Effects of an estrogen treatment on the tissue specific expression of steroid receptors in the bovine gastrointestinal tract: Quantification of androgen receptor (AR), progesterone receptor (PR), estrogen receptors (ER) α and β mRNA with real-time RT-PCR.

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INTRODUCTION

Steroid hormones, like estrogens, androgens and gestagens, play an important role in the cell and tissue differentiation as well as in the regulation of metabolic processes. The steroid hormone effect in the target cell is mediated by its cytoplasmic receptor which binds the steroid with high affinity and high selectivity. As a consequence of the steroid-hormone-receptor interaction the transcription of specific genes is being activated and led to an increased synthesis of specific proteins in the target cell.

Goal of this study was to evaluate the deviating tissue sensitivities and the influence of estrogens on the regulation of steroid receptors in ten compartments of the bovine gastrointestinal tract: in four stomachs (*rumen, reticulum, omasum, abomasum*) and in six different gut regions (*duodenum, jejunum, ileum, caecum, colon, rectum*).

Following receptor types of the steroid receptor family were investigated:

- Androgen receptor (**AR**)
- Estrogen receptor alpha (**ERα**) and Estrogen receptor beta (**ERβ**)
- Progestin receptor (**PR**)

The localisation and dominant expression of ERβ in both kidney regions and in the jejunum leads to the hypothesis that ERβ plays a dominant role in the gastrointestinal tract (*Pfaffl et al., 2001*). AR and ERα was already detected earlier in the bovine gastrointestinal tract (*Sauerwein et al., 1995*). ERβ might be a major actor in absorptive processes in the gut and in pathophysiological processes like gastrointestinal cancer.

As estrogen treatment we used implants with an estrogen active preparation RALGRO, which contains Zeranol (1 implant = 36 mg Zeranol). Zeranol is a derivative of the mycotoxin Zearalenone and shows strong estrogenic as well as anabolic effects. It exhibits all symptoms of hyper-estrogenism in particular reproductive and developmental disorders. It is well known that steroids lead to an increased synthesis of specific proteins and it is proposed that estradiol can stimulate via ERα its own receptor expression at least in the uterus (*Jungblut et al., 1976*).

To quantify these possible transcripts also in low abundant tissues, like the gastrointestinal tract, sensitive and reliable real-time RT-PCR quantification methods were developed and validated on the LightCycler.

MATERIAL & METHODS

Eight heifers were treated over 8 weeks with multiple RALGRO pellet implantations (0x, 1x, 3x, 10x). During treatment period and after slaughtering Zeranol concentrations were measured in plasma by enzyme-immuno-assay (*Lange et al., 2001*) (Figure 1).

Four stomachs (*rumen, reticulum, omasum, abomasum*) and six different gut regions (*duodenum, jejunum, ileum, caecum, colon, rectum*) were samples and investigated with real-time RT-PCR.

Primer pairs were designed to produce an amplification product spanning two RNA-splicing sites in the region of the receptor ligand binding domain by primer design software HUSAR. Primer sequences were designed as multi-species primers, according to the available EMBL sequences, which fit to the following species with sufficiently high precision: cattle *(Bos taurus)*, sheep *(Ovis aries)*, pig *(Sus scrofa)*, mouse *(Mus musculus)*, rat *(Rattus norvegicus)* and human *(Homo sapiens)*, produced commercially (MWG Biotech).

After tissue extraction (TriPure) and reverse transcription of total RNA in cDNA, a master-mix of the following reaction components was prepared to the indicated end concentration: 6.4 µl water, 1.2 µl $MgCl_2$ (4 mM), 0.2 µl Forward Primer (0.4 µM), 0.2 µl Reverse Primer (0.4 µM) and 1.0 µl LightCycler DNA Master SYBR Green I (1x). 9 µl of master-mix was filled in the glass capillaries and 25 ng cDNA in 1 µl volume was added as PCR template.

To improve SYBR Green I quantification a new 4th segment with an high temperature fluorescence acquisition point was included to the amplification cycle program. High temperature fluorescence acquisition melts the unspecific PCR products, eliminates the non-specific fluorescence signal derived from primer dimers and ensures an accurate quantification of only the desired steroid receptor products. For all quantitative assays an external standard curve was used based on a single stranded DNA (ssDNA) molecule calculation. Therefore RT-PCR products from *Bos taurus* were cloned in pCR4.0 (Invitrogen) vector, linearised by a unique restriction digest and dilutions of each recombinant plasmid from single copies ssDNA (<14 molecules) up to $>10^9$ ssDNA molecules were used as ssDNA standard. Assay sensitivities, reproducibility, reliabilities and multi-species applications are shown in table 1.

Figure 1: Plasma Zeranol concentrations of treated animals in comparison to control animals (background = 6.9 pg Zeranol/ml plasma).

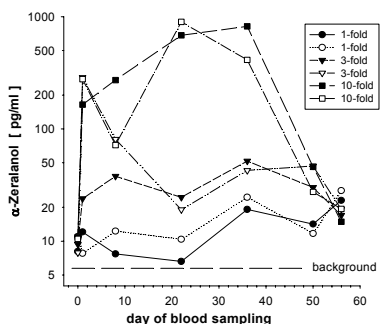


Table 1: Characterization and validation of steroid receptor real-time RT-PCR.

steroid receptors	AR	ERα	ERβ	PR
RT-PCR product length	172 bp	234 bp	262 bp	227 bp
detection limit	12 molecules	2 molecules	10 molecules	14 molecules
quantification limit	120 molecules	165 molecules	106 molecules	760 molecules
quantification range (test linearity) (*correlation*)	120 - $1.20*10^{10}$ molecules (*r = 0.998*)	165 - $1.65*10^9$ molecules (*r = 0.995*)	106 - $1.06*10^{10}$ molecules (*r = 0.996*)	760 – $7.60*10^9$ molecules (*r = 0.998*)
PCR efficiency	90.7%	81.2%	81.3%	93.9%
intra-assay variation	31.2% (n = 3)	18.7% (n = 4)	17.6% (n = 4)	5.7% (n = 4)
inter-assay variation	24.3% (n = 7)	28.6% (n = 4)	29.7% (n = 4)	25.7% (n = 4)

RESULTS

AR, PR, ERα and ERβ mRNAs were detected in all investigated gastrointestinal tissues. The AR mRNA was the major transcript (∅ 4000 – 8500 molecules/25 ng total RNA), in the 3 fore stomaches rumen, reticulum and omasum, whereas both ER subtypes were higher abundant in gut compartments ileum, colon and rectum (∅ 1750 – 2350 ERα molecules; ∅ 1050 – 2450 ERβ molecules). PR mRNA was very low abundant in all investigated tissues (< 700 molecules).

In all stomachs and duodenum the expression ratio (R) of ERα/ERβ was high (29>R>6.5) and low (R<1) in ileum and rectum, where ERβ was higher concentrated.

With increasing Zeranol treatment concentrations a significant up-regulation of ERα mRNA expression could be observed in abomasum (r=0.72; p<0.05) and a down-regulation in jejunum (r=0.77; p<0.05; Figure 2). For ERβ mRNA an up-regulation was shown in rectum (r=0.96; p<0.001), a down-regulation in jejunum (r=0.72; p<0.05) and a trend of down-regulation in reticulum (r=0.68; p=0.06). PR mRNA was up-regulated in omasum (r=0.92; p<0.001; Figure 3) and down-regulated in jejunum (r=0.73; p<0.05).

For AR mRNA expression no significant correlation with increasing Zeranol concentrations could be observed.

DISCUSSION & CONCLUSION

In view of the data provided for sensitivity, linearity and reproducibility, the steroid hormone receptor RT-PCR assays developed herein allows for the absolute and accurate quantification of low abundant steroid receptors mRNA molecules in various tissues.

In conclusion, our expression results indicate the existence of AR, PR and both ER subtypes in bovine gastrointestinal tract. The gastrointestinal tissues compartments exhibit a tissue specific expression pattern for steroid receptors and a tissue specific regulation under estrogen treatment. Therefore the bovine stomachs and the gut compartments are possible targets for steroid hormone action.

These data support the hypothesis, that ERβ may have different biological functions than ERα. However, any notations on direct physiological or pathophysiological effects of estrogens on gastrointestinal tissues and kidney remain speculative.

Figure 2: Down-regulation of ERα mRNA expression in bovine jejunum.

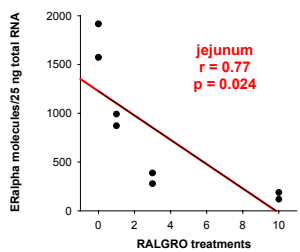
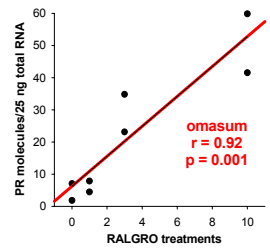


Figure 3: Up-regulation of PR mRNA expression in bovine omasum.



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