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ORIGINAL ARTICLE

The influence of trilostane on steroid hormone metabolism in canine adrenal glands and corpora lutea—an in vitro study

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Abstract Trilostane is widely used to treat hyperadrenocorticism in dogs. Trilostane competitively inhibits the enzyme 3-beta hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone (P5) to progesterone (P4) and dehydroepiandrosterone (DHEA) to androstendione (A4). Although trilostane is frequently used in dogs, the molecular mechanism underlying its effect on canine steroid hormone biosynthesis is still an enigma. Multiple enzymes of 3β–HSD have been found in humans, rats and mice and their presence might explain the contradictory results of studies on the effectiveness of trilostane. We therefore investigated the influence of trilostane on steroid hormone metabolism in dogs by means of an in vitro model. Canine adrenal glands from freshly euthanized dogs and corpora lutea (CL) were incubated with increasing doses of trilostane. Tritiated P5 or DHEA were used as substrates. The resulting radioactive metabolites were extracted, separated by thin layer chromatography and visualized by autoradiography. A wide variety of radioactive metabolites were formed in the adrenal glands and in the CL, indicating high metabolic activity in both tissues. In the adrenal cortex, trilostane influences the P5 metabolism in a dose- and time-dependent manner, while DHEA metabolism and metabolism of both hormones in the CL were unaffected. The results indicate for the first time that there might be more than one enzyme of 3β-HSD present in dogs and that trilostane selectively inhibits P5 conversion to P4 only in the adrenal gland.

Keywords Glucocorticoids · Steroid hormone biosynthesis · Radiometabolism · Trilostane · Dog

Abbreviations

3β-HSD	3-beta hydroxysteroid dehydrogenase
P5	pregnenolone
DHEA	dehydroepiandrosterone
17-OH-P5	17-hydroxy-pregnenolone
P4	progesterone
17-OH-P4	17-hydroxy-progesterone
A4	androstendione
CL	corpora lutea
TLC	thin layer chromatography

Introduction

Spontaneous hyperadrenocorticism is defined as a complex of physical and biochemical changes resulting from chronic glucocorticoid overexposure. In dogs, the most frequent cause of the disease is a tumour in the pituitary gland (85%), which secretes ACTH and stimulates the adrenal glands to produce glucocorticoids (Feldman and Nelson 2004). Treatment of hyperadrenocorticism aims at the reduction of glucocorticoid production to eliminate clinical signs caused by excess glucocorticoids. Trilostane has become the medical treatment of choice for dogs with pituitary-dependent hyperadrenocorticism. It has been used widely, effectively and safely over the

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last years (Hurley et al. 1998; Neiger et al. 2002; Ruckstuhl et al. 2002; Neiger and Lehnert 2004; Witt and Neiger 2004; Alenza et al. 2006; Vaughan et al. 2008).

The literature suggests that trilostane reduces steroid hormone biosynthesis by competitive inhibition of the 3β-hydroxysteroid dehydrogenase (3β-HSD, Potts et al. 1978). This key enzyme catalyses the conversion of pregnenolone (P5), 17-hydroxy-pregnenolone (17-OH-P5) and dehydroepiandrosterone (DHEA) to progesterone (P4), 17-hydroxy-progesterone (17-OH-P4) and androstendione (A4), respectively (see Fig. 1). The enzymatic action of 3β-HSD is thus essential for the synthesis of cortisol and all other steroids including glucocorticoids, mineralocorticoids and sex steroids.

However, literature relating to the effects of trilostane on steroid hormone biosynthesis in various species is contradictory. It is known that trilostane reduces the production of both gluco- and mineralocorticoids in rats (Potts et al. 1978). In rhesus monkeys, the drug influences adrenal, ovarial and placental steroid hormone biosynthesis in a dose-dependent manner (Schane et al. 1979), whereas in humans it mainly reduces progesterone and, minimally, cortisol production (Le Roux et al. 2002; Le Roux and Van Der Spuy 2005). A possible explanation could be the presence of different types of 3β-HSD with varying susceptibility to trilostane. It is known that humans have two isoforms of the enzyme (Thomas et al. 2004; Thomas et al. 2008), whereas mice have six and rats four (Payne and Hales 2004). Although trilostane is frequently used in medical therapy in dogs, the number of 3β-HSD enzymes and their in vitro effects on P5 and DHEA metabolism in canine adrenal tissue and corpora lutea (CL) have not yet been reported.

The aim of this study was to investigate the effects of trilostane on the metabolism of P5 and DHEA in adrenal glands and CL of dogs. As other species contain several enzymes of 3β-HSD, the study also addressed whether multiple enzymes of 3β-HSD are found in dogs too.

Materials and methods

Organs (adrenal glands & corpora lutea)

Adrenal cortex tissue from four intact male dogs, aged between 4.7 and 15.8 years (median, 9.65 years) and two female dogs, aged 1 (sexually intact) and 14.2 years (neutered) and corpora lutea tissue from two female dogs, aged 4.8 and 5 years, were used to establish the method of incubation and separation of the metabolic products. Breeds included Pomerian, Rottweiler, Large Swiss Mountain Dog, West Highland White Terrier, Polish Cattle Dog and three mixed-breed dogs.

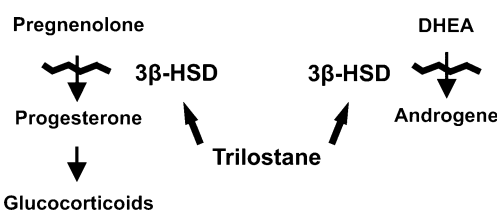

Fig. 1 Drug mechanism of trilostane according to literature

The adrenal glands were removed within 20 min after euthanasia. All dogs were euthanized with thiopental (Sandoz GmbH, 6250 Kundl, Austria) and T61 (Intervet GesmbH, 1210 Wien, Austria) intravenously. The corpora lutea were collected within 30 min of ovariohysterectomy.

The weight of the adrenal glands ranged from 1.05 to 4.4 g (median, 1.6 g); the corpora lutea weighed 3.05 g and 1.93 g. The identification of both organs was confirmed by histological examination. The samples were stained with hematoxylin and eosin. The adrenocortical zones were well distinguishable. No pathological changes were observed.

Chemicals

We used ^{3}H-P5 (NET-039, [7-^{3}H (N)]-pregnenolone; specific activity: 12.6 Ci/mmol; Perkin Elmer, Boston, USA) and ^{3}H-DHEA (NET-814; [1,2,6,7-^{3}H(N)]-dehydroepiandrosterone, specific activity: 8.1 Ci/mmol; Perkin Elmer, Boston, USA). The medium (minimum essential medium with Earl's Salts and L-Glutamine, Cat No E15-825, Lot No E82509-0249, PAA Laboratories GmbH, Pasching, Austria) was divided into portions of 11 ml tubes in advance and stored at −24°C. Trilostane (Vetoryl®) was a gift from Dr M. Hemprich, Dechra Pharmaceuticals, Shrewsbury, UK. All other chemicals were of analytical grade and so commercially available.

Preparation and incubation

The organs were cleaned from blood and fat tissue, washed twice with 1 ml 0.9% sodium chloride solution, measured and scaled. Each adrenal gland was sectioned with a blade and the medulla removed by gentle scraping to obtain only adrenal cortex tissue. The cortex was cut into small pieces and 0.1 g (if there was less tissue, 0.05 g was used) tissue was mixed with 1 ml cell culture medium and 400 µl radioactively labelled ^{3}H-P5 or ^{3}H-DHEA (250 kBq/tube) in a glass vial. Increasing doses of 200 µl trilostane (control, 0.002 µmol/tube and 0.02 µmol/tube) were added. Duplicates of samples were incubated for 240 min on a shaking water bath at 39°C. After 60, 120 and 240 min, 400 µl supernatant was transferred into new vials. Control incubations consisting of ^{3}H-P5 or ^{3}H-DHEA plus medium were also incubated for 240 min.

Quantitative analysis

10 µl of supernatant was used for counting radioactivity. It was mixed with 6 ml scintillation fluid (Quicksafe A, no.

1008000, Zinsser Analytic, Maidenhead, UK) and the radioactivity measured by liquid scintillation counting (Packard Tri-Carb 2100 TR) for 5 min while running a quench compensation programme.

The remainder of the supernatant (390 μl) was diluted with 1 ml distilled water and loaded onto a primed Sep-Pak® C18 cartridge via airflow. Cartridges were washed with 4 ml distilled water and 4 ml 10% methanol before elution of the steroid metabolites with 4 ml 100% methanol. After evaporation of the solvent, the eluate was resuspended in 100 μl methanol. An aliquot of 10 μl was used to determine the recovery after elution of radioactivity after Sep-Pak® C18 filtration. 50 μl of each sample was transferred using a linomat onto a thin layer chromatography plate (TLC, Silica Gel 60, F254, Merck, Darmstadt, Germany). In addition, a mixture of unlabelled steroid standard (8 μg androstendione, progesterone, 17α-OH-progesterone, cortisol and corticosterone) was applied to the left, the middle and the right region of the plate. First the TLC plates were developed in methanol for 1 cm. After drying, chromatography was performed using cyclohexane:ethyl acetate (1:1) as mobile phase. As soon as the solvent front reached 13 cm, the TLC plates were removed and dried at room temperature in a fume hood. The standards were visualized under ultraviolet light (254 nm), marked with a radioactive spot for subsequent detection via autoradiography and dried again. The metabolites were quantified by autoradiography (Beta Imager 2000, Biospace Mesures, Paris, France). Analysis and detection of the standards and peaks was performed with the Beta Vision+2.0 program (Beta Imager 2000, Biospace Mesures, Paris, France).

Results

Recovery

Total recovery of radioactivity was measured before and after cleaning the samples by Sep-Pak® C18 cartridges. Recovery from extraction ranged from 73 to 99.7% for P5 and from 77 to 82% for DHEA. The recovery rate decreased with increasing incubation times (120, 240 min).

Pregnenolone

All samples showed high metabolic activity. A number of metabolites were formed by the adrenal glands and CL when presented with ^{3}H-P5 as precursor. After chromatographic separation of the adrenal gland tissue samples incubated with ^{3}H-P5 ten radioactive peaks were detected (Fig. 2). In the CL samples the TLC autoradiograms distinguished up to eight distinct bands. However, the main metabolites co-eluted with the steroid standards used as references (cortisol/corticosterone, 17-OH-P4, A4, P5, DHEA and P4). The metabolites showed a higher chromatographic mobility after the cortisol standard. Additionally, there were a number of less prominent peaks that did not elute with the steroid standards. These peaks eluted between the cortisol and progesterone standards and their intensities increased with time of incubation.

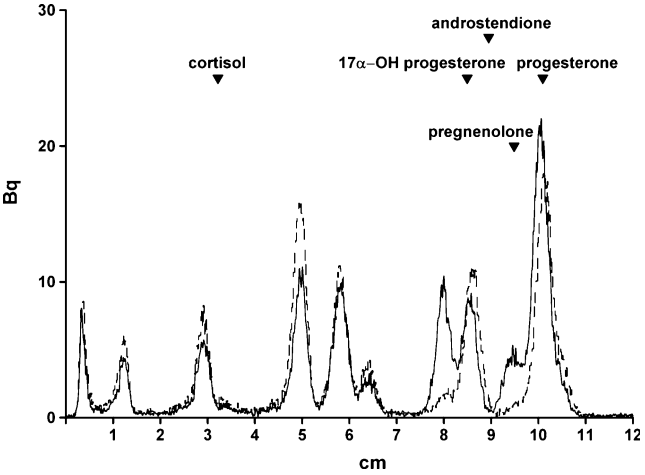

Fig. 2 2D-autoradiography (Beta Imager 2000, Biospace Measures, Paris, France) of an adrenal gland tissue sample incubated for 60 min with ^{3}H-pregnenolone as precursor (dotted line: untreated tissue, continuous line: tissue treated with the high dosage of trilostane). Open triangles mark the approximate elution positions of respective standards

Throughout all experiments, the tissue could be observed to metabolize the precursor (P5). A dominant peak at the elution position of ^{3}H-P5 was only found in the group treated with trilostane, as the P5 standard showed the same chromatographic mobility. In the samples containing trilostane, there was a notably stronger peak at the position of ^{3}H-P5 than at the position of P4. The precursor (^{3}H-P5) was thus not metabolized completely during the incubation period into other steroids, especially to P4. This finding seemed to be dose-dependent when compared to the control incubated for 60 min (Fig. 2). But with increasing incubation time (120, 240 min) more and more of the precursor was converted.

In contrast, no effect of trilostane on ^{3}H-P5 metabolism was observed in the CL (Fig. 3). Analysis of the thin layer chromatogram showed one main metabolite. The peak co-eluted with the progesterone standard.

DHEA

A number of metabolites were formed by the adrenal glands and the CL when DHEA was used as precursor (Fig. 3).

Seven radioactive peaks could be detected after chromatographic separation of ^{3}H-DHEA metabolized in the adrenal glands and nine radioactive peaks were seen in the CL incubations. Analysis of the thin layer chromatograms

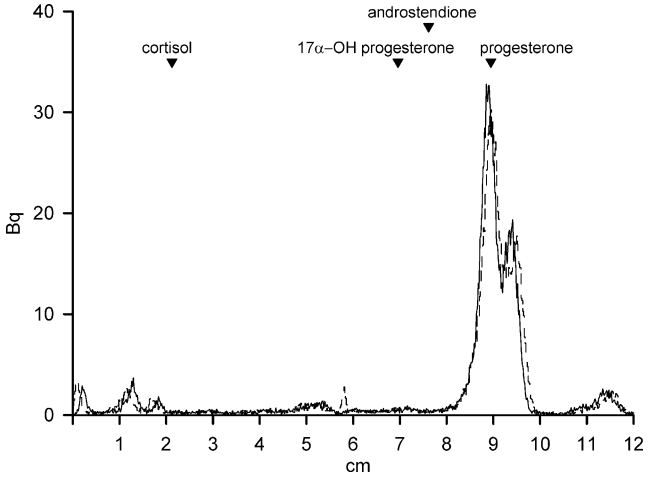

Fig. 3 2D-autoradiography (Beta Imager 2000, Biospace Measures, Paris, France) of a corpus luteum sample incubated for 60 min with ^{3}H-pregnenolone as precursor (dotted line: untreated tissue, continuous line: tissue treated with the high dosage of trilostane). Open triangles mark the approximate elution positions of respective standards

showed predominantly polar peaks. However, the main metabolites co-eluted with the steroid standards used as references (cortisol and P4).

In general, trilostane had no effect on ^{3}H-DHEA metabolism. The peaks were neither time- nor dose-dependent (Fig. 4). No radioactivity could be detected at the elution position of ^{3}H-DHEA, i.e. the precursor was completely metabolized. The main product co-eluted with the androstendione standard. In the adrenal glands, trilostane did not influence the amount of androstendione production. Same results were found in the CL, where trilostane also did not reduce the rate of conversion of ^{3}H-DHEA to androstendione. In the CL, the main metabolic product co-eluted with the progesterone standard.

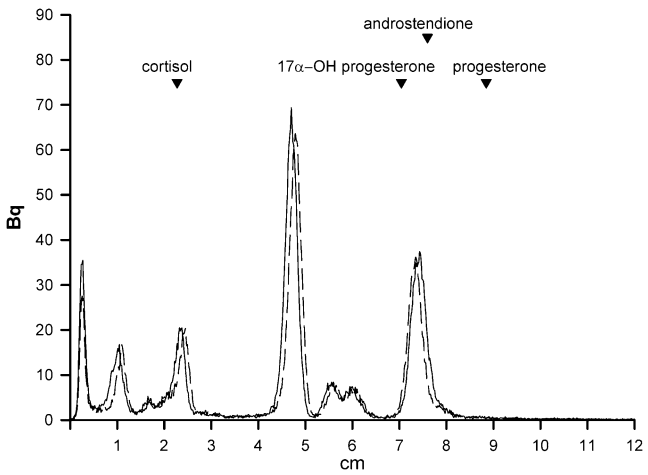

Fig. 4 2D-autoradiography (Beta Imager 2000, Biospace Measures, Paris, France) of an adrenal gland tissue sample incubated for 60 min with ^{3}H-DHEA as precursor (dotted line: untreated tissue, continuous line: tissue treated with the high dosage of trilostane). Open triangles mark the approximate elution positions of respective standards

Discussion

The decreasing recovery with increasing incubation time could be explained as tritium is removed from the steroid. So for example 3H-P5 (NET-039, [7-3H (N)] pregnenolone has its tritium label in position 7. Hydroxylation at this position can cause the dissolution of tritium from the precursor.

Trilostane is widely used to treat hyperadrenocorticism in dogs although the concrete mode of action is not fully understood. We have thus investigated whether dogs have different types of 3β-HSD, as has already been shown for humans, rats and mice.

Previous studies in humans and various other species have revealed trilostane to be a competitive inhibitor of 3β-HSD. This key enzyme is responsible for the production of both glucocorticoids and mineralocorticoids by the adrenal gland (Potts et al. 1978). Studies in vitro and in vivo have shown that trilostane inhibits steroid synthesis in the adrenal gland, the ovaries and the placenta in various species (Potts et al. 1978; Schane et al. 1979; Jungmann et al. 1982; Semple et al. 1983). Inhibition of progesterone production leads to a reduced synthesis of cortisol in the zona fasciculata, of aldosterone in the zona glomerulosa and of androstendione in the zona reticularis (Ruckstuhl et al. 2002; Sieber-Ruckstuhl et al. 2006). Treatment with trilostane decreases only the conversion of P5 to P4 in canine adrenal glands, but not the conversion of DHEA to androstendione. Therefore the drug had no detectable effects on the pathway of DHEA metabolism. These conclusions are in accordance with several reports of differences in the reduction of serum cortisol and aldosterone concentrations in dogs with pituitary-dependent hyperadrenocorticism treated with trilostane. Serum aldosterone concentration was much lower than serum cortisol concentration (Wenger et al. 2004; Galac et al. 2007). Arenas et al. (2007) also found that trilostane blocks only P4 and cortisol synthesis in vivo, although androstendione levels were unexpectedly elevated.

The most likely explanation for the results is that there is more than one enzyme of 3β-HSD in dogs, with inhibition of the isoenzyme in our project effective only in the zona fasciculata of the adrenal cortex. Another explanation might be the higher trilostane concentration in the zona fasciculata compared with other zones (zona glomerulosa, zona reticularis).

The number of 3β-HSD enzymes varies between species. Six, four and two enzymes have been identified in humans, rats and mice, respectively (Rheaume et al. 1991; Payne and Hales 2004; Malouitre et al. 2006). The

different isoforms are also tissue-specific. In rats, types 1 and 2 are found in the adrenal glands (Malouitre et al. 2006), whereas in humans only type 2 is present in the adrenal glands, with type 1 in the placenta and mammary glands (Payne and Hales 2004). In human adrenal glands, type 2 3β-HSD is required for the production of cortisol and aldosterone (Rainey et al. 2002). Trilostane may work differently in dogs than in rodents or humans and may also have different effects on different isoforms. No previous studies have investigated the number and tissue distribution of 3β-HSD isoenzymes in dogs.

Trilostane decreases the metabolism of P5 in canine adrenal gland tissue in a time-dependent matter. The reported duration of action of trilostane is generally short, with enzyme activity quickly returning to normal levels in in vivo studies (Neiger and Hurley 2001; Bell et al. 2006). A temporary suppression of enzyme activity could be also observed in the present study. Nevertheless, the brief suppression appears to provide good long-term control of the clinical signs of hyperadrenocorticism in most dogs (Neiger et al. 2002; Ruckstuhl et al. 2002).

Consistent with other reports (Braddock et al. 2003; Alenza et al. 2006; Galac et al. 2007), we found that suppression is also dose-dependent. There is a marked variation in optimal dose between dogs (breeds) with pituitary-dependent hyperadrenocorticism (Hurley et al. 1998; Neiger et al. 2002; Braddock et al. 2003; Alenza et al. 2006). A possible explanation could be a significant variation between individuals with regard to the 3β-HSD activity in the adrenal glands (Reusch 2005). Larger dogs need smaller doses than smaller ones. A very interesting finding was that Yorkshire Terriers need much higher doses than other dogs (median, 13.4 mg/kg, Alenza et al. 2006). The differences could be caused by differences in the metabolism or by the fact that small dogs have higher values of plasma corticoids than large-breed dogs (Reimers and Lawler 1990). A variation in 3β-HSD activity in adrenal glands seems also possible, resulting from variable absorption or variable conversion into active metabolites such as ketotrilostane.

In dogs, the key enzyme 3β-HSD is found both in the adrenal gland and in the corpus luteum. We tested whether trilostane affects steroid formation in the ovaries, where the main steroid pathway is from pregnenolone to progesterone, the central metabolite for the maintenance of pregnancy. As trilostane has been shown to inhibit progesterone synthesis in the ovaries and to cause abortion in rats, monkeys and humans (Potts et al. 1978; Schane et al. 1979; Le Roux et al. 2002; Le Roux and Van Der Spuy 2005; De Gier et al. 2011) we assessed whether it also causes abortion in dogs. Our study did not reveal any effect of trilostane on steroid synthesis in canine CL, possibly because of the dose we used or because of a selective inhibition of the enzyme. Le Roux et al. (2002) found that trilostane decreased the level of progesterone level but not of cortisol in pregnant women. Similar results were found in studies investigating epostane, another 3β-HSD inhibitor, low concentration of which inhibit only type 1 but not type 2 enzymes (Thomas et al. 2004). In rats, trilostane also inhibits adrenal steroidogenesis at doses lower than those required to inhibit gonadal steroidogenesis (Potts et al. 1978). It seems to operate by exerting a selective blockade on 3β-HSD.

In conclusion, this study provides essential information about the metabolism of P5 and DHEA in canine ovarial and adrenal steroid hormone biosynthesis under the influence of trilostane in dogs. The results show for the first time the existence of at least two 3β-HSD enzymes in dogs and represent an important basis for further investigations.

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