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Tissue-specific expression pattern of estrogen receptors (ER): Quantification of ERα and ERβ mRNA with real-time RT-PCR

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We have examined the tissue-specific mRNA expression of ERα and ERβ in various bovine tissues using real-time RT-PCR. The goal of this study was to evaluate the deviating tissue sensitivities and the influence of the estrogenic active preparation RALGRO on the tissue-specific expression and regulation of both ER subtypes. RALGRO contains Zeranol (α-Zearalanol), a derivative of the mycotoxin Zearalenon, shows strong estrogenic and anabolic effects, and exhibits all symptoms of hyperestrogenism, in particular reproductive and developmental disorders. Eight heifers were treated over 8 weeks with multiple-dose implantations (0×, 1×, 3×, 10×) of Zeranol. Plasma Zeranol concentration, measured by enzyme immunoassay, of multiple treated heifers was elevated. To quantify ERα and ERβ transcripts also in low-abundant tissues, sensitive and reliable real-time RT-PCR quantification methods were developed and validated on the LightCycler. Expression results indicate the existence of both ER subtypes in all 15 investigated tissues. All tissues exhibited a specific ERα and ERβ expression pattern and regulation. With increasing Zeranol concentrations, a significant downregulation of ERα mRNA expression could be observed in jejunum ($p<0.001$) and kidney medulla ($p<0.05$). These data support the hypothesis that ERβ may have different biological functions than ERα, especially in kidney and jejunum.

Key words: ERα; ERβ; expression pattern; real-time RT-PCR; estrogen treatment.

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Cloning and sequencing of ERβ in human (1), rat (2), and mouse (3) has provided the first example of a steroid hormone receptor existing in two isoforms, each of which is encoded by a separate gene. The ERβ protein is smaller than the previously identified ERα (4, 5), but possesses the modular structure of distinct functional domains (A-F) characteristic of members of the nuclear receptors. The DNA-binding domain of ERα and ERβ is highly conserved over several species (>95% homology in *Homo sapiens, Rattus norwegicus, Mus musculus, Bos taurus*) and the ligand-binding domain shows ~60% conserved residues (6–8). ERβ seems to be an important additional factor in the mechanism of estrogen action and has an overlapping but nonidentical tissue distribution in comparison to ERα. It is expressed in humans and rodents in the central nervous system, the cardiovascular system, the immune system, and in the gastrointestinal tract (9–11). Within the same organ it often appears

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that the ER subtypes are expressed differentially, supporting the hypothesis that both receptors may have different biological functions. Ligand-binding experiments have shown specific binding of estradiol-17β and transactivation of ERβ with an affinity similar to ERα (12). But the mechanism of activation by endogen steroids, phytoetrogens, xenosteroids and related synthetic drugs was shown to be highly complex (6, 13, 14). Knowledge of the distribution and regulation of ERβ in various tissues of ruminants is lacking at present. The available publications about ERβ expression in bovids are limited to cattle reproductive organs (8, 15) and the sheep hypothalamus (16, 17). However, a more detailed study of the tissue distribution of both ER subtypes is essential to continuing investigations of their regulation and physiological function. It is well known that steroids lead to an increased synthesis of specific proteins (18) and it is proposed that estradiol can stimulate via ERα its own receptor expression at least in the uterus (19). The goal of this study was to evaluate the deviating tissue sensitivities and the influence of the estrogen-active preparation RALGRO® (Mallinckrodt Veterinary, Inc., Mundelein, IL, USA) on the tissue-specific expression pattern and regulation of both ER subtypes. One RALGRO implant contains 36 mg α-Zearalanol (Zeranol), a derivative of the mycotoxin Zearalenon, and shows strong estrogenic and anabolic effects in farm animals. Besides this, it exhibits all the symptoms of hyperestrogenism, in particular reproductive and developmental disorders. Herein we describe a reverse transcription followed by a real-time polymerase chain reaction (RT-PCR) using the LightCycler system (Roche, Basel, Suisse) to detect and quantify these mRNA transcripts from both genes. For low abundancies, sensitive and reliable real-time RT-PCR quantification methods were developed and validated. RT-PCR real-time assays with an external calibration curve are quantitative and therefore an absolute comparison of ERα and ERβ within one tissue RNA preparation will be possible.

MATERIAL AND METHODS

Animal experiment and sampling

Six Holstein Friesian heifers were treated with the implant preparation RALGRO® (1×=36 mg Zeranol) 8 weeks before slaughter. Two animals served as control, two were given single, two 3-fold and two 10-fold doses of the preparation. Blood samples were taken on day 0, 1, 8, 22, 36, 49 and 56 of the treatment period. During sampling, an anticoagulative agent (6 mM EDTA and 0.02% acetylsalicylic acid final) was added. Plasma was prepared by centrifugation and decantation. Aliquots were stored at −20°C until processing.

Gynaecological inspection of animals after slaughter

During dissection of the inner organs the genital tract of the heifers was removed completely and prepared for morphological inspection. The inspection comprised the following: **1**) An estimation of the oestrus cycle day, according to the criteria of Ireland et al. (20); **2**) Uterus condition (size, existence and appearance of mucus); **3**) Corpus luteum (existence of possible cysts, post- or preovulatory status and any unphysiological appearance); **4**) Number of follicles, divided into large dominant (>10 mm in diameter) or small (3–10 mm). The corpus luteum and follicle data represent the gynaecological results from both ovaries.

Detection of Zeranol in plasma

The plasma contents of Zeranol were analysed by enzyme immunoassay (EIA) after liquid-liquid extraction. 0.5 ml plasma was thoroughly shaken overnight with 2 ml tertiary-butyl methyl ether (TBME)/petroleum ether 70/30 (v/v). After separation of the organic and the plasma phase the samples were deep frozen at −60°C for 30 min. The supernatant was decanted and evaporated to dryness in a shaking water bath at 60–80°C. The residue was re-dissolved in 0.25 ml 40% methanol and 20 µL of this extract was analysed in duplicate by a specific enzyme immunoassay (rabbit antiserum 1:1,400,000, antigen: zeranol-16-carboxy-propylether-BSA; 2.9 ng zeranol-16-carboxybutyl ether-horseradish peroxidase per well). The working interval ranged from 10 pg/ml (85% displacement of labelled antigen) to 1740 pg/ml plasma (15% displacement of labelled antigen). To validate the detection method, aliquots of a pool of negative plasma samples were fortified with 0 pg, 100 pg, 200 pg or 400 pg Zeranol/ml and analysed according to the standard protocol. The EIA assay showed a mean recovery of 106%, an intra-assay variation of 3.1%, an inter-assay variation of 11%, a limit of detection of 12 pg/ml, and a cross-reaction with other Zeranol metabolites and steroid hormones of maximal 6.5% (21).

Total RNA extraction

0.5 g frozen tissue was homogenised in 4 M guanidinium thiocyanate buffer to destroy RNase activity (22). In the following steps, the RNA-Clean protocol (AGS RNA-Clean; AGS, Heidelberg, Germany) with phenol/chloroform extraction for total RNA was

used. In order to quantify the amount of total RNA extracted, the optical density was determined with an Ultraspec 3000 photometer (Pharmacia) at three different dilutions of the final RNA preparations at 260 nm, corrected by the 320 nm background absorption. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD_{260}/OD_{280} nm absorption ratio >1.85.

ERα and ERβ specific primer design

The primers of both subtypes were derived from the bovine and ovine sequences [EMBL Ac. no. AF110402; Y18017; Z49257; AF177936]. Both primer pairs were designed to produce a ERα (234 bp) and ERβ (262 bp) amplification product spanning two RNA-splicing sites in the region of the receptor ligand-binding domain, where only ~60% homology is given. Primer design and optimisation was done with the Mac DNASIS primer design software (Hitachi Software, Yokohama, Japan). Primer sequences were additionally designed as multi-species primers (ERα forward primer: 5′-AGG GAA GCT CCT ATT TGC TCC -3′; ERα reverse primer: 5′-CGG TGG ATG TGG TCC TTC TCT-3′; ERβ forward primer: 5′-GCT TCG TGG AGC TCA GCC TG-3′; ERβ reverse primer: 5′-AGG ATC ATG GCC TTG ACA CAG A-3′; produced by MWG Biotech, Ebersberg, Germany), which fit to the following species with high precision (>90%): cattle (*Bos taurus*), sheep (*Ovis aries*), human (*Homo sapiens*), pig (*Sus scrofa*), mouse (*Mus musculus*) and rat (*Rattus norwegicus*).

Reverse transcription (RT)

1 μg total RNA from sample preparation was reverse transcribed in 40 μl as followed: M-MLV RT buffer (Promega, Mannheim, Germany) and 300 μM dNTPs (MBI Fermentas) were denaturated for 5 min at 65°C in an Mastercycler Gradient (Eppendorf, Germany). The subsequent RT was done at 37°C for 60 min by adding 2.5 mM Random Hexamer primers (Pharmacia), 200 U of M-MLV Reverse Trancriptase (Promega), 12.5 U of RNAsin RNase inhibitor (MBI Fermentas). The samples were then heated for 1 min at 99°C to terminate RT.

Optimisation of ER subtype-specific RT-PCR

Conditions for RT-PCRs were optimised in a gradient cycler with regard to Taq DNA Polymerase (Roche), PCR water, pH, primers (MWG), $MgCl_2$ (Roche) concentrations and various annealing temperatures. Amplification products were separated on by 4% high resolution NuSieve agarose (FMC Bio Products) gel electrophoresis and analysed with the Image Master system (Pharmacia). Optimised results were transferred according to the following LightCycler PCR protocol.

LightCycler PCR master mix

For the LightCycler reactions 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 (1×). 9 μl of LightCycler master mix was filled in the LightCycler glass capillaries and 25 ng reverse transcribed total RNA in 1 μl was added as PCR template. The capillaries were closed, centrifuged in a microcentrifuge, and placed in the LightCycler rotor (Roche).

LightCycler PCR time and temperature profiles

A conventional LightCycler amplification cycle contains three segments: In the 1st segment DNA is denaturated at 95°C. In the 2nd annealing segment the primer annealing takes place and the chosen temperature should be as high as possible to improve specificity. Within the following 3rd elongation segment at 72°C, the elongation time should be adapted to the length of the desired product, which is limited by Taq Polymerase processing rate (~1000 bp/min elongation time). To improve SYBR Green I® quantification a new 4th segment with a high temperature fluorescence acquisition point was included in the amplification cycle program. The following LightCycler protocol was used for ERα and ERβ real-time PCR: *denaturation program* [95°C for 30 s], a 4 segment *amplification and quantification program* repeated 50 times for **ERα** [95°C for 3 s; 64°C for 10 s; 72°C for 20 s; 82°C for 3 s with a single fluorescence acquisition point], and for **ERβ** [95°C for 3 s; 64°C for 10 s; 72°C for 20 s; 87°C for 3 s with a single fluorescence acquisition point], *melting curve program* [60°C to 95°C with a heating rate of 0.1°C/s and a continuous fluorescence acquisition] and a final *cooling program* down to 40°C. The display mode and the fluorimeter gains of channel 1 were set to 5.

Calibration curves

For both quantitative assays, an external calibration curve was used, based on a single-stranded DNA (ssDNA) molecule calculation. ERα and ERβ RT-PCR products from *Bos taurus* were cloned separately in pCR4.0 (Invitrogen, Leek, The Netherlands), linearised by a unique restriction digest, and dilutions of each plasmid preparation from single copies of ssDNA up to 10^{10} ssDNA molecules were used in calibration curves.

RESULTS

Gynaecological status of treated heifers after section

The gynaecological status of both untreated control animals dated on day 14 to 16 of the oestrus cycle showed physiological uterus and ovary conditions. Heifers treated with 1-fold

Zeranol were in day 18–20 of the oestrus cycle and the uterus showed a normal constitution with cervical mucus. On the ovaries both animals had one dominant and up to 10 small follicles. Besides this, one of them showed a corpus luteum. Heifers treated with multiple implantation (3× and 10×) had a very small uterus, cervical mucus, and the number of follicles was limited to 6.

Zeranol concentrations in plasma

The Zeranol concentrations measured in plasma of control animals were constant at a level of 6.9±1.9 pg/ml plasma (n=14). In treated animals, the plasma levels were elevated (Fig. 1) over the first half of the treatment period, corresponding to the multiple Zeranol treatment concentrations. At slaughter on day 56, the plasma concentrations dropped to values below 30 pg/ml, but were elevated in comparison to background concentrations.

Confirmation of primer specificity and sequence analysis

For exact length verification, RT-PCR products were separated by 4% high resolution gel electrophoresis. Amplified gradient MasterCycler and LightCycler PCR products showed a single band and the expected length of 234 bp for ERα and 262 bp for ERβ (Fig. 2). Specificity of the desired products was additionally documented with melting curve analysis of LightCycler Software 3.39 (Roche). Melting temperature of the high specific products is species and receptor subtype dependent (Table 1) between 85.0°C to 86.0°C for ERα and 88.8°C to 89.3°C for ERβ. Sequence analysis (MWG Biotech, Ebersberg, Germany) of cloned RT-PCR products from *Bos taurus* showed 100% homology to the published sequences.

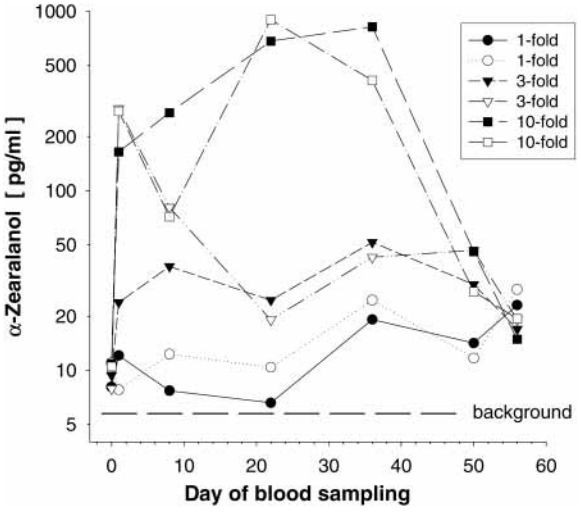

Fig. 1. Zeranol concentrations in plasma from six heifers treated with multiple dose of the estrogen-active preparation RALGRO® (1×, 3× and 10×) during 56 d treatment period. Mean Zeranol background (6.9 pg/ml; n=14) was measured in both control animals.

Real-time RT-PCR assay validation

Both real-time RT-PCR were ERα and ERβ product specific, and effective PCR amplification kinetics was shown by high PCR efficiency per cycle (Table 1). Assay sensitivities were confirmed by detection limits down to 10 ssDNA molecules and linear quantification ranges between 10^2 to 10^9 molecules. Intra- and inter-assay variations of <19% to <30%, respectively, were determined over the entire quantification range. The advantage of a high temperature fluorescence acquisition in the 4th segment during the amplification program results in reliable and sensitive ER subtype-specific quantification with high linearity (Pearson correlation coefficient; r>0.995) over seven orders of magnitude. High temperature fluorescence acquisition melts the unspecific PCR products at 82°C and 87°C, respectively, eliminates the non-specific fluorescence signal derived from primer dimers, and ensures an accurate quantification of the desired products (Fig. 2).

Tissue-specific ERα and ERβ mRNA expression

ERα and ERβ mRNA expression could be observed in all 15 investigated tissues. Table 2 summarizes the mean mRNA expression rates of the ER subtypes and their ERα/ERβ ratio for all eight investigated animals with variation coefficient (VQ). Expression data were pooled, because in most tissues (13 of 15) no effect of Zeranol treatment on the ER expression profiles could be detected. The classical tissues for estrogen action such as uterus, udder and liver showed high ERα expression. High ERα expression could also be observed in muscles such as neck muscles > hind leg muscles > longissimus dorsi > shoulder muscles. High ERβ was observed in uterus > kidney > and spleen > longissumus dorsi > udder > liver. In comparison

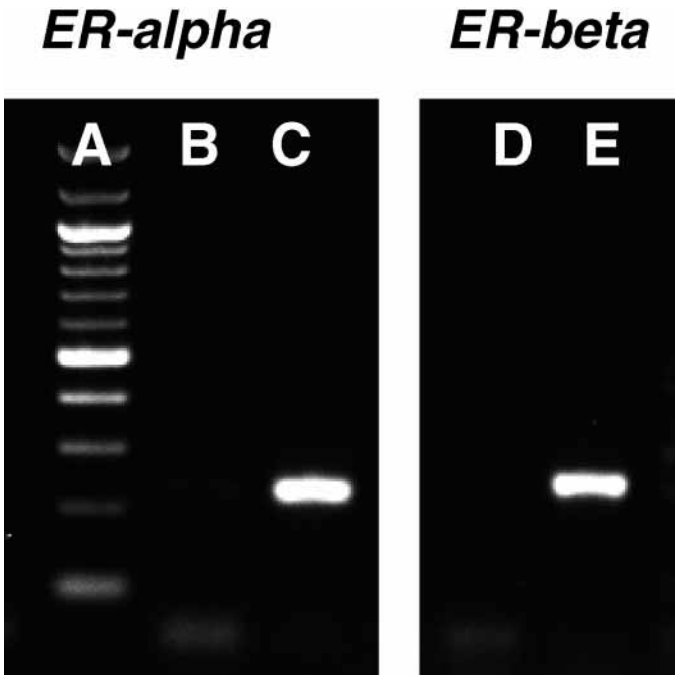

Fig. 2. LightCycler 234 bp ERα (C) and 262 bp ERβ (E) RT-PCR products in comparison with the 100 bp ladder (A) and the no template controls (B and D).

to ERα and ERβ, the expression rates (on a moleculer basis per 25 ng) were lower in most tissues, except in kidney and jejunum, where the ERα/ERβ ratios were <1. High ERα/ERβ ratios were examined in some muscle parts, liver, udder and uterus. To make the individual tissue expression pattern evident, all mean ERα and ERβ expression rates were shown (Fig. 3) in molecules per 25 ng total RNA with bidirectional error bars (mean±s.e.m.). Each tissue possesses an ERα and ERβ expression pattern which stays relatively stable even during Zeranol treatment and resulted in an ERα/ERβ expression cluster (Fig. 3). Highest expression rates of ER subtypes were observed in uterus and low concentrations in tissues of the gastrointestinal tract. A significant relationship between increasing estrogen concentrations and decreasing ERα expression was observed in kidney medulla ($p<0.001$) and jejunum ($p<0.05$) (Fig. 4), not in uterus. Liver, jejunum, abomasum, mammary gland, spleen, lung, kidney cortex and longissimus dorsi showed only a trend to decreased expression. In the remaining

TABLE 1. *Characteristics and validation parameters of multispecies ERα and ERβ real-time RT-PCR in the LightCycler system. PCR product-specific melting temperature (T_{melt} in °C) of ERα and ERβ of the investigated species*

	ERα		ERβ	
Product length	234 bp		262 bp	
Detection limit	2 molecules		10 molecules	
Quantification limit	165 molecules		106 molecules	
Quantification range (test linearity)	165–1.65×10^9 molecules (r=0.995)		106–1.06×10^{10} molecules (r=0.996)	
PCR efficiency	1.812		1.813	
Intra-assay variation	18.7% (n=4)		17.6% (n=4)	
Inter-assay variation	28.6% (n=4)		29.7% (n=4)	
Investigated species and specific T_{melt} (°C)	*Rattus norvegicus*	*85.0*	*Bos taurus*	*89.3*
	Bos taurus	*85.3*	*Ovis aries*	*88.8*
	Ovis aries	*85.4*		
	Sus scrofa	*86.0*		
	Homo sapiens	*86.0*		

TABLE 2. *Expression pattern of ERα and ERβ mRNA and expression ratio (ERα/ERβ) in 25 ng bovine total RNA. Mean expression (in mRNA molecules) and variation coefficient (VQ) of eight animals*

	ERα	VQ	ERβ	VQ	Ratio ERα/β
Uterus	980,000	53%	80,000	140%	12
Mammary gland	205,000	105%	7,250	64%	28
Liver	200,000	87%	6,250	179%	32
Lung	5,400	167%	900	94%	6
Spleen	12,000	65%	10,400	104%	1.2
Heart muscle	6,000	82%	4,200	56%	1.4
Kidney medulla	10,200	52%	35,100	65%	0.3
Kidney cortex	4,200	57%	29,300	51%	0.14
Rumen	4,060	53%	1,350	109%	3
Abomasum	4,600	84%	650	157%	7
Jejunum	1,550	72%	2,150	152%	0.7
Longissimus dorsi	79,400	61%	8,650	22%	9
Hind leg muscles	100,000	47%	5,850	76%	17
Shoulder muscles	60,500	83%	2,600	93%	23
Neck muscles	145,000	79%	2,250	133%	64

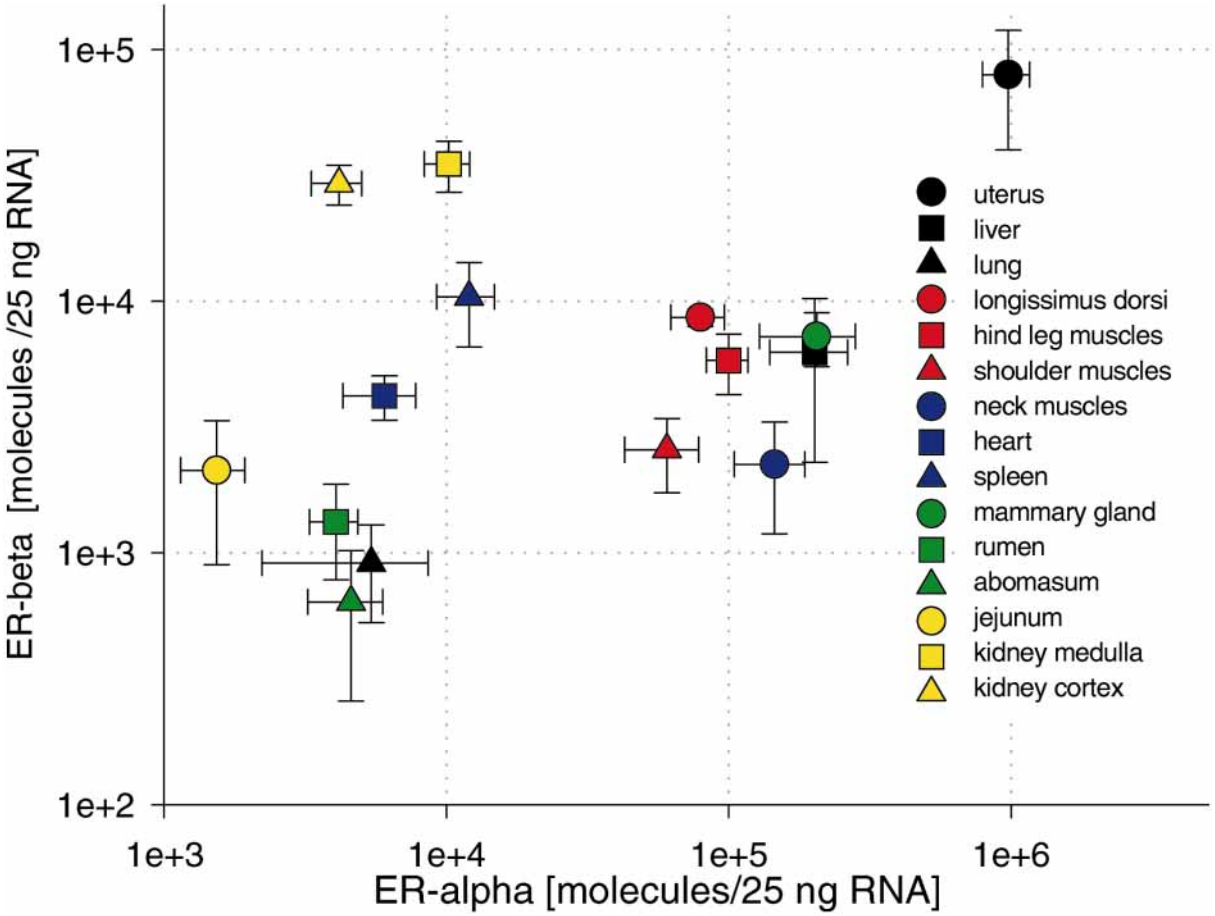

Fig. 3. Tissue-specific ERα and ERβ mRNA expression cluster from 15 tissues. Transcripts were measured on a moleculer basis with real-time RT-PCR in 25 ng total RNA (n=8) and quantified results are shown as mean with bidirectional error bars (s.e.m.).

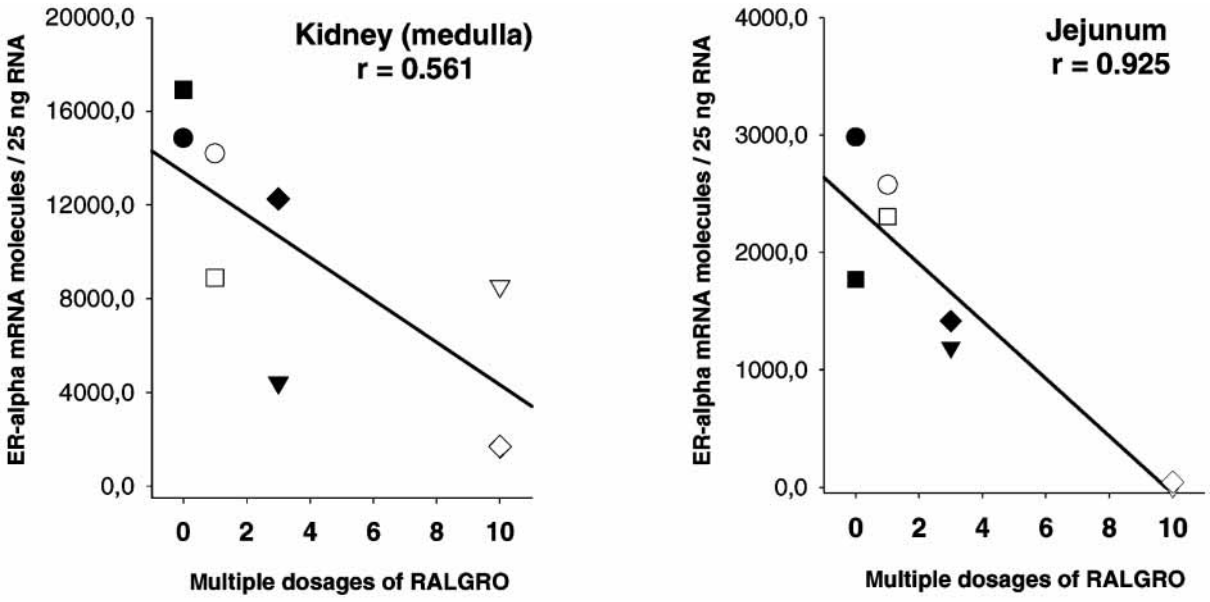

Fig. 4. Relation between multiple treatment with estrogen-active preparation RALGRO® (1×=36 mg Zeranol) and ERα expression in 25 ng total RNA in kidney medulla ($p<0.05$) and jejunum ($p<0.001$) in heifers (n=8).

tissues no trends towards change in either ERα or ERβ expression levels could be detected.

DISCUSSION

The cloning of ERβ has introduced a new level of complexity of estrogen action. Since it was discovered, no definite statement regarding its expression pattern in various tissues and its unique function has been made. This paper focuses on the tissue distribution and expression pattern of both subtypes (ERα and ERβ) in major tissues and under estrogenic active RALGRO treatment in *Bos taurus.*

α-Zearalanol (Zeranol) possesses estrogenic potencies that are approximately 50–60% compared to that of estradiol-17β. Estrogenicity was evaluated using either a competitive receptor binding assay in fish models (23) or an enzyme-linked receptor assay (24). Therefore, the 1-fold RALGRO dosage represents approximately a physiological estrogen concentration in comparison to estradiol-17β occurring at cattle oestrus. After application of a simple RALGRO dose the oestrus cycle is obviously arrested at the preovulatory stage. The multiple dosage resulted in an influence on or even a possible blockage of the oestrus cycle, accompanied by distinctively small uterus sizes, as described earlier (25). Estradiol-17β directly regulates the gondotropin-releasing hormone (GnRH) expression at the level of GnRH neurons and may exert its neuroendocrine control through direct interaction with specific receptors expressed in these cells (26). This clearly indicates the negative feedback mechanism on the gonadotropic axis caused by the high estrogen concentrations.

Plasma Zeranol measurement by EIA was a sensitive and reliable quantification methodology. Concentrations in RALGRO-treated animals resulted in measurable and elevated Zeranol levels in comparison to the control group, whereas a quantification with radioimmunoassay (RIA) did not give different results between the 36 mg Zeranol-treated and untreated groups (27). The decrease in Zeranol levels in plasma in the 10-fold group after day 35 might be due to a reduced drug absorption from the implants, which were degraded during this period.

To detect ERα and ERβ mRNA transcripts a real-time RT-PCR quantification on a LightCycler was used. RT followed by real-time PCR is a sensitive method to quantify low amounts of mRNA molecules (28, 29) and offers important insights into the local expression and para- and autokrine regulation. The reliability of such an assay depends on the sensitivity, the inter-test variations, and on a sufficiently wide linear quantification range. In this

paper we describe the design and validation of a real-time RT-PCR according to the demands of a fast-cycle PCR (30). RT-PCR real-time assays with an external calibration curve are directly quantitative and therefore an absolute comparison of ERα and ERβ mRNA molecules is possible. As demonstrated herein, ERα and ERβ LightCycler RT-PCR meets these test parameter requirements with excellent performance. We have used this ER subtype-specific mRNA quantification system to determine the expression levels, the tissue distribution, and to compare the tissue-specific expression pattern of both ER subtypes in *Bos taurus.* Methods such as multispecies quantification tests were developed and can be used in other species such as humans, sheep, pig and rat with sufficiently high homologies of the primer recognition sites. ERβ was previously detected in a few species and tissues (9, 11, 13, 31), but a direct comparison and quantification of both ER subtype mRNA within one tissue is not available so far. A comparison and co-expression of ERα and ERβ study had already been done in normal mice and ERα knockout mice (ERKO mice) by ribonuclease protection assay (RPA) (32). RPA with following densitometric analysis of band intensities is a comparative, not a quantitative methodology. The assay detected the relative ERα and ERβ mRNA expressions in various organs of the female and male reproductive tracts. In further publications, ERα and ERβ expression and regulation in rat ovary was investigated by Northern Blotting and RT-PCR (33, 34). Quantification of the ER-specific subtypes was done semiquantitatively via RT-PCR with the following densitometric analysis of ethidium bromide-stained gels. In uterus, ERα was the major transcript, whereas in ovary granulosa cells, the ERβ transcript is dominant. ERα/ERβ expression ratio (R) was determined to be in the range 0.5 to 1.0 over the rat ovarian cycle with very close ERα/ERβ ratios. From our own studies in bovine ovarian tissues (data not published) we can confirm the expression of both subtypes and the dominant ERβ expression in granulosa cells (R=0.10) and theca cells (R=0.85). ERβ is functional in granulosa cells, regulated by ovulatory doses of gonadotropins, and seems to be a modulator of ERα action during follicular development (34). In contrast, in bovine corpus luteum, ERα is the dominant estrogen receptor subtype with a wide expression ratio (R=16.7; data not published).

As presented herein, we detected and quantified ERα and ERβ transcripts in all 15 investigated tissues. The results of the multiple implantation of the estrogen-active compound showed a dose-dependent downregulation of ERα expression only in kidney medulla and jejunum. The remaining tissues were not significantly influenced by estrogen treatment, only trends towards a down- or upregulation were observed with high variations. The hypothesis that estrogens (19) can stimulate ERα expression via its own receptor could not be confirmed in this study. The opposite hypothesis must even be reached that excessive Zeranol will downregulate the ERα expression in kidney medulla and jejunum. Estradiol-17β-treated mice showed increased uterus size, but decreased ERβ expression (35). The authors conclude that ERβ plays an important role in modulation of the effects of ERα and, in addition, or as a consequence of this, has a regulatory function in the uterus. In isolated rat granulosa cells, a significant downregulation of ERα under estrogen-active DES (diethylstilboestrol) treatment could be shown (36). However neither Zeranol nor DES will act like the physiological ligand estradiol-17β.

As a result of the mainly nonsignificant relationship (except in kidney-medulla and jejunum) between Zeranol treatment and ERα and ERβ expression levels, all expression data within one tissue were pooled. The derived mean expression concentrations and variations are characteristic for all investigated tissues and the relation between the two ER subtypes results in a tissue-specific expression cluster. More detailed studies of this ERα/ERβ cluster with its tissue-specific variations will lead us to a better understanding of receptor regulation and physiology. Data show high ERα expression rates in the classical estrogen-sensitive tissues and a wide ERα/ERβ ratio. ERβ transcripts were mainly expressed at a lower level. The dominant ERα expression (R=2.6) could also be shown in various brain regions, including hypothalamus, pituitary gland and six different brain lobes (data not published). No expression of ERα could be observed in the bovine endothelial cell preparation, whereas the ERβ transcripts showed very low-abundant expression (data not

published). The dominant role of ERα, especially in uterus, explains why it was the first cloned ER as most purification and cloning and attempts were based on uterine tissue (7).

Both subtypes could also be detected in the muscular tissues with a very wide expression ratio, especially in muscle parts which are involved in cattle allometric growth such as neck and shoulder muscularity. The molecular basis for this sexually dimorphic muscle growth pattern might be attributed to relatively higher sensitivities to sexual steroids in these muscles (37). Over and above that, the present study implies that local differences in ERα expression might be one promoting factor for higher growth velocities and might play a key role in allometric growth.

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 these tissues. ERα had already been detected in the bovine gastrointestinal tract (38), but ERβ might play the major role in the absorptive processes. However, whilst any notions as to the direct physiological effects of estrogens on gastrointestinal tissues and kidney remain speculative, there are some indications that estrogens might influence the calcium transport in rats (39).

CONCLUSION

In view of the data provided for sensitivity, linearity and reproducibility, the RT-PCR assay developed here allows the absolute and accurate quantification of ERα and ERβ mRNA molecules with a sufficiently high sensitivity even for tissues with low abundancies down to a few molecules. Our expression results indicate the existence of two ER subtypes in various bovine tissues, their different expression pattern and co-expression, as well as their tissue-specific regulation during estrogen treatment. These different expression patterns of ERα and ERβ can be regarded as supporting the hypothesis that ER subtype proteins may have different biological functions, especially in kidney and jejunum where there is an inverse ERβ expression ratio in comparison to the other investigated tissues. In the future more detailed studies of ERα and ERβ must be carried out in all kidney cell types and all parts of the gastrointestinal system as part of continuing investigations into ER regulation and its physiological function.

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