te hebben. Tevens wil ik je bedanken voor de mogelijkheden die ik, dankzij jouw inzet, op de Faculteit heb gekregen.

Mijn co-promotor, Dr. Hans Kooistra
Beste Hans. Onze samenwerking en vriendschap gaan ver terug in de tijd. Jouw briljant wetenschappelijk denken en je tomeloze inzet voor de endocrinologie op alle velden hebben mijn bewondering. Ik wil je bedanken voor je vertrouwen, voor al je inspanningen, voor de kansen, voor je coaching binnen en buiten het onderzoek, voor

je steun en voor je begrip. Jij hebt een grote invloed op mijn carrière gehad en ik hoop dat wij samen nog vele endocrinologische uitdagingen zullen aangaan.

Mijn co-promotor, Dr.ir. Jan Mol
Beste Jan. Onder jouw deskundige leiding werden alle hormoonanalyses en het moleculair onderzoek gedaan. Jouw bijdrage is zó waardevol en onmisbaar. Zeer bedankt ook voor je vakkundige commentaar op mijn manuscripten. Gelukkig houdt onze samenwerking niet op als dit promotietraject is beëindigd.

Auke Schaefers-Okkens
Beste Auke. Al was je dit keer niet direct betrokken bij het promotieonderzoek, je luisterend oor was er altijd voor me. Ik waardeer het enorm. Jij weet wat balans in het leven is en het was goed dat ik op bepaalde momenten hieraan herinnerd werd.

Mijn paranimf, Jeffrey de Gier
Beste Jeffrey. Wij zijn kamergenoten vanaf mijn eerste werkdag op de Kliniek. Wat ik bijzonder fijn vind is dat ik door onze vele persoonlijke gesprekken het leven in Nederland beter heb leren begrijpen. In 2005 hebben onze eerstgeborenen, Renzo en Zala, besloten om op dezelfde dag ter wereld te komen. Sindsdien delen wij natuurlijk ook alle `ups` van het ouderschap. Ik verheug me al op de `film` (jouw promotie) die wij in de nabije toekomst gaan bekijken.

Mijn paranimf, Lars Slingerland
Beste Lars. Onze vriendschap ontstond tijdens je promotieonderzoek op de Kliniek. Ik vind het heerlijk om met jou over het onderzoek te praten. Je begrijpt mij goed. Ik mis je op de werkvloer en tegelijkertijd voelt het goed om te zien dat je geniet van je nieuwe baan. Ik ben bijzonder blij dat je mijn paranimf wil zijn.

Mijn collega bij de discipline Endocrinologie, Jenny Buijtels
Beste Jenny. Ik wil je bedanken voor het overnemen van de poli`s endocrinologie en de telefonische spreekuren. Dankzij jou kon ik met een gerust hart de nodige tijd aan het promotieonderzoek besteden. Ik wens je veel succes met de afronding van je eigen proefschrift.

De chirurgen Freek van Sluijs en Elaine Naan
Beste Freek en Elaine. Ik bewonder jullie geduld en chirurgische vaardigheden. Degene die ooit gezegd geeft dat de bijnier(tumor) niet gemaakt was om verwijderd te worden, heeft jullie niet zien opereren. Zonder jullie expertise zou het eerste gedeelte van dit proefschrift niet tot stand zijn gekomen.

Björn Meij
Beste Björn. Met jouw hulp is het ectopische ACTH secretie syndroom bij de hond voor het eerst bewezen. Dank je voor je inzet in de puzzelfase van deze casus, voor het vertrouwen en je opbouwend commentaar op het manuscript.

Miriam Kool
Beste Miriam. Wij leerden elkaar kennen tijdens je onderzoekstage die uitgegroeid is tot een promotieonderzoek. Ik wens je heel veel succes en verheug me op onze toekomstige samenwerking. Ik wil je bedanken voor je hulp met de Western blots.

Adri Slob
Beste Adri. De creativiteit in het lab is op je lijf geschreven. Met de in vitro experimenten moesten wij enkele tegenslagen incasseren; uiteindelijk zijn wij er alleen maar wijzer van geworden. Dank je dat je mij *(een olifant*) in de *glazen kast* liet werken.

Monique van Wolferen
Beste Monique. Dank je dat je bereid was op korte termijn de StAR analyse uit te voeren zodat het manuscript op tijd klaar was.

Jeanette Wolfswinkel
Beste Jeanette. Samen met Monique en Adri heb je `mijn` studenten tijdens hun onderzoekstages begeleid. Ik ben zeer dankbaar voor je betrokkenheid en interesse.

Studenten en collega-dierenartsen: Vera Kars, Viktor van Marrewijk, Debbie Arts en Margit van den Heiligenberg
Beste Vera, Viktor, Debbie en Margit. Jullie hebben een belangrijke bijdrage aan mijn proefschrift geleverd. Ik heb met veel plezier met jullie gewerkt en ik heb prettige herinneringen aan onze discussies, in het bijzonder over de resultaten. Bedankt voor jullie werk in het lab.

Afdeling Diagnostische beeldvorming
Beste allemaal. Jullie zijn een belangrijke steen in de mozaïek van dit proefschrift.
Beste George, Edoardo, Susanne, Leonie, Natasja, Kim en Stefanie. Telkens als ik (weer) met een vraag over de bijnieren/hypofyse langs kwam, kon ik op jullie deskundigheid rekenen. Mijn dank hiervoor.

Afdeling Anesthesiologie
Beste allemaal. Altijd adequaat in het toedienen van `slaapmiddelen` en altijd attent. Bedankt voor jullie super service.

Dierverzorgers (IZA en verpleegafdeling) en receptiemedewerkers
Beste allemaal. Ik wil jullie bedanken voor de goede zorgen voor de patiënten en de eigenaren.

Medewerkers van het laboratorium UVDL
Bedankt voor jullie hulp tijdens de trilostane studie, vooral voor het zorgvuldig noteren en opslaan van het materiaal. Ondanks de vele monsters en het extra werk ben ik steeds met een glimlach ontvangen en dat doet mij goed.

Bruce Belshaw
Dear Bruce. We learned to know each other when I visited Utrecht for the first time. I still remember your lectures, your notes and I am grateful for your coaching. I have learned so much from you. Besides that, you corrected my English language and I could not think of a better person to do that. Thank You.

Prof.dr. Vojteh Cestnik
Spoštovani Profesor. Najina naklonjenost k endokrinologiji naju je povezala v času mojega delovanja na Veterinarski fakulteti v Ljubljani. Bil ste moj mentor, strokovna opora in zaveznik. Veseli me, da ste se odzvali povabilu na zagovor doktorata in hvaležna sem vam, da ste bili vedno na moji strani.

Borut: pesnik, prijatelj in filozof
Grafi, slike in slovenski tekst so šele s tvojo pomočjo dobili obliko, ki jo je bilo mogoče *na ogled postavit*. Hvala Ti.

Moji prijatelji: Tanja in Dušan, Urška in Jožko, Andrej in Maja, Nataša in Valter
Kljub temu, da se ne vidimo pogosto, je vsako naše srečanje samo nadaljevanje prejšnjega. Lepo je imeti takšne prijatelje.

Moja starša, Ria in Ivo
Verjetno ni lahko, ko se edina hči odloči, da bo živela 1200 km stran in si bo tam ustvarila družino. Vendar me pri tem nikoli nista poskušala ustaviti, nikoli postavljala vprašanj. Vedno samo pomagala in vzpodbujala. V imenu ljubezni, za katero sem vama neskončno hvaležna.

Zala in Tajda, mijn meisjes
Materinstvo in doktorat ni enostavna kombinacija. Trudila sem se, da ne bi zamudila nobenega vajinega podviga, nobene vajine lumparije in biti z vama prav vsak trenutek, ko sta me potrebovali. Vedita, da od vseh nazivov, ki sem jih in jih morda še bom, dobila v življenju, mi je najdražji ta, s katerim me kličeta samo vidve: MAMA.

Jeroen, moja Ljubezen

Met jou kan ik lachen. Met jou wil ik alles delen. Aan jou vraag ik als ik het niet meer weet. Aan jou kan ik alles vertellen. Met jou ga ik oud worden. Met jou wil ik leven. Jij *bent* mijn leven.

Curriculum Vitae

The author of this thesis was born in Slovenj Gradec, Slovenia, on January 16th 1968. She attended primary school in Mežica and Novo mesto and graduated from the Gymnasium in Novo mesto in 1986. In the same year she started to study veterinary medicine at the Veterinary Faculty in Ljubljana, Slovenia. After graduation in 1993, she completed an internship at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine in Utrecht. Thereafter she returned to Slovenia and was employed at the University Clinic for Small Animals in Ljubljana until 2001. During this period she completed a PhD thesis about the use of a progesterone receptor antagonist in dogs. Thereafter she moved to The Netherlands again to start a residency in companion animal internal medicine (2001-2004) at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine in Utrecht. In 2009 she became a staff member of this department.
The author is married to Jeroen van Veenendaal. In 2005 she became mother of Zala and in 2008 her daughter Tajda was born.

De schrijfster van dit proefschrift werd op 16 januari 1968 geboren in Slovenj Gradec (Slovenië). Zij doorliep de lagere school in Mežica en Novo mesto en na het behalen van haar diploma op het Gymnasium in Novo mesto in 1986, startte zij met de studie Diergeneeskunde aan de Veterinaire Faculteit in Ljubljana, Slovenië. Na voltooiing van de studie in 1993 werd zij roulant bij het Departement Geneeskunde van Gezelschapsdieren van de Faculteit Diergeneeskunde in Utrecht. Daarna keerde zij terug naar Slovenië en werkte op de Universtiteitskliniek voor Gezelschapsdieren in Ljubljana tot eind 2001. Tijdens deze periode heeft de schrijfster een proefschrift over het gebruik van progesteron receptor antagonisten bij de hond afgerond. Nadien heeft zij de opleiding tot specialist interne diergeneeskunde doorlopen (2001-2004) bij het Departement Geneeskunde van Gezelschapsdieren van de Faculteit Diergeneeskunde in Utrecht. Sinds 2009 is zij verbonden als staflid aan dit departement.
De schrijfster is getrouwd met Jeroen van Veenendaal en heeft twee dochters, Zala (2005) en Tajda (2008).

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