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The impact of stress on the prevalence of prednisolone in bovine urine: A metabolic fingerprinting approach

Nathalie De Clercq[a], Lieven Van Meulebroek[a], Julie Vanden Bussche[a], Siska Croubels[b], Philippe Delahaut[c], Lynn Vanhaecke[a,*]

[a] Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health & Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium
[b] Ghent University, Faculty of Veterinary Medicine, Department of Pharmacology, Toxicology and Biochemistry, Laboratory of Pharmacology and Toxicology, Salisburylaan 133, B-9820 Merelbeke, Belgium
[c] CER Groupe, Département Santé, Rue du Point du Jour 8, B-6900 Marloie, Belgium

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ABSTRACT

Recent studies support the hypothesis that the glucocorticoid prednisolone can be formed from cortisol under influence of stress. To evaluate this hypothesis, urine samples of supposedly non-stressed bovines (at the farm) and bovines subjected to two different forms of stress, i.e. upon slaughter (natural stress) or following administration of a synthetic analog of the adrenocorticotropic hormone (pharmacologically-induced stress) were analysed, and their urinary cortisol and prednisolone levels evaluated. At the farm, none of the examined samples exhibited urinary prednisolone levels higher than the CCα (0.09 $\mu g\,L^{-1}$). Upon slaughter or following synthetically induced stress, significantly positive correlations between cortisol and prednisolone could be demonstrated, 0.52 and 0.69, respectively. Of all prednisolone-positive urine samples ($n = 84$), only one showed a prednisolone levels (i.e. 6.45 $\mu g\,L^{-1}$) above the threshold level of 5 $\mu g\,L^{-1}$ suggested by the European Reference Laboratories. Subsequently, an untargeted analysis was performed (metabolic fingerprinting) to characterize the urinary metabolite patterns related to the three different cattle groups. In this context, multivariate statistics assigned a total of 169 differentiating metabolites as playing a key role in the urinary pattern in response to stress. Three of these ions were defined as steroids using an in-house created database. As a result, the metabolic fingerprinting approach proved to be a powerful tool to classify unknown bovine urine samples that tested positive for prednisolone, while providing information about the stress status of the animal.

1. Introduction

The main glucocorticoid cortisol and its precursor cortisone have been acknowledged to fulfil a wide range of physiological functions, being amongst others related to stress responses, homeostatic effects, and anti-inflammatory actions [1,2]. Based on these functions, more potent synthetic analogs such as dexamethasone, betamethasone, prednisolone, and methylprednisolone have been introduced and are routinely used in veterinary practice for the treatment of diverse inflammatory diseases and metabolic disorders [3]. Besides their therapeutic actions, glucocorticoids have also been associated with growth-promoting effects, (alone or in combination with anabolic steroids) for fattening purposes as well [4]. However, the therapeutic use of synthetic glucocorticoids is been strictly regulated in the European Union [5] in order to protect consumers against potential harmful residues, present in animal derived food products. More specifically, maximum residue limits (MRLs) have been set for betamethasone, dexamethasone, methylprednisolone, and prednisolone in diverse tissues of animal origin [6]. Moreover, the use of synthetic glucocorticoids is completely prohibited for the sole purpose of increasing the body weight of bovines. For this reason, national residue monitoring plans are implemented in the various member states to detect any misuse of glucocorticoids, whereby urine is considered as the preferred matrix.

In this context, the European Commission declared more non-compliant urine samples for prednisolone in the past few years, which has been reported in their annual Commission Staff Working Document on the implementation of the established national glucocorticoid monitoring plans. Moreover, there was no direct evidence of unauthorized use. These findings, in essence originating from the increased analytical sensitivity of the applied screening

* Corresponding author. Fax: +32 9 264 74 92.
E-mail address: Lynn.Vanhaecke@ugent.be (L. Vanhaecke).

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methods, led to the hypothesis of an endogenous prednisolone origin. In addition, since most of the prednisolone positive urine samples were collected at the slaughterhouse, it has been suggested that stress may induce the involved metabolic processes [7]. Indeed, various studies report on the detection of prednisolone residues in urine samples that were either collected at the slaughterhouse [7–9] or after therapeutic stress inducement [10]. In contrast, during a field survey, no prednisolone was detected in the majority of urine samples from untreated cattle [11]. These observations are supported by the underlying mechanisms, associated with the physiological stress response. In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is stimulated whereby the hypothalamus produces corticotropin-releasing hormone (CRH), which in turns triggers the secretion of adrenocorticotropic hormone (ACTH). This latter hormone may affect the adrenal gland, as such promoting the synthesis and release of cortisol [1,12] (Fig. 1). Since cortisol (Δ^4-pregnene-11β,17α,21-triol-3,20-dione) is only differing from prednisolone ($\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione) by a single ring double bond, the formation of prednisolone from cortisol may be assumed. This has recently been evidenced by the study of Rijke et al. [13], whereby a significant decrease of cortisol and formation of prednisolone within 6 h was observed during an in vitro incubation experiment with bovine S9 liver enzyme extract. Based on the possible endogenous formation of prednisolone, the European Reference Laboratories have suggested a threshold level for prednisolone in bovine urine of 5 μg L^{-1}, thereby taking into account various potential endogenous origins and influencing factors [13,14].

In this study, the influence of stress on the prevalence of prednisolone and prednisone in bovine urine was evaluated to respond toward the inconsistent data reported in literature and confirm the validity of the above-mentioned threshold level. To this extent, 12 healthy bovines were subjected to a treatment of intramuscular injection with a synthetic analog of ACTH, i.e., tetracosactide hexaacetate, to induce pharmacologically-induced stress. In addition, bovine urine was collected from 144 meat and milk producing bovines under real life conditions differing in degree of stress imposed, i.e. under normal housing conditions at the farm and upon slaughter. The collected urine samples were analysed by usage of full-scan high-resolution orbitrap mass spectrometry to acquire the samples' metabolic fingerprints, allowing the identification of metabolite patterns that are characteristic for bovines under well-defined stress conditions. Parallel to this untargeted metabolic fingerprinting, a targeted strategy was considered as well, thereby determining the concentration levels of relevant glucocorticoids and derived products; i.e. cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone, 20α-dihydroprednisolone and 20β-dihydroprednisolone.

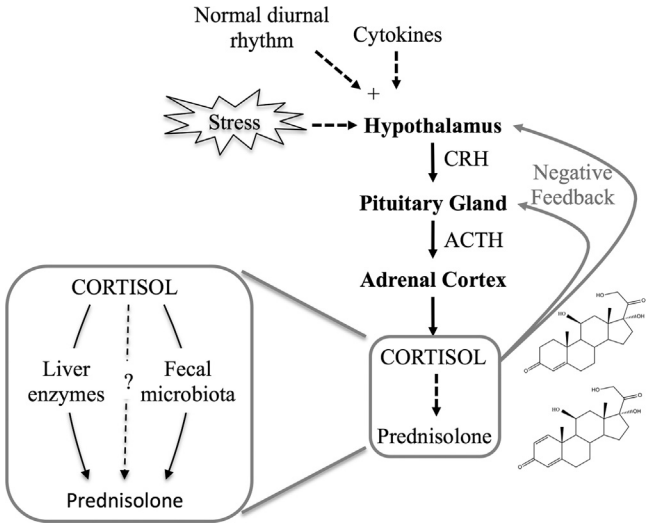

Fig. 1. Systematic regulation of circulating cortisol levels by the hypothalamic-pituitary-adrenal (HPA) axis and the possible pathways of endogenous prednisolone formation.

2. Material and methods

2.1. Bovine urine collected during ACTH treatment

2.1.1. Test animals

Twelve healthy cows of a mixed breed were subjected to the ACTH in vivo study and housed in the animal facilities of the Centre d'Economie Rurale (CER, Marloie Belgium) under controlled experimental conditions. Age of the selected animals ranged from 2 to 6 years and body weight was between 360 and 570 kg. Animals were fed a commercially available diet, commonly applied in zootechnical practice, with ad libitum access to water and hay. During the entire study, animals were kept in three separate and equal-sized groups, all housed in a half-covered pen. Prior to the actual ACTH treatment, an initial acclimatization of 18 days was considered. This study was approved by CER's Ethical Committee (CE/Sante/ET/004).

2.1.2. Experimental protocol for ACTH treatment

Subsequent to the acclimatization period, all test animals received at 8 am a daily 2 mg intramuscular (IM) injection of tetracosactide hexaacetate (Pharmacy Department, Faculty of Veterinary Medicine, Utrecht University), which corresponds to 200 IU of ACTH, and this for 4 consecutive days.

2.1.3. Urine sample collection

During the acclimatization period, urine samples were collected daily in the morning at 8 am by a veterinarian with the help of a probe (to prevent faecal contamination) and immediately stored in the dark at −80 °C until analysis [15]. During the ACTH treatment period, urine samples were collected twice a day, one sample prior to IM injection of ACTH and a second 4 h (on the first and second day) or 6 h (on the third and fourth day of ACTH administration) post-administration. Until four days after the termination of the ACTH treatment, daily urine samples were collected in the morning at 8 am (post-treatment samples).

2.2. Bovine urine collected at the farm

2.2.1. Test animals

Urine samples were collected from 11 clinically healthy Belgian Beef cows and 31 milking cows, covering an age range between 5 months and 7 years. The animals were housed at four different farms (CER, $n = 11$; JPW, $n = 8$; PDW, $n = 12$; DD, $n = 11$) located in Belgium, whereby the respective breeders stated that cows had not been subjected to any drug treatment 30 days prior to effective urine sampling.

2.2.2. Urine sample collection

Urine samples were collected during feeding (between 8 and 11 am), thereby waiting for spontaneous micturition, while carefully avoiding fecal contamination. These samples were stored within 2 h at −80 °C in the dark, until analysis.

2.3. Bovine urine collected at slaughter

2.3.1. Test animals

Urine samples at the slaughterhouse (Flanders Meat Group, Zele) were collected from 102 healthy bovines, originating from 43 different farms. The selected animals comprised 64 cows and