Na^+,K^+-ATPase content in skeletal muscle of dogs with pituitary-dependent hyperadrenocorticism

B.A. Schotanus[1], B.P. Meij[2], I.H.C. Vos[1], H.S. Kooistra[2], M.E. Everts [1]

Departments of Pathobiology[1] and Clinical Sciences of Companion Animals[2], Faculty of Veterinary Medicine, Utrecht University, 3508 TD Utrecht, the Netherlands.

Running head:

Na^+,K^+-ATPase in muscle of dogs with Cushing's disease

Address for reprint requests and other correspondence

I.H.C. Vos, Department of Pathobiology, Division of Anatomy and Physiology, Faculty of Veterinary Medicine, Utrecht University, PO Box 80.158, 3508 TD Utrecht, the Netherlands.

Phone: +31 30 2535313

Fax: + 31 30 2539233

E-mail: I.Vos@vet.uu.nl

ABSTRACT

Several hormones regulate Na^+,K^+-ATPase content in the muscle cell membrane, which is essential for maintaining muscle cell excitability. Chronic glucocorticoid excess is associated with muscle weakness and reduced endurance. We hypothesized that chronic glucocorticoid excess affects Na^+,K^+-ATPase content in canine skeletal muscle, and contributes to reduced endurance and muscle weakness associated with pituitary-dependent hyperadrenocorticism (PDH) in dogs. Therefore Na^+,K^+-ATPase content in skeletal muscle was evaluated before and after hypophysectomy and hormone replacement (cortisone and L-thyroxin) in dogs with PDH (n=13) and healthy controls (n=6). In addition, baseline and exercise-induced changes in plasma electrolyte concentrations and acid-base balance were evaluated before and after hypophysectomy in dogs with PDH. Na^+,K^+-ATPase content of gluteal muscle in dogs with PDH was significantly lower than in control dogs (201±13 vs. 260±8 pmol/g wet weight; $P<0.01$). Similar differences were found in palatine muscle. After hypophysectomy and on hormone replacement, Na^+,K^+-ATPase was increased (234±7 pmol/ g wet weight). Both plasma pH and base excess in dogs with PDH (7.44±0.01; 1.7±0.6 mmol/l, respectively) were significantly higher ($P<0.05$) than after hypophysectomy and hormone replacement (7.41±0.01; -0.2±0.4 mmol/l, respectively). Exercise induced respiratory alkalosis, but did not result in hyperkalemia in dogs with PDH. In conclusion, chronic glucocorticoid excess in dogs with PDH is associated with decreased Na^+,K^+-ATPase content in skeletal muscle. This may contribute to reduce endurance in canine PDH, although dogs with PDH did not exhibit exercise-induced hyperkalemia. Na^+,K^+-ATPase content normalized to values statistically not different from healthy controls after hypophysectomy and hormone replacement.

Keywords: *Cushing's disease, Hypophysectomy, Glucocorticoids, K^+ homeostasis, Exercise*

INTRODUCTION

Pituitary-dependent hyperadrenocorticism (PDH, Cushing's disease) is a common endocrinopathy in dogs and is an interesting model for Cushing's disease in humans (1). The central hallmark of PDH is chronic excess of glucocorticoids, caused by excessive secretion of adrenocorticotropin (ACTH) by a pituitary adenoma. Clinical manifestations in dogs with PDH include polyuria, polydipsia, polyphagia, reduced endurance, and muscle weakness.

Na^+,K^+-ATPase is an important electrogenic pump for maintaining cellular excitability in the plasma membrane of skeletal muscle cells. Action potentials are associated with influx of Na^+ and efflux of K^+, thereby disturbing the Na^+,K^+-gradients and decreasing cellular excitability. Activated Na^+,K^+-ATPase restores muscle cell excitability by exchanging intracellular Na^+ ions for extracellular K^+ ions. If activation or concentration of Na^+,K^+-ATPase is insufficient, muscle excitability will decrease and hyperkalemia may develop (2), in particular during prolonged exercise, leading to the inability to produce force (3).

Concentration and activation of Na^+,K^+-ATPase is regulated by hormonal and non-hormonal factors (3). Thyroid hormones are potent up-regulating factors of the Na^+,K^+-ATPase content in human (4) and rat (5,6) muscle. Hypothyroidism in dogs is associated with decreased Na^+,K^+-ATPase content in muscle (2). Elevated plasma aldosterone concentration (7,8) and K^+ deficiency (7) decrease Na^+,K^+-ATPase content in skeletal muscle of rats, whereas growth hormone (GH) increases the Na^+,K^+-ATPase content in K^+ deficient and control rats (9), but not in hypophysectomized rats (10). Treatment with glucocorticoids in humans (11,12) and rats (13) is associated with a rise in Na^+,K^+-ATPase content in muscle. There are no reports on the effect of glucocorticoids on the content of Na^+,K^+-ATPase in muscle of dogs.

Based on previous studies in humans and rats, we hypothesized that chronic glucocorticoid excess increases Na^+,K^+-ATPase content in canine skeletal muscle in dogs with PDH. To test this hypothesis, the content of Na^+,K^+-ATPase in skeletal muscle was evaluated before and after hypophysectomy and hormone replacement (14) in dogs with PDH and healthy controls. In addition, baseline and exercise-induced changes in plasma Na^+ and K^+ concentration and acid-base balance (2) were evaluated before and after hypophysectomy and hormone replacement in dogs with PDH.

MATERIALS AND METHODS

Dogs.

Thirteen dogs (four males and nine females) with PDH were included in this study. The dogs were of various breeds and body weight and age ranged from 6 to 50 kg, and from 3 to14 years, respectively. Diagnosis of hyperadrenocorticism was based upon history, physical examination, biochemistry and elevated urinary corticoid/creatinine ratios (UCCR) (range 13.5 to 598 $x10^{-6}$, reference < 10 x 10^{-6}) in two consecutive morning urine samples. After collection of the second urine sample, the dogs received three oral doses of 0.1 mg dexamethasone per kg body weight at 8h intervals. The next morning, a third urine sample was collected. The UCCR in the third urine sample was less than 50% of the mean UCCR in the first two samples in eleven dogs and PDH was diagnosed (15). Suppression in the third urine sample was less than 50% in two dogs; diagnosis of PDH was confirmed by elevated plasma ACTH concentrations (251 and 412 pg/ml, respectively), ultrasonography of the adrenals, and pituitary imaging.

All dogs underwent transsphenoidal hypophysectomy as described previously (14). Hormone replacement therapy consisted of substitution with L-thyroxin (15 μg/kg twice daily) and cortisone acetate (1 mg/kg twice daily). The dosage of cortisone acetate was gradually reduced over a period of four weeks to 0.25 mg/kg twice daily. Desmopressin, a synthetic arginine-vasopressin (AVP) analogue, was administered subconjunctivally for two weeks (16), and continued for up to ten weeks in five dogs because of prolonged central diabetes insipidus.

The dogs returned for follow-up investigation ten weeks after hypophysectomy. Three dogs were excluded from follow-up. One dog had incomplete pituitary removal based on a UCCR of 7.6 x 10^{-6}, measured 24h after final cortisone acetate medication and two dogs died within four

weeks after surgery. The UCCR in the remaining dogs (n=10) had decreased to $0.8 \pm 0.2 \times 10^{-6}$ and all clinical signs of hyperadrenocorticism had resolved.

Healthy dogs (three males and three females, weight 16 to 23 kg, age 2 to 10 years) of various breeds served as controls for the Na^+,K^+-ATPase content in skeletal muscle.

The Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, approved the protocol of the study. Owners of the dogs agreed by written consent.

Hormone determinations.

Blood samples were taken from the jugular vein and transferred to ice-chilled EDTA-coated tubes for measurement of plasma cortisol, ACTH, aldosterone, plasma renin activity (PRA), insulin-like growth factor-1 (IGF-1), and GH. For determination of plasma concentrations of thyrotropin (TSH) and thyroxine (T_4) blood was transferred into ice-chilled heparin-coated tubes. Samples were centrifuged for 12 min by 4°C and stored at –25°C until assayed. In hypophysectomised dogs blood was sampled 12h and 24h relative to the last dosage of L-thyroxin and cortisone acetate, respectively.

Plasma cortisol concentration was measured with a radioimmunoassay (RIA) validated for the dog (Diagnostic Products Corporation (DPC), Los Angeles, CA). Intra- and inter-assay coefficients of variation ranged from 3.0 to 5.1% and from 4.0 to 6.4%, respectively. The sensitivity of the assay was 5.5 nmol/l.

Plasma ACTH concentration was measured using a two-site immunoradiometric assay (IRMA) (Nichols Institute, Wijchen, Netherlands). The antiserum is highly specific for $ACTH_{1-39}$. Intra- and inter-assay coefficients of variation were 3.2 and 7.8%, respectively, and the sensitivity was 1 pg/ml. The antiserum neither cross reacts with α-MSH nor with ACTH precursors (17,18).

Aldosterone was extracted from 1 ml plasma with dichloromethane. The extract was evaporated, redissolved in assay buffer, and aldosterone was quantitated by RIA (ICN Pharmaceuticals Inc, Costa Mesa, CA) (19).

PRA was measured in the presence of inhibitors of angiotensinases and angiotensin-1 converting enzyme. After incubation, the samples were deproteinized with acetone/ammonia 4mol/l (9:1, v/v) and centrifuged. Supernatants were evaporated and dissolved in assay buffer. Angiotensin-1 was measured by RIA (using an antibody from Peninsula Laboratories Inc, Belmont, CA and a tracer from NEN Life Sciences Products, Boston, MA) (20). Aldosterone/PRA ratio was calculated to discriminate between renin-dependent and independent changes of aldosterone.

Plasma IGF-1 concentration was measured by a heterologous RIA as described previously (21) with intra- and inter-assay coefficients of variation of 4.7 and 15.6%, respectively.

Plasma GH concentration was measured by a homologous RIA as described previously (22). Intra- and inter-assay coefficients of variation were 3.8 and 7.2%, respectively. The lowest detectable amount of GH was 0.5 ng/ml.

Plasma TSH concentration was determined by a homologous solid-phase, two-site chemiluminescent enzyme immunometric assay (Immulite canine TSH, DPC) according to the manufacturer's instructions. Intra-assay coefficients of variation were 5, 4, and 3.8% at TSH levels of 0.20, 0.50, and 2.6 ng/ml, respectively. Inter-assay coefficients of variation were 6.3 and 8.2% at TSH levels of 0.16 and 2.8 ng/ml, respectively. The lowest detectable amount of TSH was 0.03 ng/ml. Upper limit of the reference range for plasma TSH concentration in euthyroid dogs is 0.6 ng/ml (23).

Plasma total T_4 concentration was determined by a homologous solid-phase, chemiluminescent enzyme immunoassay (Immulite canine Total T_4, DPC) according to the

manufacturer's instructions. The assay was validated for the dog. Intra-assay coefficients of variation were 13.8 and 8.2% at T_4 levels of 8 and 25 nmol/l, respectively. The lowest detectable amount of T_4 was 1.5 nmol/l (24).

Urinary corticoid concentration was measured by RIA as described previously (15). 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 (Jaffé kinetic method, initial rate reaction) and the UCCR was calculated (15).

Muscle biopsies.

Muscle biopsies were taken from the medial gluteal and palatine muscle in six healthy dogs and all dogs with PDH. Muscle biopsies were collected surgically (palatine muscle) or with a biopsy needle (gluteal muscle; 14 gauge, 16 cm; Medical Device Technologic) during the hypophysectomy procedure, and stored at –80ºC. A second needle biopsy of the medial gluteal muscle was collected ten weeks after hypophysectomy in ten dogs that were under anaesthesia for post-operative pituitary imaging.

[^{3}H]-ouabain binding.

Muscle biopsies, weighing 2-13 mg, were used to determine the concentration of Na^+,K^+-ATPase by ^{3}H-ouabain binding as described previously (25). The justification to apply this method to quantify the Na^+,K^+ pump content as an estimate of Na^+,K^+-ATPase activity in dog skeletal muscle is based on the methodological studies reviewed in Hansen and Clausen (26) and its previous validation on muscle tissue of hypothyroid dogs (2). During the measurement biopsies were kept in baskets with a gas inlet attached to the bottom allowing continuous gassing with air to ensure agitation. Biopsies were pre-washed twice for 10 min at 37ºC in unlabeled tris-

vanadate-sucrose buffer (tris-base-vanadate (1 mM), trisbuffer (20 mM), $MgSO_4$ (3 mM) and sucrose (250 mM, pH 7.3). Biopsies were incubated for 120 min in this buffer with 0.6 μCi/ml ^{3}H-ouabain ([^{3}H(G)]-ouabain, 18 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA; purity by radiochemical analysis 95%) and unlabeled ouabain (Sigma-Aldrich Chemie, Steinheim, Germany) added to a total ouabain concentration of 1 μM. To correct for non-specifically bound ^{3}H-ouabain, samples were also incubated in buffer containing 1 mM unlabeled ouabain. Washout occurred during four periods of 30 min at 0ºC in ice-cold, unlabeled buffer. After washout, biopsies were blotted on a filter, weighed and put in a pony vial. An aliquot of 0.5 ml 5% trichloroacetic acid with 0.1 mM unlabeled ouabain as a carrier was added. The biopsies were allowed to soak overnight at 4ºC. The next day scintillation cocktail (3 ml) was added and specific activity of ^{3}H-ouabain was measured by liquid scintillation counting (LSC). Specific activity measured by LSC was used to calculate the amount of ^{3}H-ouabain taken up and retained after washout. From that the concentration Na^+,K^+-ATPase was calculated, corrected and expressed as pmol/g wet weight.

Exercise test.

Dogs with PDH were subjected to an exercise test, before and 10 weeks after hypophysectomy to investigate exercise mediated plasma changes. Exercise consisted of a 5-min-walk on a treadmill at a speed of 4.5 km per hour**.** Prior to the exercise test, all dogs were familiarized with the treadmill (2). At 2 and 0 min before exercise, and 0, 2 and 10 min after exercise blood samples were taken from the jugular vein and collected in tubes preheparinized with 60 IU dry electrolyte balanced lithium/sodium heparin (Radiometer Medical A/S, Copenhagen, Denmark). Plasma concentrations of Na^+ and K^+, pH, pO_2, pCO_2, base excess (BE),

and active bicarbonate were measured with the combined blood gas and electrolyte analyser (ABL505, Radiometer Copenhagen, Denmark).

Statistics.

Statistical analysis was performed using SigmaStat version 2.0 (SPSS Inc, Chicago, IL, USA). Plasma hormone concentrations were calculated from two blood samples obtained with an interval of one hour. Differences in plasma hormone concentrations before and after hypophysectomy were assessed by two-tailed Student's *t*-test for paired observations. Wilcoxon Signed Rank Test was used for nonparametric data, i.e., plasma concentrations of ACTH, GH, and IGF-1. The ouabain binding capacity of skeletal muscle of dogs with PDH before and after hypophysectomy, and of the control dogs was evaluated with ANOVA with Student-Newman-Keuls correction as post-hoc test. Plasma concentrations measured in the exercise test, before and after hypophysectomy, were assessed by Student's *t*-test for paired observations and repeated measurement ANOVA. Correlations within groups were assessed by the Spearman correlation test. Undetectable values were assumed equal to the detection limit. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SEM or as median and range.

RESULTS

^{3}H-ouabain binding capacity.

Na^+,K^+-ATPase content in the gluteal muscle of dogs with PDH was significantly lower than that of control dogs (201 ± 13 vs. 260 ± 8 pmol/g wet weight; $P = 0.006$; Figure 1). Na^+,K^+-ATPase content in gluteal muscle ten weeks after hypophysectomy increased (234 ± 7 pmol/g wet weight) compared to preoperative values ($P < 0.05$), and was not different from the Na^+,K^+-ATPase content in healthy dogs (Figure 1). Na^+,K^+-ATPase content in palatine muscle was also significantly lower in dogs with PDH than in control dogs ($P < 0.001$; Figure 2). In both groups Na^+,K^+-ATPase content was significantly higher in palatine than in gluteal muscle (PDH: 340 ± 17 vs. 201 ± 13 pmol/g wet weight, $P < 0.001$ and controls: 484 ± 20 vs. 259 ± 8 pmol/g wet weight, $P < 0.01$; Figure 2).There were no significant correlations between the Na^+,K^+-ATPase content and any of the measured hormones within groups

Hormone concentrations.

Plasma ACTH and cortisol concentrations and UCCR were significantly lower after hypophysectomy compared to preoperative values (Table 1). The Ald/PRA ratio was not different before and after hypophysectomy. The plasma TSH concentration was undetectable and the plasma T_4 concentration increased significantly after hypophysectomy and on L-thyroxin substitution. Plasma GH concentration was undetectable (< 0.5 ng/ml) after hypophysectomy in all but two dogs. Plasma IGF-1 concentration was significantly lower after hypophysectomy compared to preoperative values ($P < 0.05$).

Exercise test.

The plasma Na^+ concentration was significantly higher ($P < 0.05$) in dogs with PDH compared to the values after hypophysectomy (Figure 3A), whereas plasma K^+ concentration was not different (Figure 3B). No significant changes in plasma K^+ or Na^+ concentrations were measured immediately (t=0 min) after exercise compared to baseline pre-exercise values, both before and after hypophysectomy. The plasma K^+ concentration before hypophysectomy was decreased at 2 and 10 min after exercise ($P < 0.05$), whereas after hypophysectomy, the plasma K^+ concentration was only decreased at 10 min after exercise ($P < 0.05$) (Figure 3B).

Plasma pH and Base Excess (BE) in dogs with PDH were significantly higher ($P < 0.05$) than the corresponding values after hypophysectomy (Figure 3C, 3D). Plasma pCO_2 and plasma HCO_3^- concentrations were not significantly different between the two groups (Figure 3E, 3F). Plasma pH was increased (Figure 3C, $P < 0.001$) and plasma pCO_2 was decreased (Figure 3E, $P < 0.05$) immediately after exercise in both groups. The exercise-induced changes in pCO_2 appeared to be larger in the dogs with PDH than those in dogs after hypophysectomy, however the difference was not significant ($\Delta pCO_2 = -0.8 \pm 0.2$ in dogs with PDH vs. -0.4 ± 0.1 kPa after hypophysectomy; $P = 0.17$). Following exercise, recovery to baseline pH occurred in 2 min in the dogs with PDH, whereas after hypophysectomy recovery occurred at 10 min after exercise. Exercise-induced changes in plasma K^+ concentration did not correlate with muscle Na^+,K^+-ATPase content or with exercise-induced changes in pCO_2.

DISCUSSION

The results of this study demonstrate that the Na^+,K^+-ATPase content is decreased in the gluteal and palatine muscle of dogs with pituitary-dependent hyperadrenocorticism, suggesting that in dogs glucocorticoid excess decreases Na^+,K^+-ATPase content in skeletal muscle throughout the body. The reduction in absolute number of pumps in the body may even be larger than the reduction in the Na^+,K^+-ATPase content expressed per gram wet muscle weight considering that dogs with PDH have skeletal muscle atrophy. Na^+,K^+-ATPase content was enhanced to the normal range in the dogs after hypophysectomy and hormone replacement therapy with L-thyroxin and cortisone acetate.

Hormonal regulation of Na^+,K^+-ATPase content

Long-term regulation of Na^+,K^+-ATPase is exerted by several hormones, each with a different potency (6). Plasma ACTH and cortisol concentrations were increased in dogs with PDH. Administration of exogenous glucocorticoids up to 14 days has been shown to increase the Na^+,K^+-ATPase content in skeletal muscle of humans and rodents (11-13) and it was hypothesized that the Na^+,K^+-ATPase content was increased in muscles of dogs with PDH. Unexpectedly, decreased Na^+,K^+-ATPase contents were found in skeletal muscle of dogs with PDH. The discrepancy between our findings and findings in previous studies may be explained by the fact that PDH in dogs is associated with long-term changes in the concentrations of a number of circulating hormones. First, long term exposure to glucocorticoids, as investigated in our study, may have different effects on Na^+,K^+-ATPase content than relatively short-term exposure. Similarly, GH secretion is acutely stimulated by administration of glucocorticoids such as dexamethasone (27,28). However, long term cortisol treatment suppresses GH secretion

through increased somatostatin tone (29). Second, endogenous glucocorticoids excess may also have mineralocorticoid activity (30) that would reduce Na^+,K^+-ATPase content probably due to muscular K^+deficiency (3,8). Under physiological conditions cortisol is converted, by the enzyme 11-β-hydroxysteroid dehydrogenase type II, into its keto-analogue cortisone which cannot bind to the mineralocorticoid receptor (31). However, with excess cortisol the capacity of 11-β-hydroxysteroid dehydrogenase type II may be insufficient.

Hypophysectomy and subsequent medication with cortisone acetate and L-thyroxin for ten weeks resulted in an increase of the Na^+,K^+-ATPase content. The dosage of cortisone was gradually reduced from 1 mg/kg to 0.25 mg/kg twice daily over four weeks. The reduction in plasma cortisol concentration after hypophysectomy, as compared to dogs with PDH, may have contributed to the increase in Na^+,K^+-ATPase content. However, as our treatment dosage following hypophysectomy may have resulted in slightly elevated plasma cortisol concentrations, compared to healthy dogs. This may explain why Na^+,K^+-ATPase content in dogs after hypophysectomy and on hormone replacement was not equal to that in healthy controls.

Thyroid hormone may overrule the effect of cortisol on Na^+,K^+-ATPase. This may be more evident in dogs than in humans. Chronically elevated cortisol levels have an inhibitory effect on TSH production and thyroid hormone metabolism (32). In addition, a pituitary adenoma may compress surrounding hypophyseal tissue and thus interfere with TSH secretion. Indeed, in our dogs with PDH plasma total T_4 concentration was decreased. This corresponds with a study by Ferguson and Peterson (33) describing significant decreases in total and free T_4 in dogs with hyperadrenocorticism. In contrast studies in humans give equivocal results, and for example may even show an increase in free T_4 in patients with Cushing disease, followed by a fall after surgery (34). As thyroid hormones are important regulators of Na^+,K^+-ATPase content in skeletal muscle

(2-6), the possibly stimulating effect of corticosteroids may be overruled by the chronically low thyroid hormone concentrations in dogs with PDH, ultimately resulting in decreased Na^+,K^+-ATPase contents. Similarly, because hypophysectomy and hormone replacement therapy was associated with normalization of plasma T_4 concentration, this may have contributed to restoration of Na^+,K^+-ATPase content.

Growth hormone also regulates skeletal muscle Na^+,K^+-ATPase content. Injection of human GH increased Na^+,K^+-ATPase content in skeletal muscle in normal rats (9), but had no effect in hypophysectomized rats (10). After hypophysectomy Na^+,K^+-ATPase tended to increase, despite the fact that plasma GH concentrations remained low. This suggests that GH did not play a role in restoration of the Na^+,K^+-ATPase content after hypophysectomy.

The Na^+,K^+-ATPase consists of three subunits (α, β, and γ) with different isoforms, combination of which results in different isozymes. In rat muscle, the majority of Na^+,K^+-ATPase contains the α_2-isoform with high affinity for ouabain (8,13). In the dog the relative Na^+,K^+-ATPase isozyme abundance in skeletal muscle is not known. Moreover, as hormones have variable influences on the individual isoforms of the Na^+,K^+-pump (35), this might also contribute to the discrepancy in Na^+,K^+-ATPase response of rats versus dogs. In all dogs biopsies from palatine muscle contained a higher content of Na^+,K^+-ATPase than the medial gluteal muscle. A relationship with type and function of the muscle is considered. The palatine muscle consists predominantly of fast twitch fibers, whereas the gluteal muscle appears to be mixed. This corresponds with the fact, described by Khaleeli et al.(36), that fast muscle fibre types are affected more by corticosteroid myopathy: Na^+,K^+-ATPase content decreased by 30% in the palatine muscle and by 23% in the gluteal muscle. However, the exact mechanism underlying the difference in Na^+,K^+-ATPase content between gluteal and palatine muscle needs further investigation.

Treadmill exercise test

Dogs with PDH had significantly higher plasma Na^+ concentrations than after hypophysectomy. Dehydration, due to interference of glucocorticoids with AVP secretion and its effect on the renal collecting duct cells, may explain the higher plasma Na^+ concentration in the PDH dogs (37,38).

In the present study plasma K^+ concentrations were normal, and in fact tended to be higher in the dogs with PDH. This is in agreement with a recent study of Wenger et al (39), although slight hypokalemia has been described for dogs with chronic glucocorticoid excess (30,38).

Skeletal muscle Na^+,K^+-ATPase is a main mechanism for the acute clearing of extracellular K^+ during exercise (40). In hypothyroid Beagles subjected to the same exercise protocol as in the present study a peak in plasma K^+ was seen immediately after exercise (2). This plasma K^+ peak was not observed in dogs with PDH, which may be explained in two ways. First, the 5-min exercise test, being a sub-maximal workload, may not have been sufficient to cause an exercise-mediated plasma K^+ peak. Second, this absence can be explained by the concurrent respiratory alkalosis: changes in pCO_2 due to hyperventilation are quantitatively related with an increase in pH, and decreases in HCO_3^- and K^+ (41). This was essentially the same for the exercise-mediated plasma changes found in the present study. Although changes in plasma pCO_2 were not significantly different in dogs with PDH before and after hypophysectomy, calculation with exact values revealed that the decreased pCO_2 in dogs with PDH led to decreased plasma K^+ concentration thereby flattening the plasma K^+ peak. The more pronounced respiratory alkalosis during and after exercise in dogs with PDH may be caused by enhanced panting (42) due to an enlarged abdomen, or in response to glucocorticoid-mediated body heat production (43).

Increased body temperature stimulates the thermal drive for panting, resulting in hypocapnia and alkalosis immediately after exercise in dogs (44). The exercise-mediated hypocapnia in the hypophysectomized dogs was less severe than before surgery and resembled the pCO_2 changes in healthy dogs subjected to a light work load (44).

In summary, dogs with PDH suffering from chronic changes in the circulating hormones cortisol and thyroid hormone, demonstrate a generalized decrease in skeletal muscle Na^+,K^+-ATPase content. This may contribute to muscle weakness that is characteristic of canine PDH. Correction of hyperadrenocorticism by hypophysectomy and subsequent hormone replacement therapy with L-thyroxin and cortisone acetate normalized the Na^+,K^+-ATPase content. Despite the decrease in Na^+,K^+-ATPase, dogs with PDH showed no hyperkalemia after sub-maximal exercise possibly because of the generation of respiratory alkalosis during exercise.

ACKNOWLEDGEMENTS

The technical assistance by Mr. H.G.H. van Engelen, Mr. A. Schot and Mr. B.J.L.J. Joosten, the scientific support by Dr. W.E. van den Brom, Dr. J. Mol and Dr. P. Boer (Nephrology, University Medical Centre Utrecht (UMCU)) and critical reading of the manuscript by Dr. J.A. Joles (Nephrology, (UMCU)) are gratefully acknowledged. Financial support by the "Stichting Jubileumfonds der Veeartsenijkundige Hoogeschool" of the Faculty of Veterinary Medicine, Utrecht University and the "Boelstra Olivier stichting" (grant to B.A. Schotanus) is acknowledged.

REFERENCES

1. Kemppainen RJ, Peterson ME. Animal models of Cushing's disease. Trends Endocrinol Metab 1994;5:21 - 8.

2. Schaafsma IA, van Emst MG, Kooistra HS, Verkleij CB, Peeters ME, Boer P et al. Exercise-induced hyperkalemia in hypothyroid dogs. Domest Anim Endocrinol 2002;22:113-25.

3. Clausen T. Na^+-K^+ pump regulation and skeletal muscle contractility. Physiol Rev 2003;83:1269-324.

4. Kjeldsen K, Nørgaard A, Gøtzsche CO, Thomassen A, Clausen T. Effect of thyroid function on number of Na-K pumps in human skeletal muscle. Lancet 1984;2:8-10.

5. Kjeldsen K, Everts ME, Clausen T. The effects of thyroid hormones on ^{3}H-ouabain binding site concentration, Na,K-contents and ^{86}Rb-efflux in rat skeletal muscle. Pflugers Arch 1986;406:529-35.

6. Clausen T. Long- and Short-Term Regulation of the Na^+-K^+ Pump in Skeletal Muscle. News Physiol. Sci. 1996;11:24-30.

7. Dørup I. Effects of K^+, Mg^{2+} deficiency and adrenal steroids on Na^+, K^+-pump concentration in skeletal muscle. Acta Physiol Scand 1996;156:305-11.

8. Dørup I, Clausen T. Effects of adrenal steroids on the concentration of Na^+-K^+ pumps in rat skeletal muscle. J Endocrinol 1997;152:49-57.

9. Dørup I, Flyvbjerg A, Everts ME, Orskov H. Effects of growth hormone on growth and muscle Na^+-K^+ pump concentration in K^+-deficient rats. Am J Physiol 1992;262:E511-7.

10. Everts ME, Dørup I, Flyvbjerg A, Marshall SM, Jørgensen KD. Na^+-K^+ pump in rat muscle: effects of hypophysectomy, growth hormone, and thyroid hormone. Am J Physiol 1990;259:E278-83.

11. Ravn HB, Dørup I. The concentration of sodium, potassium pumps in chronic obstructive lung disease (COLD) patients: the impact of magnesium depletion and steroid treatment. J Intern Med 1997;241:23-9.

12. Nordsborg N, Goodman CA, McKenna MJ, Bangsbo J. Dexamethasone upregulates skeletal muscle maximal Na+, K+ pump activity by muscle group specific mechanisms in humans. J Physiol 2005;

13. Thompson CB, Dørup I, Ahn J, Leong PK, McDonough AA. Glucocorticoids increase sodium pump alpha(2)- and beta(1)-subunit abundance and mRNA in rat skeletal muscle. Am J Physiol Cell Physiol 2001;280:C509-16.

14. Meij BP, Voorhout G, Rijnberk A. Progress in transsphenoidal hypophysectomy for treatment of pituitary-dependent hyperadrenocorticism in dogs and cats. Mol Cell Endocrinol 2002;197:89-96.

15. Galac S, Kooistra HS, Teske E, Rijnberk A. Urinary corticoid/creatinine ratios in the differentiation between pituitary-dependent hyperadrenocorticism and hyperadrenocorticism due to adrenocortical tumour in the dog. Vet Q 1997;19:17-20.

16. Meij BP, Voorhout G, van den Ingh TS, Hazewinkel HA, Teske E, Rijnberk A. Results of transsphenoidal hypophysectomy in 52 dogs with pituitary-dependent hyperadrenocorticism. Vet Surg 1998;27:246-61.

17. Hodgkinson SC, Allolio B, Landon J, Lowry PJ. Development of a non-extracted 'two-site' immunoradiometric assay for corticotropin utilizing extreme amino- and carboxy-terminally directed antibodies. Biochem J 1984;218:703-11.

18. Findling JW, England WC, Raff H. Emerging techniques: the use of immunoradiometric assay for the measurement of ACTH in human plasma. Trends Endocrinol. Metab. 1990;6:283.

19. Boer P, Hene RJ, Koomans HA, Nieuwenhuis MG, Geyskes GG, Mees EJ. Blood and extracellular fluid volume in patients with Bartter's syndrome. Arch Intern Med 1983;143:1902-5.

20. Boer P, Sleumer JH, Spriensma M. Confirmation of the optimal pH for measuring renin activity in plasma. Clin Chem 1985;31:149-50.

21. Nap RC, Mol JA, Hazewinkel HA. Age-related plasma concentrations of growth hormone (GH) and insulin-like growth factor I(IGF-I) in Great Dane pups fed different dietary levels of protein. Domest Anim Endocrinol 1993;10:237-47.

22. Eigenmann JE, Eigenmann RY. Radioimmunoassay of canine growth hormone. Acta Endocrinol (Copenh) 1981;98:514-20.

23. Williams DA, Scott-Moncrieff C, Bruner J, Sustarsic D, Panosian-Sahakian N, Unver E et al. Validation of an immunoassay for canine thyroid-stimulating hormone and changes in serum concentration following induction of hypothyroidism in dogs. J Am Vet Med Assoc 1996;209:1730-2.

24. Kooistra HS, Diaz-Espineira M, Mol JA, van den Brom WE, Rijnberk A. Secretion pattern of thyroid-stimulating hormone in dogs during euthyroidism and hypothyroidism. Domest Anim Endocrinol 2000;18:19-29.

25. Nørgaard A, Kjeldsen K, Hansen O, Clausen T. A simple and rapid method for the determination of the number of 3H-ouabain binding sites in biopsies of skeletal muscle. Biochem Biophys Res Com 1983;111:319-25.

26. Hansen O, Clausen T. Quantitative determination of Na+-K+-ATPase and other sarcolemmal components in muscle cells. Am J Physiol 1988;254:C1-7.

27. Veldhuis JD, Lizarralde G, Iranmanesh A. Divergent effects of short term glucocorticoid excess on the gonadotropic and somatotropic axes in normal men. J Clin Endocrinol Metab 1992;74:96-102.

28. Casanueva FF, Burguera B, Muruais C, Dieguez C. Acute administration of corticoids: a new and peculiar stimulus of growth hormone secretion in man. J Clin Endocrinol Metab 1990;70:234-7.

29. Wehrenberg WB, Janowski BA, Piering AW, Culler F, Jones KL. Glucocorticoids: potent inhibitors and stimulators of growth hormone secretion. Endocrinology 1990;126:3200-3.

30. Rijnberk A. Adrenals. In: Rijnberk A, editor. Clinical Endocrinology of Dogs and Cats. Kluwer Academic Publishers; 1996. pp. 243.

31. Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 1988;242:583-5.

32. Brabant A, Brabant G, Schuermeyer T, Ranft U, Schmidt FW, Hesch RD et al. The role of glucocorticoids in the regulation of thyrotropin. Acta Endocrinol (Copenh) 1989;121:95-100.

33. Ferguson DC, Peterson ME. Serum free and total iodothyronine concentrations in dogs with hyperadrenocorticism. Am J Vet Res 1992;53:1636-40.

34. Stratakis CA, Mastorakos G, Magiakou MA, Papavasiliou E, Oldfield EH, Chrousos GP. Thyroid function in children with Cushing's disease before and after transsphenoidal surgery. J Pediatr 1997;131:905-9.

35. Thompson CB, Dorup I, Ahn J, Leong PK, McDonough AA. Glucocorticoids increase sodium pump alpha(2)- and beta(1)-subunit abundance and mRNA in rat skeletal muscle. Am J Physiol Cell Physiol 2001;280:C509-16.

36. Khaleeli AA, Edwards RH, Gohil K, McPhail G, Rennie MJ, Round J et al. Corticosteroid myopathy: a clinical and pathological study. Clin Endocrinol (Oxf) 1983;18:155-66.

37. Biewenga WJ, Rijnberk A, Mol JA. Osmoregulation of systemic vasopressin release during long-term glucocorticoid excess: a study in dogs with hyperadrenocorticism. Acta Endocrinol (Copenh) 1991;124:583-8.

38. Baas JJ, Schaeffer F, Joles JA. The influence of cortisol excess on kidney function in the dog. Vet Q 1984;6:17-21.

39. Wenger M, Sieber-Ruckstuhl NS, Muller C, Reusch CE. Effect of trilostane on serum concentrations of aldosterone, cortisol, and potassium in dogs with pituitary-dependent hyperadrenocorticism. Am J Vet Res 2004;65:1245-50.

40. McDonough AA, Thompson CB, Youn JH. Skeletal muscle regulates extracellular potassium. Am J Physiol Renal Physiol 2002;282:F967-74.

41. Muir WW, 3rd, Wagner AE, Buchanan C. Effects of acute hyperventilation on serum potassium in the dog. Vet Surg 1990;19:83-7.

42. Meyer M, Hahn G, Buess C, Mesch U, Piiper J. Pulmonary gas exchange in panting dogs. J Appl Physiol 1989;66:1258-63.

43. Coyer P, Cox M, Rivers JP, Millward DJ. The effect of catabolic doses of corticosterone on heat production in the growing rat. Br J Nutr 1985;53:491-9.

44. Wagner JA, Horvath SM, Dahms TE. Cardiovascular, respiratory, and metabolic adjustments to exercise in dogs. J Appl Physiol 1977;42:403-7.

45. Rijnberk A, Mol JA. Adrenocortical Function. In: Kaneko JJ, editor. Clinical Biochemistry of Domestic Animals. Academic Press; 1997. pp. 553-70.

46. Javadi S, Kooistra HS, Mol JA, Boer P, Boer WH, Rijnberk A. Plasma aldosterone concentrations and plasma renin activity in healthy dogs and dogs with hyperadrenocorticism. Vet Rec 2003;153:521-5.

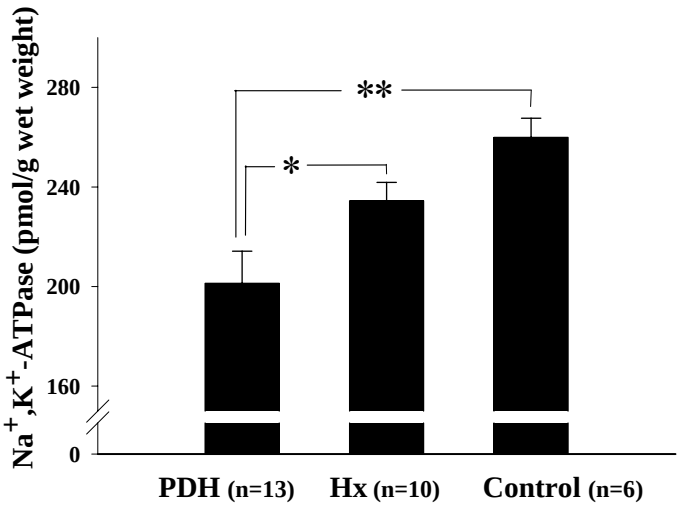
Table. 1. Urinary corticoid/creatinine ratio (UCCR), plasma hormone concentrations and plasma renin activity (PRA) in dogs with pituitary-dependent hyperadrenocorticism (PDH) before (n=13) and 10 weeks after (n=10) hypophysectomy (Hx).

Hormone Value	PDH	After Hx	Reference Value
UCCR ($x10^{-6}$)	82±43[a]	0.8±0.2[a]	<10
Cortisol (nmol/l)	193±30[a]	37±15[a]	27-188[†]
ACTH (pg/ml)	76.5 (6.5–663)[a]	11 (1.0-30.5)[a]	10-90[†]
Aldosterone (pmol/l)	100±13	194±61	118±14[‡]
PRA (fmol/l/s)	174±31	254±53	201±25[‡]
Ald/PRA	0.61±0.6	0.8±0.2	
TSH (ng/ml)	0.12±0.03[b]	<0.03[b]	0.0-0.6
tT_4 (nmol/l)	13±3[b]	22±4[b]	19-46
GH (ng/ml)	0.6 (<0.5-1.8)	0.5 (<0.5-1.2)	0-5
IGF-1 (ng/ml)	90 (51-279)[b]	51 (37-67)[b]	36-280[†#]

Values are expressed as mean ± SEM or median and range.

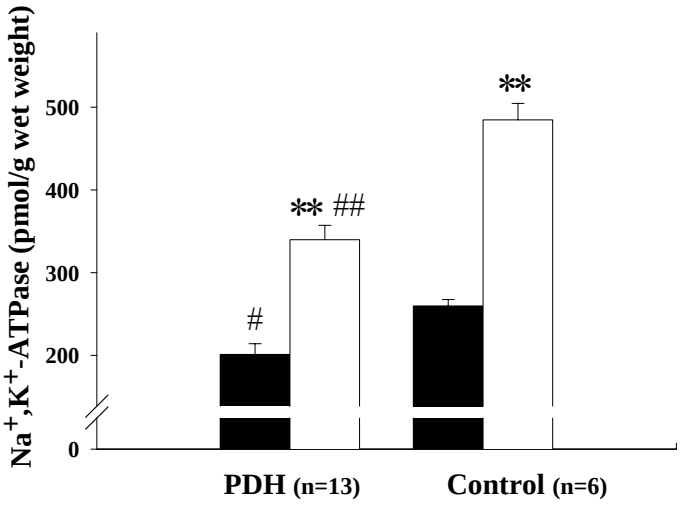
UCCR (n=13 before; n=10 after hypophysectomy), adrenocorticotropin (ACTH), aldosterone (n=12;5), thyroid stimulating hormone (TSH), total T_4 (tT_4), growth hormone (GH, n=13;9), insulin-like growth factor (IGF-1, n=13;7). Reference values were obtained from the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University, from Rijnberk, 1997[†] (45), and from Javadi et al, 2003 (46)[‡]. [#]Age and breed dependent. [a]$P < 0.01$, [b]$P < 0.05$ before vs. after Hx.

Fig. 1.


Na+,K+-ATPase (pmol/g wet weight)
280
240
200
160
0
**
*
PDH (n=13)
Hx (n=10)
Control (n=6)

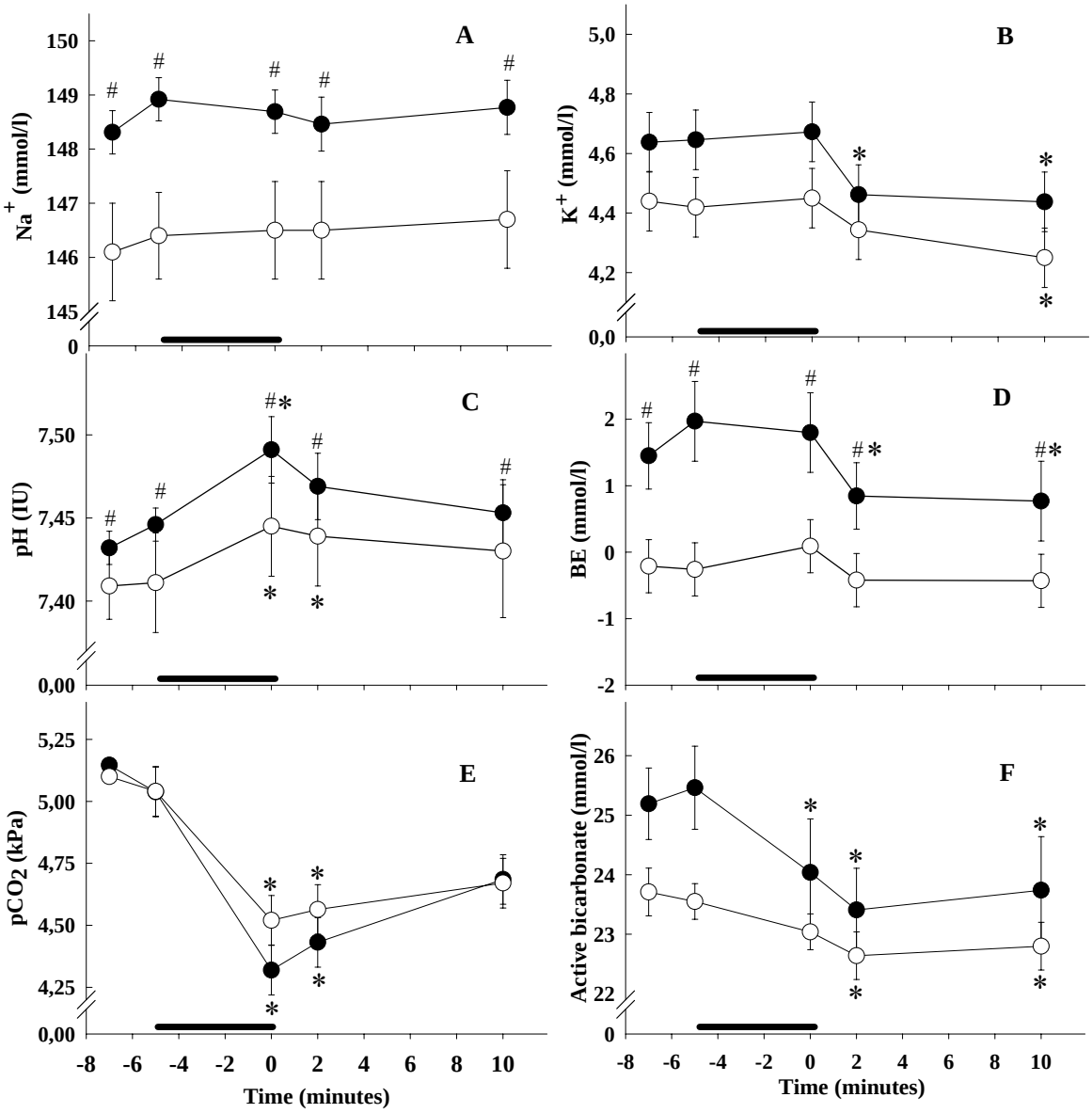
1

Fig. 2.


Na+,K+-ATPase (pmol/g wet weight)
500
400
300
200
0
#
** ##
**
PDH (n=13)
Control (n=6)

1

Figure 3.


A
B
C
D
E
F
Na+ (mmol/l)
K+ (mmol/l)
pH (IU)
BE (mmol/l)
pCO2 (kPa)
Active bicarbonate (mmol/l)
Time (minutes)
Time (minutes)

Legends to figures

Figure 1.

Na^+,K^+-ATPase content (pmol/g wet weight) in gluteal muscle of dogs with pituitary-dependent hyperadrenocorticism before (PDH) and 10 weeks after hypophysectomy (Hx), and in healthy control dogs. Mean ± SEM; **P* < 0.05. ***P* < 0.01.

Figure 2.

Na^+,K^+-ATPase content (pmol/g wet weight) in gluteal (black bars) and palatine (white bars) muscle of dogs with pituitary-dependent hyperadrenocorticism (PDH) and healthy control dogs. ***P* < 0.001 compared to gluteal muscle. $^{\#,\#\#}P$ < 0.01 and *P* < 0.001 respectively, compared to control muscle.

Figure 3A-F.

Plasma Na^+ (A), K^+ (B), pH (C), Base Excess (D), pCO_2 (E) and HCO_3^- (F) before and after a 5-min treadmill exercise test in dogs with pituitary-dependent hyperadrenocorticism before (●, n=13) and after (○, n=10) hypophysectomy.

—— = period of exercise;

Mean ± SEM;

* *P* < 0.05 within the group, compared to the mean value before exercise;

$^{\#}$ *P* < 0.05 between groups.