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Role of FKBP51 in the modulation of the expression of the corticosteroid receptors in bovine thymus following glucocorticoid administration

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Abstract

The aim of this work was to study the transcriptional effects of glucocorticoids on corticosteroid hormone receptors, prereceptors (11β-hydroxysteroid dehydrogenase 1 and 2, 11β-HSD1 and 2) and chaperones molecules regulating intracellular trafficking of the receptors (FKBP51 and FKBP52) in thymus of veal calves. Moreover, the expression of FKBP51 and FKBP52 gene were investigated in beef cattle thymus. In the cervical thymus of veal calves dexamethasone administration in combination with estradiol decreased FKBP51 expression ($P < 0.01$). The same treatment increased mineralocorticoid receptor (MR) ($P < 0.01$) and 11β-HSD1 expression ($P < 0.05$) compared to control group in the cervical thymus of veal calves. The thoracic thymus of veal calves treated with dexamethasone and estradiol showed a decreasing of FKBP51 ($P < 0.05$), FKBP52 ($P < 0.05$), glucocorticoid receptor ($P < 0.05$) and MR expression ($P < 0.05$) compared to control group in the thoracic thymus of veal calves. The gene expression of FKBP51 decreased both in cervical ($P < 0.01$) and thoracic thymus ($P < 0.01$) of beef cattle treated with dexamethasone and estradiol. Additionally, also prednisolone administration reduced FKBP51 expression in the cervical thymus ($P < 0.01$) and in the thoracic thymus of beef cattle ($P < 0.01$). The gene expression of FKBP52 increased only in the cervical thymus following dexamethasone administration ($P < 0.01$). The decrease of FKBP51 gene expression in thymus could be a possible biomarker of illicit dexamethasone administration in bovine husbandry. Moreover, so far an effective biomarker of prednisolone administration is not identified. In this context, the decrease of FKBP51 gene expression in thymus of beef cattle following prednisolone administration could play an important role in the indirect identification of animals illegally treated with prednisolone.

Keywords: thymus, bovine, FKBP51, FKBP52, glucocorticoids, real time PCR

1. Introduction

Despite the European Union ban on the use of the synthetic glucocorticoids (GCs) for growth-promoting purposes in bovine livestock [1], these molecules are illegally administered, either alone or in association with anabolic steroids, to improve quality and quantity of meat in veal calves and beef production [2]. However, the analytical methods adopted by official monitoring programs are unable to detect unknown molecules or different drugs administered in combination at very low dosages [2]. So, novel approaches, such as the target organ histology and "*omics*" techniques, have been proposed as screening tools to identify secondary markers of illicit treatments, irrespective of the molecule used [3-7]. In this respect, the thymus atrophy is considered an indirect biomarker of corticosteroid administration in bovine [8-10] and the thymus histology is officially adopted by the Italian National Program for Residue Surveillance (PNR).

The functions of GCs are mediated via their intracellular glucocorticoid (GR) and mineralocorticoid receptor (MR). The affinity of the MR for GCs is high, so that it is substantially occupied under basal conditions, whereas GR are activated with high plasma corticosteroid levels. In the absence of ligand, corticosteroid receptors remain sequestered in complex with chaperone and co-chaperone proteins including HSP90, HSP70, a 23-kDa co-chaperone (p23) and FK506 binding protein 51 (FKBP51) or FK506 binding protein 52 (FKBP52). The association of FKBP52 with receptor-chaperone complexes results in an enhancement of receptor hormone binding [11-13] and allows the nuclear translocation of the complex [14]. In the nucleus the receptor complexes promote the gene transcription, including FKBP51 gene, whose product competes with FKBP52 for the acceptor binding site on HSP90. The receptor-enhanced expression of FKBP51 moves the equilibrium towards the FKBP51-containing complexes which bind with lower affinity to GCs, resulting in impaired nuclear

translocation of the receptor [15]. Interestingly, FKBP51 but not FKBP52, is up-regulated by steroid hormones [16-19], excluding estrogens [20], rendering the FKBP51 protein as a component of an ultra-short regulatory loop in steroids signaling.

An important additional level of regulation is represented by 11β-hydroxysteroid dehydrogenases (11β-HSDs). Indeed, 11β-HSD1 regenerates the active form of GCs, whereas 11β-HSD2 metabolizes active GCs into inactive derivatives [21] thereby protecting the MR from occupation by endogenous GCs [22].

The aim of this study was to investigate how a chronic exposure to synthetic GCs, such as dexamethasone (DEX) and prednisolone (PRD), affects the molecular mechanisms modulating the response to these hormones. So, the gene expression of GR, MR, 11β-HSD1, and 11-βHSD2 was evaluated in thymus of veal calves treated with DEX or PRD. Moreover, the expression of FKBP51 and FKBP52 in the thymus of veal calves and beef cattle experimentally treated with GCs was investigated to establish whether the FKBP51 gene may be considered as a biomarker for the detection of GCs abuse in bovine husbandry.

2. Materials and methods

2.1. Animals and experimental design

The experiments were authorised through the Italian Ministry of Health and the Ethical Committee of the University of Turin. The carcasses of the treated animals were appropriately destroyed (2003/74/CE – DL 16 March 2006, No. 158).

In trial 1, twenty-two Friesian male veal calves at approximately 4 mo of age were used. The calves were housed in 10 x 15 m boxes, with concrete floors lacking litter or lateral partitions. The calves were tethered and fed with liquid milk replacer twice a day (providing per kg: 950 g

dry matter (DM), 230 g crude protein (CP), 210 g ether extract (EE), 60 g ash, 1 g cellulose, 75 mg retinol, 50 mg ascorbic acid, 5 mg Cu, 0.125 mg cholecalciferol, and 80 mg α-tocopherol). The amount of feed was increased gradually to 8 L/calf/d and then gradually increased to 16 L/calf/d. After one mo, 0.5 kg of barley straw (per kg: 900 g DM, 20 g CP, 10 g EE, 60 g ash, and 410 g crude fibre) was added to the diet, according to the recommendations of the European Commission (97/182/EC). The calves were randomly assigned to 4 experimental groups at approximately 5 mo-old: group A (n = 6) was weekly administered 5 mg/animal of estradiol benzoate intramuscular for six wk in combination with 0.40 mg/animal/d of dexamethasone (DEX) *per os* for the last 31 d of treatment; group B (n = 8) was administered 15 mg/animal/d of prednisolone (PRD) *per os* for 31 d; group K1 (n = 8) served as control. The calves were slaughtered at 3 d after the last treatment.

In trial 2, eighteen Charolaise male beef cattle (17 to 22 mo-old) were used. The animals were housed in 10 x 15 m boxes with concrete floors lacking litter or lateral partitions. All animals were fed a concentrated diet comprising corn silage, corn, hay, and a commercial protein supplement; water was supplied *ad libitum*. The beef cattle were randomly assigned to 3 experimental groups: group C (n = 6) was administered with 0.70 mg/animal/d of DEX *per os* for 40 d; group D (n = 6) was administered 15 mg/animal/d of PRD *per os* for 35 d; group K2 (n = 6) served as control. The beef cattle were slaughtered at 6 d after the last treatment. The animals resulted healthy upon intra-vitam and post-mortem examinations.

2.2. Tissue sampling and processing

Samples of cervical and thoracic thymus were obtained from each animal at slaughterhouse. The samples were immediately frozen in liquid nitrogen or placed in 5 to 10 volumes of RNAlater Solution (Ambion) and then stored at -80 °C for molecular analyses.

2.3. RNA extraction, reverse transcription and qPCR

Several milligrams of each tissue sample were disrupted using a TissueLyser II (Qiagen, Hilden, Germany) using stainless steel beads in 1 mL of TRIzol Reagent (Ambion) according to the manufacturer's protocol. The RNA concentration was spectrophotometrically determined, and the RNA integrity was evaluated using an automated electrophoresis station (Experion Instrument, Bio-Rad, Hercules, CA). Using the QuantiTect Reverse Transcription Kit (Qiagen), cDNA was synthesised from 1 µg of total RNA.

The effect of treatments on FKBP51 and FKBP52 mRNA expression in the cervical and thoracic thymus of veal calves and beef cattle was evaluated through quantitative polymerase chain reaction (qPCR). The evaluation on GR, MR, 11-βHSD1 and 11β-HSD2 mRNA expression was limited to veal calves (trial 1), because the expression of these genes in thymus of beef cattle (trial 2) was previously reported [3]. To determine the relative amounts of the specific transcripts, the cDNA obtained from retrotranscription was subjected to qPCR [23] using the IQ5 detection system (Bio-Rad) and respective gene primers in an IQ SYBR Green Supermix (Bio-Rad). Primer sequences of FKBP51 and FKBP52 were designed using Primer3 (vers. 4.0.0) based on reference sequences NM_001192862 and NM_001034322, respectively. Primer sequences of GR, MR, 11β-HSD1 and 11β-HSD2 were previously reported [3]. The peptidylpropyl isomerase A (PPIA) gene was used as a housekeeping gene, as previously described [3]. The expression level of each target gene was calculated using the $2^{-\Delta Cq}$ method, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ [24].

2.4. Statistical analysis

Statistical analyses were performed using Graph-Pad InStat (vers. 3.05) statistical software (GraphPad Inc., San Diego, CA). The analysis of gene expression was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post-test. If Bartlett's test suggested that the difference between the standard deviations of each group was significant, then the nonparametric Kruskal-Wallis test with Dunn's post-test versus the control group was applied. The Grubbs test was used to reveal potential outliers. A P value of < 0.05 was considered statistically significant. The data are shown as the mean arbitrary units ($2^{-\Delta Cq}$) ± SEM.

3. Results

In the cervical thymus of veal calves, the DEX administration in combination with estradiol (group A) increased the expression of MR (mean of mRNA arbitrary units ± SEM: $1.28 \times 10^{-4} \pm 3.48 \times 10^{-5}$) compared with the control group K1 ($4.72 \times 10^{-5} \pm 8.34 \times 10^{-6}$) ($P < 0.01$) (Fig. 1B) and 11β-HSD1 ($2.16 \times 10^{-4} \pm 1.88 \times 10^{-5}$) (Fig. 1C) compared with the control group K1 ($1.18 \times 10^{-4} \pm 2.17 \times 10^{-5}$) ($P < 0.05$). The same treatment also decreased FKBP51 expression ($7.75 \times 10^{-3} \pm 6.40 \times 10^{-4}$) compared with the control group K1 ($1.17 \times 10^{-2} \pm 6.84 \times 10^{-4}$) ($P < 0.01$) (Fig. 1E). No change of GR, 11β-HSD2 and FKBP52 expression was observed in the cervical thymus (Fig. 1A, D, F).

In the thoracic thymus the DEX administration in combination with estradiol (group A) decreased GR expression ($2.58 \times 10^{-3} \pm 7.69 \times 10^{-4}$) ($P < 0.05$) compared with the control group K1 ($5.74 \times 10^{-3} \pm 1.31 \times 10^{-3}$) (Fig. 1A), MR expression ($4.10 \times 10^{-5} \pm 4.04 \times 10^{-6}$) compared with the control group K1 ($1.04 \times 10^{-4} \pm 2.19 \times 10^{-5}$) ($P < 0.05$) (Fig. 1B), FKBP51 expression ($5.40 \times 10^{-4} \pm 8.75 \times 10^{-5}$) compared with the control group K1 ($1.87 \times 10^{-3} \pm 6.92$

$\times 10^{-4}$) ($P < 0.05$) (Fig. 1E) and FKBP52 expression ($1.65 \times 10^{-3} \pm 4.08 \times 10^{-4}$) compared with the control group K1 ($4.54 \times 10^{-3} \pm 1.24 \times 10^{-3}$) ($P < 0.05$) (Fig. 1F). No change of 11β-HSD1 and 11β-HSD2 expression was observed in the thoracic thymus (Fig. 1C, D).

The administration of DEX (group C) in beef cattle reduced FKBP51 expression in the cervical thymus ($1.44 \times 10^{-2} \pm 1.87 \times 10^{-3}$) compared with the control group K2 ($8.08 \times 10^{-2} \pm \times 10^{-2}$) ($P < 0.01$) (Fig. 2A) and in the thoracic thymus ($1.37 \times 10^{-2} \pm 2.42 \times 10^{-3}$) compared with the control group K2 ($5.18 \times 10^{-2} \pm 1.02 \times 10^{-2}$) ($P < 0.01$) (Fig. 2A). Moreover, also PRD administration (group D) reduced FKBP51 expression in the cervical thymus ($2.31 \times 10^{-2} \pm 3.03 \times 10^{-3}$) ($P < 0.01$) (Fig. 2A) and in the thoracic thymus ($1.54 \times 10^{-2} \pm 4.19 \times 10^{-3}$) compared with the control group K2 ($P < 0.01$) (Fig. 2A). The expression of FKBP52 gene increased following DEX administration only in cervical thymus ($2.97 \times 10^{-2} \pm 3.33 \times 10^{-3}$) compared to control group K2 ($1.73 \times 10^{-2} \pm 3.56 \times 10^{-3}$) ($P < 0.05$) (Fig. 2B).

4. Discussion

The administration of DEX caused a decrease of GR expression only in thoracic thymus of veal calves, whereas no change was detected in cervical thymus. These findings are partially consistent with data previously reported in thymus of beef cattle where GR expression did not change in response to DEX treatment [3].

Although many studies showed that FKBP51 expression can be induced via GCs treatment both in *vitro* [17,19] and *in vivo* [16,18], a decrease of FKBP51 expression in thymus of veal calves and beef cattle following DEX administration was detected. Similar results in bovine skeletal muscle following long term administration of DEX were reported [5].

Most of the information about FKBP51 is closely related to human and mouse, and very little information is available about this gene in other animals. Moreover, it should be emphasised that illicit schedules in veal calves and cattle substantially differ, in terms of dosage and duration of administration, from those used in human. Furthermore, unlike the *in vitro* experiments, GCs treatment was halted 3 or 6 d (trial 1 and 2, respectively) before the slaughter, when the organs were sampled for molecular analysis. During this period, it is conceivable that a restoration of the physiological conditions occurs. Indeed, Scharf and colleagues [18] observed that FKBP51 mRNA was significantly up-regulated 4 h and 8 h after a single DEX treatment in the central amygdale of mouse, but returned to baseline already after 24 h. Additionally, even GR down-regulation induced by GCs over-stimulation was reversed by DEX withdrawal [25].

Nevertheless, the subcellular localization of steroid receptors and thereby their activity is also affected by other factors, such post-translational modifications, redox milieu or protrusion of localization signals [reviewed in 26,27].

The long term administration of GCs has been related to many adverse systemic effects. In particular, the hypothalamic-pituitary-adrenal (HPA) axis is suppressed and the full recovery of the suppressed adrenal response may take more several months after the GCs withdrawal [28]. However, the degree of suppression can be affected by several factors, such as the duration of treatment, type of steroid employed and dosage, as well as by the route of the drug administration [reviewed in 29]. Then, it reasonable to suggest that a prolonged exposure to GCs leads to the suppression of the HPA axis also in calves and beef. The consequently reduction of serum cortisol influences the GR expression with a finally decrease of FKBP51 gene expression. Moreover, there is increasing evidence that epigenetic changes in non-coding regions of FKBP51 gene may influence basal and hormone-stimulated

expression. Many studies reported that GCs decreased the FKBP51 DNA methylation [16,30] and this reduction persisted for 1 to 4 wk after the GCs administration was discontinued [16]. However, experimental data suggest that the epigenetic patterns observed may be tissue-specific [16,30] and probably other epigenetic mechanisms, like chromatin modifications, would be implicated in the FKBP51 gene expression [31].

PRD treatment caused a decrease of FKBP51 expression only in the thymus of beef cattle, but not of veal calves. Differences of gene expression in thymus of beef cattle between DEX and PRD treatment have been reported [3]. The difference is not limited to changes of the regulation of individual genes alone, but is also evident at morphological and histological level. Indeed, the DEX treatment induces atrophy in the thymus and the parenchyma is replaced by fat tissue [8,9], whereas no histological lesions were observed in the thymus of the PRD-treated beef cattle [10].

These findings considered together suggest a differential response to DEX and PRD in veal calves and beef cattle, perhaps because of differences in pharmacokinetics and/or pharmacodynamics. Dexamethasone has a stronger anti-inflammatory action and a lower mineralocorticoid effect than PRD [32]. Moreover, the biological half-life of PRD is minor compared to DEX (http://toxnet.nlm.nih.gov). Because PRD has a weaker effect than DEX, the biological effects of these synthetic GCs probably differ in duration.

The increase of 11β-HSD1 and MR gene expression in the thymus of DEX-treated animals is probably due to lymphatic tissue substitution by adipocytes. Indeed, adipocytes express physiologically 11β-HSD1 and MR genes to a greater extent than lymphocytes [33]. On the contrary, in thoracic thymus the MR expression decreased, whereas no change of 11β-HSD1 was detected. The differences between the portions of thymus may be due to different rate of regeneration. Indeed, thymus preserves an intrinsic ability to regenerate after GCs

administration because the bovine thymic parenchyma and activity could be restored, as previously shown by gross and histological observations [9].

Although many articles reported that the expression of FKBP52 gene is not under steroids control, except estrogen [20,34], FKBP52 gene expression changed in thoracic thymus of veal calves and in cervical thymus of beef cattle following DEX treatment. It is possible that the prolonged treatment with DEX finally caused a change in FKBP52 gene expression. Moreover, DEX administration could directly cause no change of gene expression, but through the activation of different indirect mechanisms of regulation.

Regarding estrogens, most studies about FKBP52 are related to *in vitro* experiments using tumor cells [20,34] and very little information is available about this gene *in vivo*. However, the molecular interactions that mediate constitutive or regulated gene activity of this gene are largely unexplored [35] and further studies are needed to better evaluate the mechanism regulating FKBP52 gene expression.

In conclusion, the results of our experiments demonstrated that GCs specifically induce a decrease of the FKBP51 mRNA levels in the thymus of veal calves and beef cattle. This finding could allow the application of FKBP51 expression as a indirect biomarker in a screening test to identify the animals illegally treated with GCs in bovine husbandry. Above all, the gene expression decrease induced by PRD in thymus of beef cattle appears of particular interest because so far an effective biomarker of PRD administration is not identified. Therefore, the implications of these results have greater relevance for their potential application to food safety monitoring, considering that changes of the FKBP51 gene expression may persist for up to a week after the suspension of GC treatment.

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The authors declare no conflicts of interest.

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Figure captions

Fig. 1. Effects of dexamethasone in combination with estradiol benzoate (group A) or prednisolone (group B) on GR (A), MR (B), 11β-HSD1 (C), 11β-HSD2 (D), FKBP51 (E) and FKBP52 (F) gene expression compared with the control group K1 in the cervical and thoracic thymus of veal calves. The results are presented as the means ± SEM. The y-axes show arbitrary units representing relative mRNA expression levels. *$P < 0.05$, **$P < 0.01$ versus the control group K1.

Fig. 2. Effects of dexamethasone (group C) or prednisolone (group D) on FKBP51 (A) and FKBP52 (B) gene expression compared with the control group K2 in the cervical and thoracic thymus of beef cattle. The results are presented as the means ± SEM. The y-axes show arbitrary units representing relative mRNA expression levels. *$P < 0.05$, **$P < 0.01$ versus the control group K2.