Horm Mol Biol Clin Invest 2010;2(2):257–265 © 2010 by Walter de Gruyter • Berlin • New York. DOI 10.1515/HMBCI.2010.029

Effect of trenbolone acetate plus estradiol on transcriptional regulation of metabolism pathways in bovine liver

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Abstract

Background: The use of anabolic steroids is forbidden for food producing animals in the EU. Owing to the advantages of anabolics for production profitability, illegal application is appealing. Anabolics are known to influence gene expression of several tissues. We focused on the liver because of its important role in nutrient and hormone metabolism. The aim of the present study was to find differentially regulated metabolic pathways, which might be used as treatment biomarkers.
Material and methods: A total of 18 Nguni heifers were allocated equally to a control group and a treatment group and were implanted with Revalor H. Expression of 34 target genes was measured using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).
Results: Upregulation of androgen receptor and insulin-like growth factor 1 (IGF-1) and downregulation of IGF-2, insulin-like growth factor binding protein 2, steroid hormone binding globulin, insulin receptor α, insulin receptor β, tyrosine aminotransferase, 17β-hydroxy steroid dehydrogenase 2,3-hydroxy-methylglutaryl-coenzym-A-synthase, cathepsin B, hepatocyte growth factor, steroidogenic acute regulatory protein, apolipoprotein 2 and tumor necrosis factor α was demonstrated.
Conclusion: Several biochemical pathways showed different regulations on mRNA level under the influence of trenbolone acetate plus estradiol. The inhibition of nutrient metabolism and protein breakdown seems to support growth processes. IGF-1 plays an important role in growth and development and thus the upregulation of IGF-1 could be responsible for the stimulation of growth in treated animals. The upregulation of IGF-1 could also be revealed as a possible risk factor for the generation of artherosclerotic plaques, which are known as long-term side effects following the use of anabolic steroids. Principal components analysis of RT-qPCR results showed that both groups arrange together and can be clearly separated. Therefore, these might be used as possible biomarkers in bovine liver.

Keywords: anabolic steroids; estradiol; liver; mRNA gene expression; principal components analysis; trenbolone acetate; reverse transcriptase quantitative polymerase chain reaction.

Introduction

Anabolic agents are used in animal husbandry owing to their capacity to increase growth rate, the amount of lean body mass and feed efficiency (1). According to the profitability in lean meat production, the application of these drugs is admitted and wide spread in countries such as the USA and Canada. In the European Union (EU), the use of anabolic agents is only permitted for zootechnical purposes such as estrus synchronization and estrus induction, but is forbidden in food production since 1988 because the potential health risks for the consumer can hardly be estimated. Steroid hormones are known to influence many primary and secondary dependent tissues in the organism. Liver plays an important role in the metabolism of steroid hormones, e.g., hormone decomposition, and production of cholesterol as a precursor of steroid hormones. Additionally, many metabolic pathways in liver are controlled by steroid hormone action. Therefore, the liver has been chosen to be the main objective in this investigation. For this study, target genes participating in these pathways were identified and analyzed to reveal changes due to anabolic action. Target genes have been assigned to different functional groups.

After binding to intracellular receptors, reproductive hormones act indirectly on the muscle cell by stimulation of growth factors in the liver and several other organs (2). This observation provides the basis for the selection of different hormone receptors such as the androgen receptor (AR), estrogen receptors (ERα, ERβ), growth hormone receptor (GHR), insulin receptors (IRα, IRβ) and insulin-like growth factor 1 receptor (IGF-1R), as well as growth factors such as insulin-like growth factors (IGF-1, IGF-2) and insulin-like growth factor binding proteins (IGFBP-1, IGFBP-2). In addition, steroid hormone binding globulin (SHBG), which is responsible for the humoral transport of steroid hormones, has been investigated (3).

The rate of muscle growth is affected by variation of the rate of protein synthesis, the rate of protein degradation or both. This can be controlled by various hormones, e.g., estro-

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Received March 4, 2010; accepted April 26, 2010; previously published online June 11, 2010

gens and androgens (4). To study the influence of anabolic hormones on protein metabolism, the catabolic enzymes cathepsin L (CTSL), cathepsin B (CTSB) and tyrosine aminotransferase (TAT) have been investigated.

The decrease in protein synthesis appears to be mediated in part by proinflammatory cytokines, such as tumor necrosis factor α (TNFα). Its suppression of the IGF-1 functional complex would mediate their catabolic effects (5–9).

The liver is the center of steroid hormone metabolism. Enzymes, which are involved in the biological inactivation of steroid hormones, could be influenced by the exogenous application of anabolic steroids. Therefore, different 17β-hydroxy steroid dehydrogenases (HSD17B2, HSD17B4, HSD17B8) have been examined (10, 11). In addition to steroid hormone elimination, the liver also plays an important role in steroidogenesis as the starting product cholesterol is synthesized in the liver. Thus, the following factors affecting cholesterol metabolism were investigated: 3-hydroxy-methyl-glutaryl-coenzym-A (HMGCoA-) synthase, HMGCoA-reductase and farnesyldiphosphate-farnesyltransferase (FDFT), which are key enzymes in the synthetic pathway of cholesterol formation from acetyl-CoA. Particular focus should be brought to the expression of FDFT as it catalyzes the first reaction only leading to cholesterol (12). Sterol regulatory element binding proteins (SREBP-1, SREBP-2) and their activator SREBP cleavage activating protein (SCAP) are important factors in the feedback regulation of cholesterol synthesis. The rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane. This process is regulated by steroidogenic acute regulatory protein (STAR) (13, 14). The first reaction in the synthesis of steroid hormones is the conversion of cholesterol to pregnenolone. This reaction is catalyzed by cytochrome P450 11A1 (CYP11A1).

In humans, misuse of anabolics often leads to severe cardiac incidents due to atherosclerosis. Anabolic steroids are known to have an atherogenic influence by decreasing the levels of high-density lipoprotein (HDL)-cholesterol. This impairment of the plasma lipid level is associated with an increased risk for cardiovascular diseases (15). The drop-down in the HDL-fraction possibly originates from an accelerated HDL-catabolism, which is mediated by hepatic lipase (LIPC) and lipoprotein lipase (LPL) (16, 17). Different apolipoproteins (APOA1, APOC2) are also associated with an increased risk of atherosclerosis.

Sex hormones have been implicated in the generation of certain forms of cancer and liver tumors (18, 19). The protooncogenes myelocytomatosis cellular oncogene homolog (c-myc) and myeloblastosis viral oncogene homolog (v-myb) were analyzed. Carcinogenesis is usually linked with a dysregulation of cell proliferation. Thus, a member of one class of transcription factors, namely CAAT enhancer binding proteins (CEBPA), and the proliferation factor hepatocyte growth factor (HGF), which have an impact on the regulation of liver cell proliferation, were investigated.

Revalor H was chosen for treatment in the present study because it is an anabolic supplement widely used and licensed in the Republic of South Africa. Several investigations showed its efficacy in cattle and the beneficial effect on meat quality and quantity (20). In addition, the activity of this combination on day 42 has been proven, as the intended effect is persistent up to day 105 after implantation and residues of the applied drug are detectable in blood up to day 90 (21).

The aim of the present study was to identify transcriptional changes in metabolic pathways of liver, which could point to functional changes in liver physiology due to anabolic action. Additionally, it should be attempted to find gene expression patterns, which could be used as possible biomarkers for anabolic treatment. The application of appropriate biostatistical tools should help to extract the required information from the measured gene expression patterns (22).

Experimental

Experimental design

A total of 18 healthy non-pregnant, cyclic Nguni heifers were divided into a control group and a treatment group with nine animals in each group. Animals were housed under controlled conditions in a fenced loose barn without any access of unauthorized individuals. The treatment group was implanted with Revalor H, an anabolic preparation licensed in South Africa, containing 140 mg trenbolone acetate plus 20 mg estradiol-17β (Intervet, Spartan, South Africa) into the middle third of the pinna of the ear, according to the manufacturer's instructions.

Animal attendance was done by the Onderstepoort Veterinary Institute (Onderstepoort, Pretoria South Africa). The animals were housed and fed according to practice.

At slaughter on day 42, liver samples were collected. The tissue was conserved in RNA later (Applied Biosystems, Darmstadt, Germany), immediately after the removal and stored until analysis at −80°C.

Determination of progesterone values

Plasma samples for analysis of progesterone were taken during the course of the animal trial on days −11 and −5 before treatment and on days 2, 7, 9, 16, 22, 24, 29, 32 and 39 after the application of the anabolic preparation. Plasma progesterone levels were determined using an enzyme immunoassay as previously described (23).

Extraction of total RNA

Total RNA was extracted with the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Total RNA concentration was measured using the NanoDrop (peqLab Biotechnologie GmbH, Erlangen, Germany). $OD_{260/230}$ and $OD_{260/280}$ ratios were checked considering sample purity.

Analysis of RNA integrity

Control of RNA integrity was performed with the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The eukaryotic total RNA Nano Assay (Agilent Technologies) was used for sample analysis and the RNA integrity number (RIN) served as RNA integrity parameter. The RIN is calculated based on a numbering system from 1 to 10, with 1 being most degraded and 10 being most intact (24).

Primer design

Primer pairs were newly designed using published bovine nucleic acid sequences of GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) or previously established primer sequences were used. Newly designed primers were ordered and synthesized at Eurofins MWG (Ebersberg, Germany).

Primer sequences of the reference genes (RGs) and the target genes are summarized in Table 1.

Reverse transcription (RT)

RNA samples were converted to cDNA using M-MLVH$^-$ reverse transcriptase (Promega, Regensburg, Germany). In total, 500 ng RNA was diluted to a final volume of 13 μL. The mastermix for the reverse transcription was prepared as follows: 4 μL 5× reaction buffer (Promega), 1 μL random primers (Invitrogen, Karlsruhe, Germany), 1 μL dNTP (Fermentas, St. Leon-Rot, Germany), 1 μL M-MLVH$^-$ reverse transcriptase (Promega). After adding 7 μL of the mastermix to the diluted sample, the plate was inserted into the Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany) and temperature protocol was started: 21°C, 10 min; 48°C, 50 min; 90°C, 2 min; 4°C hold. After cDNA synthesis, all samples were diluted 1:1 to a final volume of 60 μL. Reverse transcription was done in replicates and twice for the same biological sample and a pool of both reactions was created for every sample to gain a sufficient volume for analysis.

Quantitative PCR (qPCR)

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) by a standard protocol recommended by the manufacturer. All components necessary for real-time RT-qPCR were mixed in the reaction wells. The mastermix was prepared as follows: 7.5 μL 2× MESA Green qPCR MasterMix, 1.5 μL forward primer (10 pmol/μL), 1.5 μL reverse primer (10 pmol/μL), 3.0 μL RNase free water (5Prime, Hamburg, Germany). Per well, 13.5 μL mastermix plus 1.5 μL containing 12.5 ng cDNA was added. The plate was sealed, placed in the iQ5 Cycler (Bio-Rad, München, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; annealing temperature according to Table 1, 60 s) and melting curve analysis.

Data evaluation

Expression data were analyzed using relative quantification. Suitable RGs for normalization of gene expression data were evaluated using the geNorm and Normfinder algorithm in GenEx v. 5.0.2.8 software (multiD Analyses AB, Gothenburg, Sweden). Optimal number of RGs was selected using pairwise variation analysis integrated in geNorm algorithm implemented in GenEx. The geometric mean of two selected and validated RGs (Ubiquitin, Histon H3) was used as reference gene index. Data were normalized and relatively compared to the control group according to the ΔCq model (25) with the following formulas:

$$\Delta Cq = Cq_{(\text{target gene})} - Cq_{(\text{reference gene index})}$$
$$\Delta\Delta Cq_{(\text{treatment})} = \Delta Cq_{(\text{treatment})} - \text{mean } \Delta Cq_{(\text{control})}$$

The expression ratio of an RG compared to the control group is expected to be $2^{-\Delta\Delta Cq}$.

Relative expression data were statistically evaluated using Sigma Stat 3.0 (SPSS Inc., Chicago, IL, USA). The Student t-test was used to determine p-values. Results with $p < 0.05$ were considered as statistically significant.

To visualize the multivariate response of the selected classifier genes to the treatment, the principal components analysis (PCA) method was employed using GenEx v. 5.0.2.8 (multiD Analyses AB). PCA involves a mathematical procedure that transforms a number of variables (here normalized expression values) into a smaller number of uncorrelated variables called principal components. By this, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot, here two dimensions. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible (26).

Results

Plasma progesterone levels

Analysis of plasma progesterone levels (Figure 1) showed a decrease in the treatment group compared to the control group. Significant differences between the control and treatment group could be observed at days 2, 22, 24, 29, 32 and 39 showing a clear initial response to the anabolic treatment and documenting the ongoing effectiveness of the anabolic preparation Revalor H until the end of the animal trial.

RNA integrity

All 18 samples were examined using the Agilent 2100 Bioanalyzer. The RNA samples showed good RNA quality with a mean RIN of 8.34 ± 0.36, which is perfectly suitable for PCR analysis (27).

Gene expression analysis

Out of 34 investigated genes, 11 were significantly regulated in the treatment group compared to the control group, four genes did not reach the significance level, but a trend for regulation could be observed ($p < 0.1$). Significant regulations could be obtained for genes from the receptors and endocrine factors group, the cholesterol metabolism group, the hormone decomposition and the protein metabolism group. The x-fold regulations of the significantly regulated genes are shown in Figures 2 and 3 as whisker-box plots. The box area of the plot represents 50% of all observations between the lower and upper quartile, the dotted line shows the sample median and whisker the outer 50% of the data.

In the endocrine group, an upregulation of AR and IGF-1 and a downregulation of IGF-2, IGFBP-2, SHBG, IRα and IRβ was observed. AR was upregulated in the treatment group in comparison to the control group by a mean factor of 1.68 ($p = 0.01$). IGF-1 was upregulated by a mean factor of 1.89 ($p = 0.012$). IGF-2 was downregulated by a mean factor of 0.77 ($p = 0.013$). IGFBP-2 showed a significant

Table 1 Sequences of the used primers with product length [bp], annealing temperature (T_M) and accession number, primers without accession numbers have been obtained from coworkers.

Functional group	Gene	Sequence [5′→3′]		Product length [bp]	T_M [°C]	Accession no.
Receptors	AR	FOR	CCT GGT TTT CAA TGA GTA CCG CAT G	172	60	AY862875
		REV	TTG ATT TTT CAG CCC ATC CAC TGG A			
	ERα	FOR	AGG GAA GCT CCT ATT TGC TCC	233	60	NM_001001443
		REV	GGT GGA TGT GGT CCT TCT C			
	ERβ	FOR	AAT CCA TCC TAC CCC TGG AG	164	64	NM_174051
		REV	ATT TGG GCT TGT GGT CTG TCG A			
	GHR	FOR	CCA GTT TCC ATG GTT CTT AAT TAT	137	60	NM_176608
		REV	TTC CTT TAA TCT TTG GAA CTG G			
	IRα	FOR	TCC TCA AGG AGC TGG AGG AGT	89	62	AJ488553
		REV	TTT CCT CGA AGG CCT GGG GAT			
	IRβ	FOR	TCC TCA AGG AGC TGG AGG AGT	111	60	AJ320235
		REV	TAG CGT CCT CGG CAA CAG G			
	IGF-1R	FOR	CCC AAA ACC GAA GCT GAG AAG	200	60	XM_606794
		REV	TCC GGG TCT GTG ATG TTG TAG			
Endocrine factors	IGF-1	FOR	CAT CCT CCT CGC ATC TCT TC	239	62	NM_00177828
		REV	CTC CAG CCT CCT CAG ATC AC			
	IGF-2	FOR	ACC CTC CAG TTT GTC TGT GG	166	62	BC126514
		REV	ACA CAT CCC TCT CGG ACT TG			
	IGFBP1	FOR	ACC AGC CCA GAG AAT GTG TC	244	64	NM_174554
		REV	GTT TGT CTC CTG CCT TCT GC			
	IGFBP2	FOR	AGC ATG GCC TGT ACA ACC TC	157	60	NM_174555
		REV	CCC TGC TGC TCG TTG TAG AA			
	SHBG	FOR	ACT TGG GAT CCA GAG GGA GT	188	62	NM_174555
		REV	TCC CCA TGG ATC TTC ACT TCC			
Protein metabolism	CTSB	FOR	GGT TGC AGA CCG TAC TCC AT	391	62	NM_174031
		REV	CAT TGT CAC CCC AAT CAG TG			
	CTSL	FOR	CAC TGG TGC TCT TGA AGG ACA	177	62	NM_174032
		REV	TAA GAT TCC TCT GAG TCC AGG C			
	TAT	FOR	ACC CTT GTG GGT CAG TGT TC	165	60	NM_001034590
		REV	ACA GGA TGG GGA CTT TGC TG			
Transcription factors/ proliferation	c-myc	FOR	TCT TGC GCC TAA ATT GAC CTA TTG	153	60	NM_001046074
		REV	GCC AAG GTT GTG AGG TTG TTC			
	v-myb	FOR	CTG TCA AGT CCA CCC CAG TTA	186	63	NM_176635
		REV	TTC TGA GGA TGG TTG GTG GAG			
	HGF	FOR	GAT GTC CAT GGG AGA GGA GA	170	62	NM_001031751
		REV	TCA GGA ATT GTG CAC CCA TAA TTA G			
	CEBPA	FOR	CCA AGA AGT CCG TGG ACA AG	184	62	NM_176784
		REV	AGT TCG CGG CTC AGT TGT TC			
Immune factors	TGFβ	FOR	ACG TCA CTG GAG TG TGC GG	155	63	NM_001166068.1
		REV	TTC ACG CCG TGA ATG GTG GCG			
	TNFα	FOR	TCT GCC ATC AAG AGC CCT TGC C	185	64	NM_173966
		REV	GCG ATG ATC CCA AAG TAG ACC TGC C			
Cholesterol metabolism	HMGCoA-Synthase	FOR	GAT GGT CGC TAT GCA CTG GT	132	60	BC112666.1
		REV	GCC CTC TCT CGA GGA CCA GA			
	HMGCoA-Reductase	FOR	CTC TCT AAA ATG ATC AGC AT	246	60	
		REV	TCA ACT TTT CTT TCT CTG TTT			
	FDFT	FOR	GAA ATG CGC CAT GCA GTA	198	60	
		REV	GGA GAT CGT TGG GAA GTC CT			
	SREBP-1	FOR	CCA GCT GAC AGC TCC ATT GA	67	60	
		REV	TGC GCG CCA CAA GGA			
	SREBP-2	FOR	CAG GTC CTG GTA CAG CCT CA	158	60	
		REV	GCT CTT ACC GGA ACT TGC AG			
	SCAP	FOR	GGT CAC TTT CCG GGA TGG	179	60	NM_001101889.1
		REV	TGG GTA GCA GCA GGC TAA GA			
Steroid metabolism	STAR	FOR	TGG AAA AGA CAC GGT CAT CA	154	63	NM_174189
		REV	CTG GGG CAT CTC CTC ATA GA			

(Table 1 continued)

Functional group	Gene	Sequence [5′→3′]		Product length [bp]	T_M [°C]	Accession no.
	CYP11A1	FOR	CGG AAA GTT TGT AGG GGA CAT C	177	63	NM_176644
		REV	ACG TTG AGC AGA GGG ACA CT			
	HSD17B2	FOR	CAT CTC AGG CAC GAG TCA AAT G	160	62	NM_001075726
		REV	CAC TGG GGA GAT GTC TGG ATC			
	HSD17B4	FOR	CGG ATG ACC CAA AGC ATT TTG C	176	62	NM_001007809.1
		REV	TCT GTC TCA CAA GGG CTC CAA			
	HSD17B8	FOR	GGG CAT CAC CAG AGA TGA AT	228	62	NM_001046324
		REV	CAA TCA CTC CAG CCT TGG AT			
Lipid metabolism	APOA1	FOR	TTT GGG AAA ACA GCT CAA CCT GAA	215	63	NM_174242
		REV	GCC ACT TCT TCT GGA ACT CG			
	APOC2	FOR	GGG TTT CTC ATC CTC CTG GT	240	63	NM_001102380
		REV	AAT CCC TGC ATA GGT GGT CAC			
	LIPC	FOR	CGC CAT TCA CAC CTT TAC CT	186	62	NM_001035410
		REV	TCA TGG GCA CAT TTG ACA GT			
	LPL	FOR	CTC CTG ATG ATG CGG ATT TTG TA	196	62	NM_001075120
		REV	ACC AGC TGA TCC ACA TCT CC			
Reference genes	H3	FOR	ACT CGC TAC AAA AGC CGC TC	232	60	NM_001014389
		REV	ACT TGC CTC CTG CAA AGC AC			
	UBQ	FOR	AGA TCC AGG ATA AGG AAG GCA T	426	60	NM_174133
		REV	GCT CCA CCT CCA GGG TGA T			

downregulation ($p = 0.001$) in the treatment group compared to the control group by 0.27. A downregulation by a mean factor of 0.66 was also observed in SHBG ($p = 0.0002$). IRα and IRβ were significantly downregulated ($p_{IR\alpha} = 0.02$; $p_{IR\beta} = 0.011$) by a mean factor of 0.77 and 0.69, respectively.

One gene in the steroid hormone decomposition group showed a significant regulation. HSD17B2 was downregulated by a mean factor of 0.73 ($p = 0.039$). STAR was not significantly regulated ($p = 0.06$) but showed a trend for downregulation by a mean factor of 0.64.

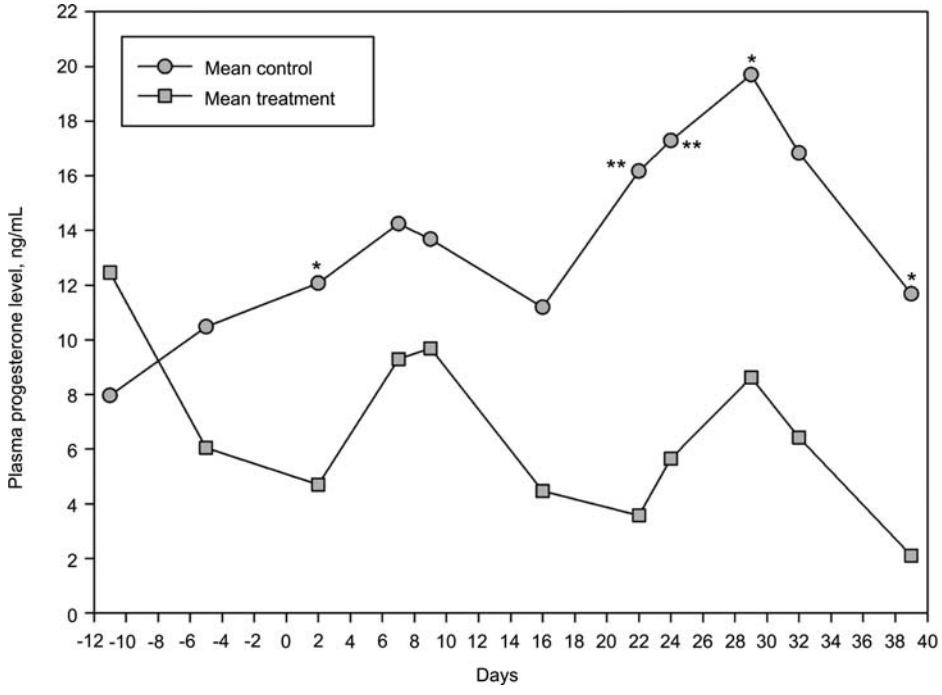

Figure 1 Plasma progesterone levels determined during the course of the study, showing significant differences between the control group and the treatment group at day 2, 22, 24, 29 and 39. *$p \leq 0.05$, **$p \leq 0.01$.

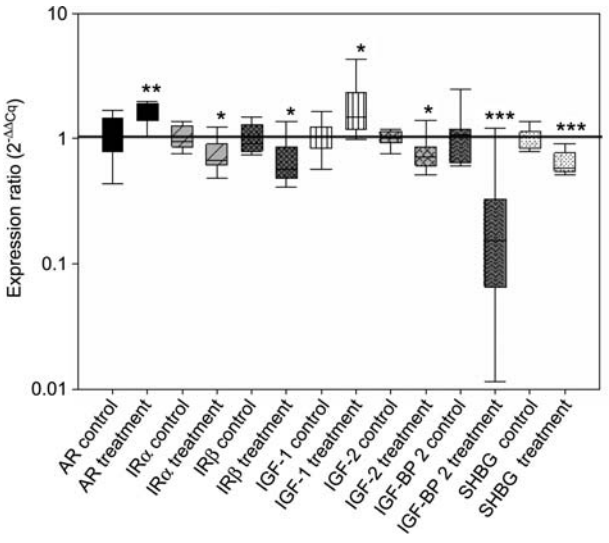

Figure 2 Expression ratio of regulated target genes under hormone treatment. *p < 0.05, **p < 0.01, ***p < 0.001.

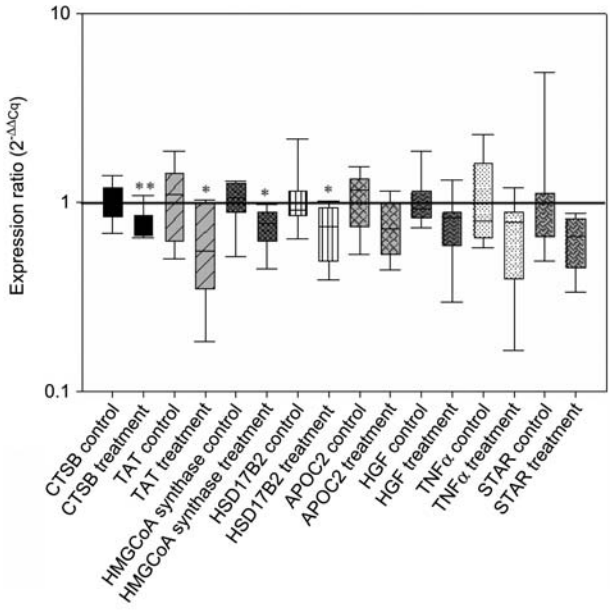

Figure 3 Expression ratio of regulated target genes under hormone treatment. *p < 0.05, **p < 0.01.

In addition, one gene in the cholesterol metabolism group was significantly regulated. For HMGCoA-synthase, a downregulation by a mean factor of 0.74 was observed (p = 0.023). APOC2 showed a trend for downregulation by a mean factor of 0.75, but this difference was not significant (p = 0.059).

In the protein and amino acid group, significant downregulations were shown for CTSB and TAT. CTSB was downregulated (p = 0.009) in the treatment group in comparison to the control group by a mean factor of 0.77. TAT was downregulated (p = 0.028) by a mean factor of 0.61. For TNFα, no statistically different expression could be determined, but a trend for downregulation by a mean factor of 0.70 was visible (p = 0.082).

The proliferative factor HGF showed a trend for downregulation by a mean factor of 0.78, but the difference was not significant (p = 0.07).

PCA was produced as shown in Figure 4 by plotting the expression results of regulated genes of all samples of both groups by their first end second principal component. Gray crosses represent samples of the control group, black triangles display the samples of the treatment group. A distinct control group could be seen separated from the treatment group, showing that there was a multitranscriptional response to the treatment.

Discussion

Steroid hormones are known to alter expression of various genes by acting as transcription factors and thereby mediate their physiological effect. In the current study, the influence of the anabolic combination trenbolone acetate plus estradiol on gene expression in liver of Nguni heifers was investigated. It is generally known that the effect of the anabolic combination Revalor H is consistent during an experimental period as in the present trial (21) and also the actual measurement of the plasma progesterone levels showed a significant difference between the control and the treatment with the most distinct differences at days 22–39 proving the effectiveness of Revalor H until the end of the experimental course. Progesterone levels were lower in the treatment group compared to the control group due to negative feedback mechanisms following the application of exogenous hormones. Therefore, it can be assumed that gene expression changes are due to the anabolic treatment.

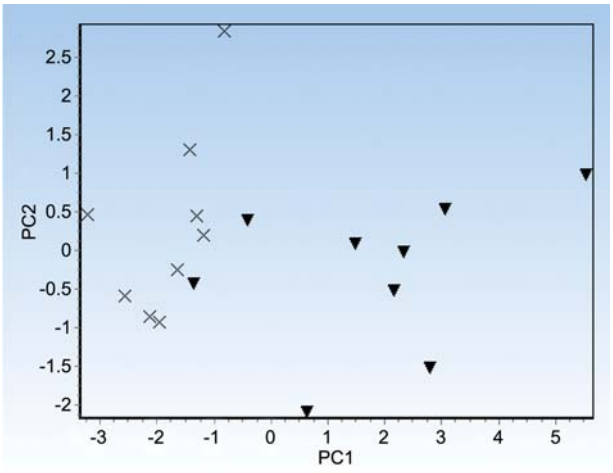

Figure 4 PCA of regulated target genes with control animals represented by grey crosses and treated animals represented by black triangles.

The actual analysis showed an upregulation of IGF-1. This is in agreement with the well-established hypothesis that steroid hormones increase IGF-1 gene expression and also with investigations of other groups, which showed an upregulation for IGF-1 in feedlot cattle and rats (28–30). Several other publications (30–32) showed in the case of an upregulation of IGF-1 mRNA also increased concentrations of circulating IGF-1 protein levels in lambs and steers, thus it can be assumed that regulation of IGF-1 levels really occurs on the level of transcription by directly affecting the IGF-1 gene. A higher synthesis of IGF-1 in the liver, which is the main source for IGF-1, could be partially responsible for the higher serum levels (32). Owing to the para- plus endocrine action of IGF-1, it can be assumed that the growth promoting effect of anabolics on muscle tissue is mediated by circulating IGF-1 produced in liver.

In addition to skeletal muscles, IGF and IGFBP are also known to have an influence on smooth muscles. IGF-1 and IGF-2 have been shown to stimulate the migration and proliferation of smooth muscle cells, whereas IGF-1 is more potent in the postnatal phase than IGF-2 in the prenatal phase (33, 34). This response can be modulated by binding of IGF to IGFBP. In particular, IGFBP-2 can completely suppress the effect of IGF-1. The migration of smooth muscle cells is one of the initial events in the formation of atherosclerosis, which is the major reason for cardiovascular side effects after misuse of anabolic steroids in humans (16). In the present study, an upregulation of IGF-1 and a downregulation of IGFBP-2 was recognized. This observation could be a sign of an augmented migration of smooth muscle cells. These changes on the gene expression level and the known disturbed lipid profile (35) could cause the increased risk of atherosclerosis following abuse of anabolics.

The endocrine factor SHBG contributes to the humoral transport of steroid hormones and is mainly synthesized in the liver. The expression of this factor has never been examined in correlation with the action of anabolic steroids, neither in human nor in bovine, but the literature describes a decreased protein level of SHBG in human users of anabolic steroids (3). In calves, the potential of SHBG binding level to be used as a biomarker for anabolic treatment has also been tested (36). These findings are in line with our results of a downregulation of SHBG mRNA in liver. During the exogenous application of anabolic steroids, the organism could reduce the synthesis of SHBG on the mRNA level to restrict the transport of steroid hormones and thereby facilitate degradation of unbound steroids.

The biosynthesis of steroid hormones requires cholesterol and its biosynthesis is in part controlled by genetic expression of the enzymes HMGCoA-Synthase, HMGCoA-Reductase and FDFT. In our experiment, HMGCoA-Synthase was downregulated; but owing to the fact that we could not detect any regulation for HMGCoA-Reductase or FDFT, it is difficult to make any statement concerning the influence of a treatment with trenbolone acetate plus estradiol on cholesterol biosynthesis.

Cholesterol is the initial component in the synthesis of steroid hormones, thus its synthesis should be reduced with the application of exogenous hormones. After its synthesis, cholesterol is transported to the inner mitochondrial membrane for the generation of bile acids or steroid hormones. This transfer is mediated by STAR and is thereby the first step in steroid hormone generation (14). STAR showed a trend for downregulation in the current study. This leads to the hypothesis that steroid hormone synthesis is blocked under the influence of exogenous hormones. However, CYP11A1, the enzyme catalyzing the first and rate-limiting step in steroid syntheses from pregnenolone to progesterone showed no significant regulation. Thus, this block could be introduced prior to the actual pathway of steroid synthesis at the level of formation and processing of the initial product, which is visible in the downregulation of HMGCoA-Sythase and STAR.

HSD17B2 belongs to the group of oxidative steroid dehydrogenases, which direct their activity towards the inactivation of steroid hormones. HSD17B2 itself catalyzes the reaction from estradiol to estrone and testosterone to Δ4-androstendione (11). The finding of a downregulation of HSD17B2 in the current study was unexpected. This enzyme should be upregulated under the influence of exogenous hormones to accelerate the degradation of the applied substances. However, no precise information on physiological responses could be given based on gene expression results. So possibly, the inactivation of the applied hormones is regulated on another level.

The liver plays a pivotal role in glucose metabolism and homeostasis. Glucose uptake and storage as glycogen by hepatocytes is mediated by the interaction of insulin with hepatic insulin receptors. In the present study, IRα as well as IRβ were downregulated leading to an impaired insulin sensitivity of the liver. This mechanism could be responsible for the reduction of glycogen synthesis, lipogenesis and protein synthesis due to the action of anabolic steroids. Because these nutrients can be less metabolized by the liver, they are kept in the blood and are available for proliferation processes in the skeletal muscles.

This is consistent with the observed downregulation of TAT and CTSL. TAT is an enzyme that is mainly located in the liver catalyzing the first step in the degradation pathway of the amino acid tyrosine (37). The downregulations of the protease CTSB and the aminotransferase TAT could be responsible for an anti-catabolic effect and for protein plus nitrogen retention. Together with the upregulation of IGF-1, these effects could be responsible for muscle growth under the influence of anabolic steroids.

Unlike the natural androgen testosterone, which mediates its anabolic action by binding to the AR and also, after its conversion by an aromatase to estradiol, to ER (ERα and ERβ), the xenobiotic androgen trenbolone acetate is able to bind to AR, GR and progestin receptor (2). AR and GR as well as both ER subtypes were investigated in this study. As just an upregulation of AR occurred, it could be assumed that the anabolic effects of the applied treatment are mainly due to trenbolone acetate by its binding to AR. However, it should be noted that there are also other regulation levels of physiological function such as post-transciptional modifica-

tions, which can participate in the regulation of the mentioned receptors that could not be shown to be significantly regulated in this investigation. In addition to the regulation of gene transcription, steroid hormones are known to influence the stability and degradation of existing mRNA and especially to regulate the concentrations of their own receptors (38, 39). Thus, changes in gene expression on the transcriptional level of certain target genes could only be a part of changes in biochemical pathways.

In biomarker research, the application of valid bioinformatical and statistical tools for data processing is important to extract the required information. In most of the fields that biomarkers are searched for, it is general that not only one factor is used but a variety of factors are integrated to a biomarker pattern. Therefore, in particular methods for dimensionality reduction are needed to transform the high-dimensional data sets (22). In the present study, PCA has been applied as a multivariate analysis method, which can be used, if more than three components should be taken into account. The PCA of all regulated genes showed that the treatment differs from the control animals although single genes are distributed within similar ranges. It could be observed that both groups arrange together and that a difference between control and treatment groups can be monitored. In addition to discussed regulations of single genes, this illustration could help to reveal the transcriptional shift in the treatment group, which could be an indication for functional changes in liver physiology following anabolic treatment. Also, this observation is a first indication that it is possible to elaborate a gene expression pattern making it is possible to develop a screening method to control the misuse of anabolic hormones in cattle.

In conclusion, several biochemical pathways, which were differentially regulated on mRNA level under the influence of trenbolone acetate plus estradiol, have been identified. Most changes seem to support growth processes in the organism. The metabolism of nutrients such as glucose in the liver could be suppressed by the decrease of the insulin sensitivity mediated by the downregulation of IRα and IRβ. These nutrients are kept in the blood flow and could be available for skeletal muscle growth. An inhibition of the protein breakdown was observed. Also, IGF-1 could be responsible for the stimulation of growth in treated animals (28). Additionally, the upregulation of IGF-1 could also be revealed as a possible risk factor for the generation of artherosclerotic plaques, which are known long-term side effects following the use of anabolic steroids.

PCA of RT-qPCR results showed that both groups arrange separately and that a difference between control and treatment groups becomes obvious. Therefore, the measured gene expression pattern could possibly be used as biomarkers for anabolic treatment with trenbolone acetate plus estradiol in bovine liver.

Acknowledgements

We thank the Onderstepoort Veterinary Institute, Pretoria, South Africa for supporting this study. Special thanks to Azel Swemmer and Kobus van der Merwe for animal study performance.

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