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Corticosteroid Hormone Receptors and Prereceptors as New Biomarkers of the Illegal Use of Glucocorticoids in Meat Production

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ABSTRACT: Despite the European ban on the use of growth promoters in cattle, veterinary surveillance reports indicate that the illicit use of corticosteroids persists both alone and in combination with anabolic hormones and β-agonists. Current control strategies should be informed by research into the effects of corticosteroids on bovine metabolism and improved through the development of specific, sensitive diagnostic methods that utilize potential molecular biomarkers of corticosteroid treatment. The actions of corticosteroids on target tissues are principally regulated by two receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). The effects of these steroids are modulated by prereceptor enzyme-mediated metabolism: the two isoforms of the 11β-hydroxysteroid dehydrogenase (11β-HSDs) enzyme catalyze the interconversion between active glucocorticoids, such as cortisol, into inactive compounds, such as cortisone. This study aimed to determine whether the expression of the prereceptor system and of the corticosteroid receptors could be regulated in different target tissues by the administration of dexamethasone and prednisolone in cattle. It was observed that greater up-regulation of the GR and MR genes followed dexamethasone treatment in the muscle tissues than in the kidney, liver, and salivary glands; up-regulation of GR and MR expression following prednisolone treatment was higher in adipose tissue than in the other tissues. The thymus seemed to respond to dexamethasone treatment but not to prednisolone treatment. Both treatments significantly down-regulated 11β-HSD2 gene expression in the adrenal tissues, but only dexamethasone treatment down-regulated 11β-HSD2 expression in the bulbourethral and prostate glands. Together, these data indicate that the combination of GR, MR, and 11β-HSD2 could provide a useful biomarker system to detect the use of illicit glucocorticoid treatment in cattle.

KEYWORDS: glucocorticoid-responsive genes, real-time PCR, growth promoters, cattle

INTRODUCTION

Corticosteroids are often illegally used as growth promoters in livestock production, either alone or in combination with anabolic agents, especially at low dosages and primarily through oral administration.[1,2] These steroids can enhance the feed conversion ratio and meat quality, resulting in higher tenderness and paler color, two traits particularly appreciated by consumers.[3] A better understanding of the effects of corticosteroids on bovine metabolism and the development of molecular biomarkers for use in specific, sensitive diagnostic methods to detect illegal corticosteroid treatment are issues of fundamental importance for public health.

Glucocorticoids are major permissive hormones required in the functioning of the machinery of most cells and tissues. Until recently, it was believed that the major determinants of corticosteroid action on target cells were the following: (1) circulating hormone levels, (2) plasma and tissue concentrations of binding proteins such as corticosteroid-binding globulin (CBG),[4] and (3) the presence and relative density in a particular tissue of two receptor types, the glucocorticoid (GR) and mineralocorticoid (MR) receptors.[5,6] Moreover, glucocorticoid receptors can interfere with the transcriptional activities of other factors such as the AP-1 complex by direct protein−protein interactions.[7]

It is now well recognized that an important additional level of regulatory complexity exists and that it functions through prereceptor enzyme-mediated metabolism by either ligand activation or inactivation.[8] This additional level of control is regulated by 11β-hydroxysteroid dehydrogenase (11β-HSD; EC 1.1.1.146), an enzyme that controls the access of physiological glucocorticoids (cortisol, corticosterone) to both MR and GR in peripheral tissues.[9]

Two isoforms of this enzyme, 11β-HSD1 and 11β-HSD2, interconvert active glucocorticoid, that is, cortisol, and inactive cortisone and have been characterized and cloned from human[10−12] and bovine tissues.[13] The 11β-HSD1 enzyme is believed to act in vivo predominantly as an oxo-reductase with NADP(H) as a cofactor to generate cortisol, whereas 11β-HSD2 acts exclusively as a NAD-dependent dehydrogenase that inactivates cortisol by converting it into cortisone, thereby protecting the MR from occupation by cortisol (Figure 1). In peripheral tissues, both enzymes control the ability of cortisol to bind to corticosteroid receptors.[14]

The regulation of glucocorticoid concentrations within specific tissues is an important feature of stress hormone physiology. A critical physiological problem derived from the properties of MR-expressing tissues is that these receptors display a high affinity for active glucocorticoids (cortisol or corticosterone) that is equal to their affinity for the true mineralocorticoid aldosterone.[15,16] Because glucocorticoids tend to circulate at higher levels than does aldosterone, in the absence of regulation by 11β-HSDs, the glucocorticoids inappropriately stimulate

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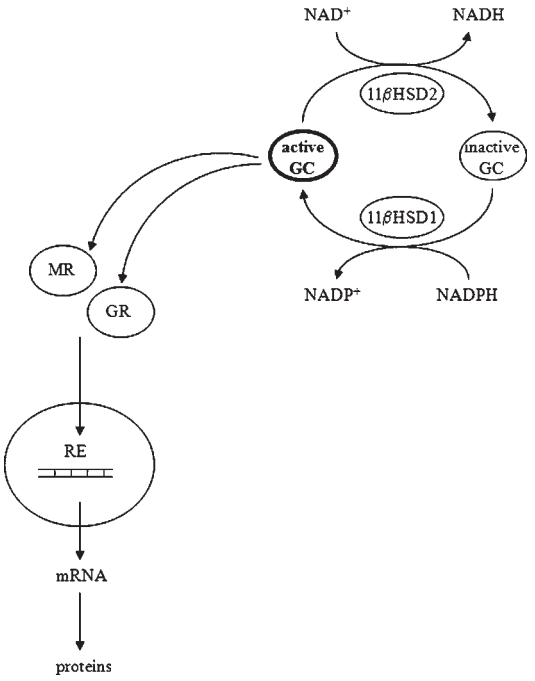

Figure 1. Diagram of prereceptor enzyme-mediated metabolism of glucocorticoids (GC). 11β-Hydroxysteroid dehydrogenase (11β-HSD1 and 2) catalyzes the interconversion of inactive and active glucocorticoids: the inactive form shows little or no effect on the intracellular corticosteroid receptors, whereas the active GC binds to nuclear receptors and, therefore, to the DNA promoter sequence (or responsive element, RE).

mineralocorticoid responses in MR-expressing tissues, resulting in an apparent excess of mineralocorticoids.[9] Additionally, elevated glucocorticoid levels can produce deleterious effects in some GR-expressing tissues,[17,18] particularly the brain.[19−22]

The two isoenzymes display different tissue localizations: 11β-HSD1 is widely expressed, most notably in the liver, lung, adipose tissue, vascular smooth muscle, skin, gonads, bone, and the central nervous system (CNS),[23] whereas 11β-HSD2 is highly expressed in adult mineralocorticoid target tissues such as the kidney, colon, salivary glands, placenta, and adrenal glands.[24]

From this brief description, it is clear that the 11β-HSDs, GR, and MR are closely related and that their synthesis and enzymatic activity might be altered by the presence of an excess of exogenous glucocorticoids. In the present study, we aimed to investigate how the prereceptor system and GR and MR react in beef cattle experimentally treated with glucocorticoids. Specifically, we investigated how the expression of these receptor genes is regulated in several tissues in which the physiological gene expression levels of 11β-HSD1 and 2 differ.[24]

MATERIALS AND METHODS

Animals and Experimental Design. Eighteen male beef cattle (age range from 17 to 22 months) were randomly assigned to three groups of 6 animals each. Group A (n = 6) was treated with dexamethasone per os (0.7 mg/day/animal for 40 days); group B (n = 6) received prednisolone per os (15 mg/day/animal for 35 days); and group K (n = 6) served as a control. The animals were euthanized 7 days after the last treatment. Dosages were decided on the basis of previous literature.[25] The animals were housed in boxes measuring 10 m × 15 m with a concrete floor lacking litter or lateral partitions. All animals were stabled in separate boxes and fed a concentrated diet consisting of corn silage, corn, hay, and a commercial protein supplement; water was supplied ad libitum. The design and protocol of the experiment were authorized by the Italian Ministry of Health and the Ethics Committee of the University of Turin. The carcasses of the treated animals were appropriately destroyed.

Tissue Sampling and Processing. Samples of the adrenal and salivary glands, kidney, liver, lung, bulbourethral gland, prostate, testis, thoracic thymus, cervical thymus, subcutaneous fat, and muscle were collected from each animal and immediately placed in 5−10 volumes of RNAlater Solution (Ambion). Samples were stored at 4 °C overnight and, after removal of the supernatant, were kept at −80 °C for long-term storage.

Total RNA Extraction and Quantitative Expression Analysis of 11β-HSDs, GR, and MR by q-PCR. A few milligrams of each tissue was disrupted using a TissueLyser II (Qiagen) with stainless steel beads in 1 mL of Trizol (Invitrogen); total RNA was purified from any residual genomic DNA with a DNA-free kit (Ambion). The RNA concentration was determined by spectrophotometry, and RNA integrity was evaluated using an automated electrophoresis station (Experion Instrument, Bio-Rad). cDNA was synthesized from 1 μg of total RNA using ImProm-II Reverse Transcriptase (Promega) and a mix of random and oligo dT primers (Promega). To determine the relative amount of specific 11β-HSD1, 11β-HSD2, GR, and MR transcripts, the cDNA was subjected to quantitative polymerase chain reaction (qPCR)[26] using the IQ 5 detection system (Bio-Rad) and respective gene primers in an IQ SYBR Green Supermix (Bio-Rad). Primer sequences were designed using Primer Express (vers. 1.5) (Table 1). The cyclophilin A gene was used as a housekeeping gene control, as reported in previous literature.[27] The amount of relative gene expression was calculated using a relative quantification assay according to the comparative Ct method (ΔΔCt method) when the primer efficiencies were similar; before each qPCR, we performed a validation experiment to demonstrate that the amplification efficiencies of the target and reference genes were approximately equal. The absolute value of the slope of the log input amount versus the ΔCt value was required to be <0.1. Once this was established, the relative abundance of each transcript, normalized to the endogenous housekeeping gene transcript (cyclophilin A) and relative to the control sample, was given by $2^{-\Delta\Delta Ct}$ (fold increase), where ΔΔCt = ΔCt-(treated sample) − ΔCt(control sample), and ΔCt is the Ct of the target gene subtracted from the Ct of the housekeeping gene. The comparative Ct method is essentially an abbreviated version of the relative standard curve method. When the difference between the primer efficiencies of the target and the housekeeping genes was too great, the relative standard curve method was applied, and the expression level of each gene was corrected for its efficiency.[28,29]

Statistical Analysis. Statistical tests were performed using GraphPad InStat (vers. 3.05) statistical software (GraphPad Inc., San Diego, CA). The statistical analysis of 11β-HSD1, 11β-HSD2, GR, and MR gene expression was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test; when Bartlett's test suggested that the difference between the standard deviations of each group was significant, the nonparametric Kruskal−Wallis test with Dunn's post test was applied.

The Grubbs test was used to reveal potential outliers. A p value of <0.05 was considered to be statistically significant.

RESULTS

Molecular Analysis. qPCR specific for GR, MR, 11β-HSD1, and 11β-HSD2 was performed to examine the expression levels of these genes in each tissue. The order of tissues in Tables 2 and 3 reflects the level of 11β-HSD expression under physiological conditions. In group A animals (Table 2), overexpression of

Table 1. Primer Sequences for qPCR

gene	primer	sequence	GenBank no.
GR	forward	5′-CCATTTCTGTTCACGGTGTG-3′	AY238475
	reverse	5′-CTGAACCGACAGGAATTGGT-3′	
11β-HSD1	forward	5′-ACTCTGCGCCAAGATGAAGT-3′	AF548027
	reverse	5′-TAGCCCTCAGGAAGTGCCTA-3′	
11β-HSD2	forward	5′-CCTAGACCGGATCCTTCTCC-3′	AF074706
	reverse	5′-ACCTTGGGGGTCAGAATACC-3′	
MR	forward	5′-GGGTCTATCTGTAGCCCTGGAA-3′	NM_001191349
	reverse	5′-TGTCTGGACTGGGAACCACAT-3′	
cyclophilin A	forward	5′-GCCCCAACACAAATGGTT-3′	NM_178320
	reverse	5′-CCCTCTTTCACCTTGCCAAAG-3′	

Table 2. Fold Gene Expression Changes of the Four Genes in Group A Animals (Dexamethasone Treated) versus Controls (Group K, Which Was Assigned a Control Value of 1)

	normalized fold expression[a] ($2^{-\Delta\Delta Ct}$)			
tissue	11β-HSD1	11β-HSD2	GR	MR
adrenal	0.66 ± 0.44	0.44 ± 0.29*	1.04 ± 0.56	1.19 ± 0.52
salivary glands	0.7 ± 1.42	0.36 ± 0.52	2.35 ± 4.4*	0.75 ± 0.95
kidney	0.67 ± 0.7	0.29 ± 0.47	2.57 ± 2.41***	1.32 ± 1.86
liver	1.4 ± 1.1	0.42 ± 0.21	3.11 ± 1.93**	3.62 ± 1.95*
lung	0.87 ± 0.77	0.58 ± 0.47	1.31 ± 1.5	1.18 ± 1.05
bulbourethral glands	0.57 ± 0.52	0.30 ± 0.22*	0.82 ± 0.93	1 ± 0.7
prostatic gland	0.48 ± 0.88	0.24 ± 0.36**	1.05 ± 1.58	0.62 ± 0.91
testicles	1.5 ± 0.57	0.91 ± 0.21	1.44 ± 0.47	1.18 ± 0.37
thoracic thymus	0.9 ± 1.3	4.00 ± 3.7*	0.65 ± 0.55	4.1 ± 2.95**
cervical thymus	0.73 ± 1.74	6.98 ± 1.68***	0.42 ± 1.02	4.53 ± 7.41***
subcutaneous adipose	0.51 ± 0.5	0.64 ± 0.68	1.75 ± 1.92	2.28 ± 2.19
muscle	1.31 ± 0.79	0.74 ± 0.55	16.36 ± 7.9**	6.61 ± 3.39

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3. Fold Gene Expression Changes of the Four Genes in Group B Animals (Prednisolone Treated) versus Controls (Group K, Which Was Assigned a Control Value of 1)

	normalized fold expression[a] ($2^{-\Delta\Delta Ct}$)			
tissue	11β-HSD1	11β-HSD2	GR	MR
adrenal	1.69 ± 0.73	0.51 ± 0.29*	1.48 ± 0.75	1.34 ± 0.79
salivary glands	0.76 ± 1.32	0.29 ± 0.55	1.32 ± 3.08	0.47 ± 0.71
kidney	0.82 ± 0.63	0.86 ± 0.49	1.34 ± 0.73	0.99 ± 1.02
liver	0.91 ± 0.5	0.31 ± 0.18	1.99 ± 1.03	2.28 ± 1.48
lung	1.17 ± 1.21	0.55 ± 0.26	1.05 ± 0.59	1.01 ± 0.53
bulbourethral glands	0.92 ± 0.86	0.93 ± 0.99	0.92 ± 0.75	1.58 ± 1.44
prostatic gland	0.64 ± 0.69	0.47 ± 0.34	0.83 ± 0.66	0.66 ± 0.56
testicles	1.48 ± 0.95	0.89 ± 0.22	1.14 ± 0.51	1.36 ± 0.48
thoracic thymus	2.19 ± 1.15	0.61 ± 0.32	1.14 ± 0.65	1.24 ± 0.62
cervical thymus	1.36 ± 0.51	0.8 ± 0.41	1.72 ± 0.72	1.04 ± 0.3
subcutaneous adipose	3.9 ± 3.77	4.74 ± 4.11	5.04 ± 3.79***	8.56 ± 7.01**
muscle	0.51 ± 0.26	0.64 ± 0.29	3.35 ± 7.9	1.27 ± 0.94

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

11β-HSD2 and the MR genes was observed in the cervical and thoracic thymus. Additionally, expression of the GR gene in the muscle was up to 16-fold higher than in the control samples. Both corticosteroid receptor genes were overexpressed by approximately 3-fold in the liver, whereas only the GR gene was found to be up-regulated in the salivary glands and kidney (approximately 2.5-fold higher versus the controls). In contrast, expression of the 11β-HSD2 gene in the gonads and adrenals was 2−4-fold lower than in the controls. As recently described by Cannizzo et al.,[30] the thymuses of group A animals were atrophic and the thymus parenchyma was replaced by fat tissue. Histologically, group A animals showed severe thymic cortical atrophy, whereas the medullary framework was still present, though reduced, showing a pronounced rarefaction of lymphocytes. In contrast, no histological changes were observed in the thymus of the group B animals versus the controls.

In the group B animals (Table 3), the gene expression profile was similar to that of group A only in the adrenal samples, in which the 11β-HSD2 gene was expressed at a level approximately 2-fold lower than the controls, and no significant changes in GR gene expression were detected in muscle tissue. The expression of GR was significantly up-regulated by approximately 5-fold in the subcutaneous adipose tissue, and the MR gene was up-regulated by 8.56-fold versus the controls.

DISCUSSION

Corticosteroids (glucocorticoids and mineralocorticoids) have multiple actions in organisms that are mediated via specific intracellular receptors. It is known that, in peripheral tissues, an important control of glucocorticoid action relies on prereceptor metabolism by 11β-HSD1 and 2. These enzymes catalyze the interconversion of the active glucocorticoids corticosterone and cortisol to inert 11 keto-products (11-dehydrocorticosterone and cortisone), thus regulating the access of these glucocorticoids to their receptors.[8] Specifically, 11β-HSD2 is a glucocorticoid-inactivating enzyme that, when colocalized with otherwise nonselective mineralocorticoid receptors (MR), ensures selective access for aldosterone in vivo.[23]

Thus, the prereceptor system of the 11β-HSDs determines glucocorticoid bioavailability, whether exogenous or endogenous in origin, and the synthesis of 11β-HSDs is very likely regulated by exogenous glucocorticoids.

Dexamethasone is a synthetic steroid similar to cortisol. Because ACTH release is reduced after administration of dexamethasone in normal organisms, cortisol levels are also reduced.[31] In human and veterinary medicine, this property is exploited by the dexamethasone suppression test to diagnose Cushing's syndrome.[32] The type 2 isoenzyme (11β-HSD2) converts cortisol into its inactive form, cortisone, and down-regulation of this isoenzyme probably results from reduced substrate availability. In this study, the group A animals showed down-regulation of this enzyme in several tissue types, particularly in the prostate, bulbourethral gland, and adrenals, where 11β-HSD2 is physiologically prevalent. In contrast, expression of this gene was up-regulated in both the thoracic and cervical thymus following dexamethasone treatment, along with a 4-fold increase in MR gene expression. In this case, histological changes were noted in the thymus, confirming the results of a similar study by Cannizzo et al. on veal calves.[25,30] This increase in 11β-HSD2 and MR gene expression in the thymus of dexamethasone-treated animals is probably due to lymphatic tissue substitution by adipocytes. Indeed, physiologically, adipocytes express 11β-HSD2 and MR genes to a greater extent than lymphocytes.[33] In the group A animals, GR and MR gene up-regulation was principally observed in muscle tissue, which showed 16- and 6-fold increases in expression, respectively. The up-regulation of these genes in the liver of the controls was only approximately 3-fold greater than in the livers of the controls following dexamethasone treatment. These data indicate that this synthetic glucocorticoid could stimulate the transcription of the GR gene and secondarily that of the MR gene.

In contrast, major changes in the GR and MR gene expression profiles were observed only in the adipose tissue of the group B animals following prednisolone treatment, whereas the thymuses of these animals showed no remarkable change in gene expression, maintaining a completely normal histological pattern. This finding contrasted with the severe morphological lesions induced by dexamethasone administration.

Prednisolone inhibited 11β-HSD2 gene expression only in the adrenal glands, and no changes in expression of this gene were observed in other organs.

These results suggest a differential response to dexamethasone and prednisolone in beef cattle. This difference is not limited to changes in the regulation of individual genes alone because the tissues also responded differently, perhaps because the dose−response relationships of the two glucocorticoids differ or because their metabolisms follow alternative pathways. Dexamethasone has a strong anti-inflammatory action and a minor mineralocorticoid effect relative to prednisolone. Compared to cortisol, the relative anti-inflammatory potencies of dexamethasone and prednisolone are 30 and 4, respectively, whereas the relative mineralocorticoid potency is 0.05 for dexamethasone and 0.5 for prednisolone.[34] The biological half-life of prednisolone is 8 h (8−24 h), compared to 32 h for dexamethasone (32−72 h). Because 1 half-life of dexamethasone is equal to 4 half-lives of prednisolone, the biological equivalence of the two compounds is approximately 40 mg of prednisone to each milligram of dexamethasone.[35] Our results may also be explained by the fact that, because prednisolone has a weaker effect than dexamethasone, the biological effects of these synthetic glucocorticoids will differ in duration. Moreover, glucocorticoid treatment was halted 1 week prior to sacrifice, when the organs were sampled for molecular analysis; therefore, it can be expected that the biological effects of the two glucocorticoids had been significantly attenuated by that time. The implications of these results have even greater relevance for their potential application to veterinary public health, as our experimental circumstances simulated the situation when animals are treated in the field where, generally, the breeder can discontinue the illegal treatment several days prior to the slaughtering of the animals.

From these preliminary findings, we conclude that dexamethasone and prednisolone act on the 11β-HSD prereceptor system and the downstream receptors in cattle. Specifically, not all tissue types exhibited similar changes in the levels of expression of the four genes, indicating that the effects of dexamethasone and prednisolone on metabolism are very different. This factor may also explain the histological differences observed in the thymus (data not shown). The GR may provide an excellent biomarker for detecting glucocorticoid treatment in cattle and allow the selection of the appropriate tissue types for analysis: muscle tissue for dexamethasone treatment and subcutaneous adipose tissue for prednisolone treatment, both of which can be easily sampled at the slaughterhouse. The only tissue that responded to

both treatments was the adrenal glands, where the gene expression of 11β-HSD2 decreased by approximately 2-fold in both groups A and B. Finally, before reliable screening tests capable of detecting corticosteroid-treated animals can be validated to support the well-established histological testing of the thymus, further research is necessary to test animals treated with different drug dosages, periods of treatment suspension, and, possibly, drug associations to evaluate the gene expression profiles of the corticosteroid receptors and 11β-HSDs in a variety of tissue types.

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