Hepatic tyrosine aminotransferase and glucocorticoid abuse in meat cattle

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HEPATIC TYROSINE AMINOTRANSFERASE AND GLUCOCORTICOID ABUSE IN MEAT CATTLE

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SHORT RUNNING TITLE: Liver tyrosine aminotransferase in glucocorticoid-treated cattle

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ABSTRACT

Besides being extensively applied as therapeutical remedies, glucocorticoids (GCs) - most notably dexamethasone or prednisolone - are also illegally used in livestock for growth-promoting purposes. This study was designed to assess the suitability of liver tyrosine aminotransferase (TAT), a gluconeogenic enzyme known to be induced by GCs, to act as a reliable candidate biomarker to screen for GC abuse in cattle. Enzyme activity was measured spectrophotometrically in liver cytosols or in cell extracts, and TAT gene expression was determined by real-time PCR. Compared to untreated veal calves, a notable scatter (20-fold) and much higher median values (3-fold) characterized TAT specific activity in liver samples from commercially farmed veal calves. A time-related increase in both enzyme activity and gene expression was detected in rat hepatoma cell lines treated with dexamethasone concentrations (10^{-8} or 10^{-9} M) in the range of those recorded in non-compliant samples from EU official controls. In experimental studies in which finishing bulls were administered GCs at growth promoting dosages, however, no such changes were recorded in dexamethasone-treated animals; a statistically significant rise in liver TAT activity (+ 57 % ) only occurred in prednisolone-treated bulls. Although further research is needed to characterize the GC-mediated response in cattle liver, TAT does not appear to be a specific and sensitive biomarker of GC abuse in the bovine species.

Keywords: Tyrosine aminotransferase, gene expression, cattle, dexamethasone abuse, biomarkers.
INTRODUCTION

Dexamethasone (DEX), a fluorinated hydro-cortisone derivative, is characterized by a remarkable glucocorticoid potency associated with a nearly complete lack of mineralocorticoid activity, making this compound the drug of choice for a wide range of therapeutic applications in both human and veterinary clinical practice (Ferguson & Hoenig, 1995). In recent years DEX and other synthetic glucocorticoids (GCs) have been illegally used in meat cattle production to improve the zootechnical performances and the carcase quality (Courtheyn et al., 2002), and/or to synergize the growth promoting effects of other illegal compounds, such as estrogens or β-agonists (Abraham et al., 2004). This has resulted in a sharp increase in bovine non-compliances for corticosteroids; interestingly, the published report referring to 2008 (EC, 2010) showed Italy ranking largely first, with a total of 86 non-compliant results, 35 of them related to DEX.

In order to achieve growth-promoting effects, DEX is usually administered by the oral route at very low dosages for an extended period of time, yielding urinary concentrations often below the “cut-off” of the most common immunoenzymatic kits used in routinary official controls (Girolami et al., 2010). Evidence has been presented indicating that, in line with other GCs, DEX undergoes limited oxidative and conjugative biotransformations in cattle (Vincenti et al., 2009), being able to build up mainly as such in meat and offal (Cherlet et al., 2005). Due to the strong biological activity, DEX residues are potentially hazardous for the consumer’s health (Botsoglou & Fletouris, 2001).

All the above reasons have prompted the researchers to develop reliable biomarkers for revealing the exposure to DEX or other GCs in cattle (Girolami et al., 2010; Nebbia et al., 2011). While some groups have focused their attention on the histological changes occurring in target organs, most notably the thymus (Cannizzo et al., 2008; Cannizzo et al., 2010), others have investigated the GC-mediated effects on blood leukocyte formula (Brambilla et al., 1998) or on blood cortisol levels (Vascellari et al., 2006;). According to the legislation in force, a biological marker should always be used in conjunction with an official analytical method to prove the non compliance of a given sample. Nonetheless, if proved feasible, the biological based approaches may facilitate the screening of more animal populations than is possible by means of the more expensive and time consuming current analytical techniques, thereby ultimately improving the effectiveness of the official controls (Mooney et al., 2009).

The metabolic enzyme tyrosine aminotransferase (E.C. 2.6.1.5, TAT) is predominantly expressed in hepatic parenchymal cells and is involved in tyrosine degradation providing ketogenic
and gluconeogenic substrates to the liver, when amino acids are used as a major source of energy (Dietrich, 1992). An extensive wealth of data indicates that, upon their binding to specific cytosolic receptors (glucocorticoid receptors, GR), GCs are involved in the regulation of gene expression of several key metabolic enzymes, including TAT (for a review, see Gessi et al., 2010). In particular, TAT is well known to be positively modulated by natural or synthetic GCs, providing a good model system for evaluating the GC signalling pathway both in vitro (Rehman et al., 2004) and in vivo (Chirino et al., 1994). As far as DEX is concerned, however, relatively little is known about the effect of the repeated administration on TAT gene expression and/or activity; the latter has been characterized in rats, in which such a biochemical phenomenon has been selected as the endpoint for the determination of NOEL (Bette & Kietzmann, 1991). Conversely, only scant information is available for cattle (Cantiello et al., 2009; Giantin et al., 2010). The present study originates from a preliminary investigation in which hepatic subfractions from either untreated or commercially farmed veal calves were assayed for TAT activity. The suitability of such an enzyme as biomarker for GCs abuse was then investigated by studying the DEX-mediated effects on both TAT gene expression and/or activity first in a rat cell line and thereafter in liver specimens from meat cattle experimentally treated with GCs or 17β-E using growth-promoting schedules. Part of the results of the present study has been presented at the 11th EAVPT International Congress held in Leipzig, and published as an abstract in the meeting proceedings (Bertarelli et al., 2009).

MATERIALS AND METHODS

**Drugs and chemicals**

Desashock® (dexamethasone sodium phosphate) was obtained from Fort Dodge Animal Health S.P.A. (Bologna, Italy). Unless otherwise stated, all other reagents were purchased from Sigma Aldrich (Milan, Italy)

**Animals, treatments, and isolation of subcellular fractions**

The study first involved hepatic cytosols (see below for the description of the isolation procedures) from thirty-eight male veal calves collected in three different abattoirs located in the Piedmont Region (northern Italy) and used in a previous study aimed at investigating the expression of two protein biomarkers for the exposure to illicit growth-promoters (Nebbia et al., 2008). In addition, cytosolic fractions from a total of thirty-five veal calves which served as controls in different experiments performed by our Department were used for comparison.
We have also examined liver samples from a number of trials previously conducted in finishing bulls in the age range 17-22 months, according to the Italian Law (DLgs 116/92) on animal experimentation. In experiment 1, Charolais bulls were allotted to two groups (n=6 each) orally receiving 0 or 1.4 mg DEX per capita/day for 40 days; slaughtering was performed 6 days after treatment withdrawal. In experiment 2, Friesian bulls either untreated (n=6) or treated with 1.4 mg DEX per capita/day for 60 days (n=6) were slaughtered 26 days after the GC treatment was discontinued. Experiment 3 involved twenty-four Charolais bulls, which were assigned to one of the following treatment groups (n=6 individuals each): a) 17β-estradiol (17β-E, 5 i.m. administrations of 25 mg each at 7 days intervals), b) DEX (0.7 mg per capita/day for 40 days), c) prednisolone (15 mg per capita/day for 35 days). A fourth group of 6 animals remained untreated and was regarded as control. All bulls were euthanatized 7 days after receiving the last treatment.

Liver cytosolic fractions were isolated by differential ultracentrifugation as detailed elsewhere (Nebbia et al., 2008), snap frozen in liquid nitrogen, and stored at –80°C until used for biochemical assays.

Cell cultures

TAT-expressing H4-II-E-C3 rat hepatoma cells were obtained from ECACC (European Collection of Cell Cultures, UK) and grown in 10-cm dishes. Cells were maintained in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 2mM L-glutamine, 1000 units/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B, and kept in a humidified chamber at 37 °C and 5% CO2. All the cell culture reagents were purchased from Sigma–Aldrich (Milan, Italy). Cells were trypsinized every 3–4 days for subculturing. Before each experiment, cells were seeded at 3 X 10^6 cells/dish and allowed to attach for 24 hours. Medium was then replaced with 10 mL of fresh medium and cells were treated with DMSO alone (used as control) or with 10^{-9} and 10^{-8} M DEX (Sigma–Aldrich) dissolved in DMSO, whose final concentration in the growth medium did not exceed 0.1% (v/v). Cells were harvested after 2, 4, 8, 16, and 24 h for real-time PCR analysis and TAT activity measurement. For each DEX concentration, three fully independent biological replicates were performed.

RNA extraction and real-time PCR

Total RNA from H4-II-E-C3 and liver specimens was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Contaminating
Genomic DNA was removed by treating each sample with DNase I (Qiagen, Milan, Italy). RNA purity and quantity were evaluated by absorbance readings at the spectrophotometer. The ratio of the optical densities measured at 260 and 280 nm were > 1.9 for all RNA samples. Two µg of total RNA was reverse transcribed into complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Sufficient cDNA was prepared in a single run to perform all the real-time PCR experiments.

Gene-specific intron spanning primers for target (TAT) and reference (Actin, ACT) genes were designed on *Bos taurus* and *Rattus norvegicus* GenBank and Ensembl mRNA sequences, using Primer Express Software (version 3.0, Applied Biosystems). Table 1 summarizes primer information including sequences, gene accession number and amplicon sizes. Each primer set efficiency was comprised between 95 and 100%. ACT was selected as the reference gene since its expression was not influenced by the DEX treatment.

Real-time PCR reactions were performed on 100 ng of cDNA, in a final volume of 25 µl consisting of the SYBR® Green PCR Master Mix (Applied Biosystems) and 150 nM of each primer set. PCR amplification was run on an ABI 7500 Real-Time PCR System (Applied Biosystems) using 96-well optical plates under the following conditions: 5 min at 95 °C for polymerase activation, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Each reaction was run in triplicate, and a no-template control was included using water instead of cDNA. mRNA relative quantification was calculated with the $2^{-\Delta \Delta Ct}$ method (Livak & Schmittgen, 2001) and data were expressed as fold change compared to control samples (*in vitro* experiments) or to the average values of control animals.

**Enzyme assays**

At each incubation time point, cells were harvested with phosphate buffer 0.125 M pH 7.6, lysed through homogenization with Dounce and centrifuged at 21000 x g for 30 min. After determining protein content using bovine serum albumin as a reference standard (Lowry *et al*., 1951), an aliquot of cell supernatants or of liver cytosolic fractions was used for measuring TAT activity with the method of Diamondstone (1966) as modified by Dundjerski *et al*., (1996). Enzyme activity was expressed as nmoles of the reaction product ($p$-hydroxybenzaldehyde) formed/min mg protein$^{-1}$ (mean ± SEM).
Data and statistical analysis

Where appropriate, statistical comparisons were carried out using non-parametric tests, and namely Mann-Whitney *t*-test or Kruskall-Wallis ANOVA followed by Dunn’s test (Instat 3.0, Graph Pad Software, Inc., San Diego, USA). A P value of 0.05 or less was considered significant.

RESULTS

Liver TAT activities in veal calves

A breakdown of all values of TAT activity that were assayed in veal calf liver preparations from either untreated individuals serving as controls in different experiments (n=35) or from commercially farmed animals (n=38) is presented in Fig. 1. The median value of the control group was 3.0 nmoles/min mg protein\(^{-1}\) while that of field specimens was thrice as high (9.1), with 50% of the samples showing values more elevated than the highest one measured in the untreated veal calves. A high statistically significant difference between the two groups was recorded (P<0.0001).

DEX-mediated increase in TAT mRNA and specific activity in cell lines

The treatment of rat hepatoma cell lines with DEX induced a time-related increase in TAT mRNA reaching the maximal values after 24 h of incubation. Such an increase was much more pronounced and straightforward with 10\(^{-8}\) M DEX (Fig. 2A) than with 10\(^{-9}\) M DEX (Fig. 2B). Enzyme activity, which was measured only after 8, 16, and 24 h of incubation, roughly paralleled the increase in TAT gene expression except for the last timepoint, where an apparent decline occurred (Fig. 2A and 2B).

Effects of DEX and other growth-promoters on liver TAT mRNA and specific activity in finishing bulls

As depicted in Table 2, there was no effect of DEX administration (1.4 mg/per capita/day for 40 days) on either TAT mRNA levels or enzyme activity in liver samples collected 6 days after the last treatment. In experiment 2, where bulls were administered DEX using the same dosage for a longer period (60 days) and applied a “withdrawal time” of 26 days, a small (around 40%) but statistically significant increase in TAT activity was recorded. As regards experiment 3, in which only the enzyme activity could be measured, neither DEX (0.7 mg/per capita/day for 40
days) nor 17β-E treatments elicited appreciable changes; by contrast, liver samples from bulls receiving PRED at a growth-promoting schedule (15 mg/per capita/day for 40 days) exhibited a notable and statistically significant increase in TAT activity (+57%). When compared with each other, no statistically significant differences in enzyme activity occurred in the groups receiving no treatments and respectively regarded as controls (Table 2).

DISCUSSION

Based on a large body of literature indicating that the increase in TAT gene expression and/or activity are considered among the most sensitive events characterizing the genomic-mediated cell response to GCs (Coutinho & Chapman, 2011), the present study has been designed to ascertain whether the measurement of the enzyme activities and/or transcript levels could represent a suitable tool to screen for the abuse of such drugs in slaughtered meat cattle.

As mentioned before, the availability of a number of veal calf liver subfractions belonging to individuals from previous experimental or field studies prompted us to investigate the variation of TAT activity. Animals serving as controls in experimental studies displayed a limited variability (less than 7 folds), despite the occurrence of possible sources of variation such as breed (i.e. crossbreds vs. Friesian), housing (i.e. individual stalls vs. collective pens) or feeding (i.e. exclusive milk feeding vs. solid feed supplementation), farm location, and season in which the experiments took place. Surprisingly, a huge scatter (around 20 folds) characterized the hepatic TAT specific activity measured in samples from commercially farmed veal calves slaughtered in different abattoirs of Piedmont (northern Italy). Despite the fact that they were declared “officially untreated”, they displayed changes in the expression of liver protein biomarkers, namely reticulocalbin and adenosine kinase (Nebbia et al., 2008), similar to those recorded in veal calves experimentally treated with 17β-estradiol, clenbuterol, and dexamethasone (Gardini et al., 2006). As will be discussed later on, many factors are reported to modulate TAT gene expression and activity, but a remarkable increase of TAT specific activity is consistently expected only after the exposure to steroids, most notably GCs (Ramakrishnan et al., 2002). This prompted us to perform further in vitro and in vivo investigations.

A crucial step was to assess whether GC concentrations of the same order of magnitude of those resulting in liver of cattle administered with growth promoting schedules were able to elicit measurable changes in TAT gene expression and activity under in vitro conditions.
Based on both the official results of EU residues control plans concerning the amount of DEX residues found in non compliant liver samples (EC, 2010) and analytical data derived from experimental work conducted on cattle (Cannizzo et al., 2011), DEX concentrations of $10^{-8}$ and $10^{-9}$ M were respectively selected for in vitro studies with a TAT-expressing rat hepatoma cell line (Johansson et al., 2005). In our work, both concentrations were able to elicit a dose- and time-related increase in TAT mRNA (up to nine- or three-fold for $10^{-8}$ M and $10^{-9}$ M DEX, respectively), which was substantially matched by a rise in TAT specific activity. Our findings are difficult to compare with those from previously published studies, which were mostly performed with rat (e.g. Schuetz & Guzelian, 1984; Schmid et al., 1987) or human primary cultures (Pascussi et al., 2001) or with murine cell lines (Visser et al., 2010). Comparable results in terms of enzyme fold inductions (specific activity) were reported by Johansson et al. (2005) using the same cell line.

The effects of DEX and other steroids administered at growth promoting schedules on liver TAT gene expression and activity have been then examined in experimentally treated finishing bulls. In line with data from previously published reports concerning veal calves (Cantiello et al., 2009) or cattle (Giantin et al., 2010), we were unsuccessful in detecting an appreciable and consistent increase in either mRNA content or enzyme specific activity in animals from any of the experiments performed, irrespective of the DEX treatment protocol. Indeed, due to the rapid kinetics of the drug in cattle (Cherlet et al., 2005; Vincenti et al., 2009), one can reasonably exclude that the limited, even if statistically significant increase in TAT activity displayed by bulls from experiment 2 as long as 26 days after the last application, could be ascribed to DEX effects (see later on the discussion about factors affecting TAT regulation). In a study aimed at defining DEX hormonal NOEL in the rat, the least effective oral dose capable of increasing liver TAT activity in a statistically significant way (+38%, P<0.05) after repeated administration (7 days) amounted to 2 $\mu$g/kg bw/day (Bette & Kietzmann, 1991). This dosage closely approximates the daily amount of DEX, expressed as per Kg bw, that was administered to bulls from experiments 1 and 2 (around 2.3 $\mu$g/kg bw/day) for a much longer period of time. The reason for such a discrepancy is puzzling. Relevant differences between the two species in certain GC-mediated responses have been reported. For example, in rats and in several other species DEX is known to enhance the transcription rate of the drug-metabolizing enzyme CYP3A in liver through a complex mechanism mainly involving the pregnane X receptor (PXR), whose expression is, in turn, regulated by GR (Pascussi et al., 2000; Goodwin et al., 2002). By contrast, no increase in the amount of hepatic CYP3A transcripts has ever been observed in
calves (Greger & Blum, 2007; Cantiello et al., 2009) or cattle (Giantin et al., 2010) repeatedly administered with DEX by the oral or the parenteral routes. More to the point, in vivo experiments performed with DEX (Shirwany et al., 1986) or methylprednisolone (Ramakrishnan et al., 2002) indicate that in the rat the extent of liver TAT induction is correlated with cytosolic GR levels, which become down-regulated as the result of the multiple exposure to GCs. Conversely, it has been reported that veal calves or cattle treated with DEX at growth-promoting dosages exhibited little variation (Giantin et al., 2010) or even an increase in the amount of hepatic GR mRNA (Cantiello et al., 2009; Divari et al., 2011). Such findings would be in contrast with the lack of a consistent rise in TAT expression observed in our study in DEX-administered bulls, although contradictory results concerning the parallelism between TAT induction and GR levels have also emerged in human (Duret et al., 2006) or murine cell lines (Visser et al., 2010).

Earlier evidence for TAT induction has been provided in rats repeatedly administered with 17β-estradiol (Braidman & Rose, 1971) and in women taking estrogen-progestogen preparations for contraceptive purposes (Rose & Cramp, 1970). By contrast, data from our study do not support a positive role of 17β-estradiol in TAT modulation. Interestingly, a synergism between 17β-estradiol and DEX in the enhancement of TAT activity has been demonstrated in the rat (Nemeth et al., 1984), but has not been observed in cattle administered with a combination of 17β-estradiol and DEX (Giantin et al., 2010).

In our study, a clear and consistent increase in TAT activity occurred only in PRED-administered bulls, thereby matching what observed in rats after the acute exposure to the GC at much higher doses (Jusko, 1995). For some of the most important GC effects (i.e. anti-inflammatory action, hyperglycemia), PRED potency has been estimated to be about 1/7 of that displayed by DEX (Punthakee et al., 2003), which is well reflected by the different schedule used in our study (15 mg/day vs. 1.4 mg/day). However, unlike DEX, PRED exhibits a slow clearance in cattle - especially when used as acetate ester, i.e. the most common form in commercial preparations - and is characterized by the generation of prednisone and possibly other metabolites still retaining GC activity (EMEA, 1999). Although such characteristics could explain the pronounced depression of the hypothalamo-pituitary-adrenal axis recorded in treated animals and lasting up to 4-6 weeks after drug discontinuation (Toutain et al., 1985), it remains to be established whether and to what extent they might also have contributed to the rise in TAT activity observed in the present investigation.
In conclusion, data from this study indicate that the measurement of liver TAT expression and/or activity would not appear to be a reliable candidate biomarker for DEX exposure in cattle and suggest that TAT regulation in the bovine species is accomplished by mechanisms different from those occurring in rats. Further studies at a molecular level are warranted to confirm such a hypothesis and to get further data concerning the possible inducing effects of PRED.

Finally, in the light of the experimental results described herein, where limited changes in TAT activity were noted also in the case of a statistically significant induction (viz PRED-treated bulls), the huge variation in enzyme activity (around 20-fold) we observed in commercially farmed veal calves is difficult to explain. Genetic polymorphisms have been demonstrated in dogs (Khan et al., 1998) and humans (Hühn et al., 1998) but, to our knowledge, not yet examined in cattle. Age has been reported to affect liver TAT inducibility, with an almost 10-fold increase in the threshold response to DEX between 1-month-old and 12-month-old rats (Chirino et al., 1994). This does not appear to be the case in veal calves, since, as mentioned before, DEX application failed to affect TAT mRNA in either 1-day-old (Greger & Blum, 2007) or in 4-month-old animals (Cantiello et al., 2009). Different kinds of stress (e.g. immobilization, cold) applied to rats have been found to increase both plasma corticosterone levels and liver TAT activity (Al-Mohaisen et al., 2000), the extent of the latter being in young adult subjects (10 months) about twice that measured in old individuals (25 months) (Wellinger & Guigoz, 1986).

As assessed by both an increase in blood cortisol levels and GR down-regulation in circulating lymphocytes, long term and short term transportation are able to elicit a stressing response in calves (Odore et al., 2004) and cattle (Odore et al., 2011), respectively. In addition, a dramatic increase (up to 7 folds) in both cortisol and cortisone urinary concentrations was recorded in slaughtered veal calves compared with pre-slaughter values (Ferranti et al., 2011). Further studies are needed to gain further insight into the role played by stress in regulating TAT expression and activity in the bovine species.

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chemical ionization-tandem mass spectrometry to monitor residue depletion kinetics.


FIGURE LEGENDS

Fig. 1: Variation of TAT activity in veal calf liver samples. Left: untreated control animals; right: commercially farmed animals slaughtered in abattoirs located in Piedmont (northern Italy). Dotted line: median values.

Fig. 2: Time-dependent increase in TAT gene expression and activity in H4-II-E-C3 cells upon incubation with different concentrations of DEX. A: $10^{-8}$ M; B: $10^{-9}$ M. Data are the average of a three independent biological replicates.
Figure 1.
Figure 2.