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Gene expression profile associated with thymus regeneration in dexamethasone-treated beef cattle

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ABSTRACT

Glucocorticoids (GCs) are illegally used as growth promoters in cattle, and the analytical methods officially applied most likely underestimate the precise frequency of the abuse. As a side effect, the administration of GCs causes fat infiltration, apoptosis, and atrophy of the thymus. However, gross and histological observations carried out previously showed that the thymus preserves an intrinsic ability to regenerate. The aim of this work was to study the transcriptional effects of GCs on genes likely involved in regeneration of the epithelial cell network in the cervical and thoracic thymus of beef cattle treated with dexamethasone (DEX) or prednisolone (PRD) in comparison with a control group. Moreover, the ratio of *bax/bcl2* genes was examined to verify a possible antiapoptotic activity occurring at the same time. In the cervical thymus, DEX administration increased the gene expression of *c-myc* ($P < 0.01$), *tcf3* ($P < 0.05$), *tp63* ($P < 0.01$), and keratin 5 (*krt5*; $P < 0.01$). In the thoracic thymus of DEX-treated cattle, the gene expression of *tcf3* ($P < 0.01$), *tp63* ($P < 0.01$), and *krt5* ($P < 0.05$) was increased. These results suggested that thymic regeneration is underway in the DEX-treated animals. However, the *bax/bcl2* ratio was decreased in both cervical and thoracic thymus of DEX-treated cattle ($P < 0.01$ and $P < 0.05$, respectively), showing an antiapoptotic effect through the mitochondrial pathway. Conversely, PRD administration caused no change in the expression of all considered genes. These results sustain the hypothesis that regeneration occurs in the thymus parenchyma 6 d after the DEX treatment was discontinued. This hypothesis is also supported by the absence of alterations in the thymus of PRD-treated beef cattle. Indeed, previous studies showed the inability of PRD to induce macroscopic and microscopic lesions in the thymus. Therefore, in this context, it is not surprising that PRD induced no alteration of genes involved in the regeneration pathway.

1. Introduction

Synthetic glucocorticoids (GCs) are largely employed as therapeutic agents in farm animals. Moreover, GCs are also illegally administered for growth-promoting purposes in beef production. Dexamethasone (DEX) is one of the most commonly administered GCs and induces fat infiltration, increases apoptosis, and causes atrophy of the thymus in cattle as side effects [1–3]. Conversely, prednisolone (PRD), another illicitly used GC, seems to be unable to induce thymus atrophy [4]. However, it is conceivable that the thymus preserves an intrinsic ability to regenerate after administration of GCs because the bovine thymic parenchyma and activity could be restored, as previously shown by gross and histological observations [3]. Nevertheless, the mechanisms controlling thymus regeneration remain largely unknown. It was previously shown that some transcription factors are overexpressed in the thymic

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stroma of mice [5]. Indeed, the thymic epithelial cells (TECs) of mice showed an upregulation of *c-myc*, *tcf3*, and *tp63* genes during DEX- or irradiation-induced atrophy and a downregulation after regeneration [5]. These transcription factors were previously shown to regulate differentiation of epithelial stem cells in various tissues [6–8], suggesting a role in reconstruction/maintenance of the epithelial cell network. Moreover, it has been demonstrated that DEX- and irradiation-induced damage of the thymus results in proliferation of a specific subset of TEC precursors expressing keratin 5 (*krt5*) [5].

Nevertheless, tissue regrowth is not only the result of cell proliferation but also the result of enhanced cell survival by means of the inhibition of apoptosis or a combination of both mechanisms [9]. Several studies have highlighted that many of the molecular pathways involved in thymus atrophy rely on the mitochondria-dependent apoptotic pathway, involving proteins of the BCL2 family [10,11]. The members of the BCL2 family are known to be key regulatory proteins in apoptotic events and can promote either cell survival or cell death. Indeed, the equilibrium between the proapoptotic and antiapoptotic members or their relative amount is crucial to sensitize the cells toward either survival or apoptosis. The antiapoptotic effect of BCL2 occurs through binding and inhibiting proapoptotic proteins such as BAX. The latter promotes apoptosis by altering mitochondrial functions and activating the release of downstream apoptogenic factors [12]. The aim of this work was to study the biological mechanisms involved in thymus regeneration of beef cattle following treatment with GCs. Therefore, an increase in transcript abundance of *c-myc*, *tcf3*, *tp63*, and *krt5* was hypothesized in the cervical and thoracic thymus during regeneration. In addition, *bax* and *bcl2* expression and their ratio were examined to evaluate a possible antiapoptotic activity occurring at the same time.

2. Material and methods

2.1. Animals

The experiment was 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 (2003/74/CE–DL 16 March 2006, No. 158).

The experimental design was already described in detail by Cannizzo et al [4]. All groups of experimental animals were kept in separate pens of 10 × 15 m and were fed a diet consisting of corn silage, corn, hay, and a commercial protein supplement; animals had ad libitum access to water. Eighteen male Charolais beef cattle of 17- to 22-mo of age were divided into three groups. Treated animals orally received either DEX-21-sodium phosphate at 0.7 mg/d for 40 d ($n = 6$) or PRD at 15 mg/d for 30 d ($n = 6$) per animal, whereas cattle from the control group ($n = 6$) were untreated. Each morning, before the distribution of feed, the animals were tied to the feed trough, and 2 trained technicians administered orally 1 capsule containing the compound using a drenching gun. The control animals were treated with a placebo. The animals were slaughtered 6 d after drug withdrawal. The gross and microscopic findings in the thymus of these animals were previously reported [4].

2.2. Samples

Thoracic and cervical thymus samples from each animal were collected and placed in RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA) to preserve the RNA integrity for molecular investigation.

2.3. Total RNA extraction and reverse transcription

Fifty milligrams of thymus was disrupted using a TissueLyser II (Qiagen, Hilden, Germany) with stainless steel beads in 1 mL of TRIzol (Invitrogen, Thermo Fisher Scientific). Total RNA was purified from any residual genomic DNA with a DNA-free kit (Ambion). The integrity of the RNA was confirmed by the Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA), and the concentration was measured by spectrophotometry. From 1 μg of total RNA, cDNA was synthesized using ImProm-II reverse transcriptase (Promega, Madison, WI) and random primers (Promega).

2.4. Selection of reference genes and analysis of reference genes' expression stability

β-Actin (*actb*), emerin (*emd*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), hypoxanthine phosphoribosyltransferase I (*hprt1*), peptidylprolyl isomerase A (*ppia*), succinate dehydrogenase complex, subunit A (*sdha*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*ywhaz*) were chosen as reference genes (RGs). The RG expression stability was determined using the BestKeeper software [13]. Genes from different functional classes were considered to minimize a potential coregulation of genes. Primer sequences of reference genes were previously reported [14,15] (Table 1).

2.5. Target gene expression analysis by qPCR

The expression of *c-myc*, *tcf3*, *tp63*, *krt5*, *bax*, and *bcl2* in the thoracic and cervical thymus was investigated by quantitative PCR (qPCR). To determine the amount of the target genes, cDNA was subjected to qPCR using the SYBRGreen method and the IQ5 (Bio-Rad) detection system. The primer sequences were designed using Primer Express v 1.5 (Applied Biosystems, Thermo Fisher Scientific) (Table 1). All samples were run in duplicate. The expression level of each target gene was calculated using the $2^{-\Delta Cq}$ method, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{housekeeping gene}}$ [16].

2.6. Statistical analysis

The data were analyzed using GraphPad InStat, version 3.00 (GraphPad Inc, San Diego, CA). The analysis of *c-myc*, *tcf3*, *tp63*, *krt5*, *bax*, and *bcl2* gene expression, the analysis of the ratio of *bax* and *bcl2* expression, and the analysis of live weight gain was performed using one-way ANOVA, followed by Dunnett's post hoc test versus the control