J. Vet. Med. A **49,** 57–64 (2002)

ISSN 0931–184X

ENDOCRINOLOGY

Institute of Physiology, Centre of Life and Food Sciences, Technical University of Munich, Germany

Effects of Synthetic Progestagens on the mRNA Expression of Androgen Receptor, Progesterone Receptor, Oestrogen Receptor α and β, Insulin-like Growth Factor-1 (IGF-1) and IGF-1 Receptor in Heifer Tissues

M. W. Pfaffl[1], A. Daxenberger, M. Hageleit and H. H. D. Meyer

Address of authors: Institute of Physiology, Centre of Life and Food Sciences, Technical University of Munich, Germany; [1]Corresponding author: E-mail: michael.pfaffl@wzw.tum.de

With 1 figure and 6 tables

Received for publication April 26, 2001

Summary

Synthetic progestagens like melengestrol acetate (MGA) are widely used for oestrus synchronization and for growth promotion in cattle production. The metabolic effects exceed its primary potency as a progestagen. It is speculated that MGA stimulates follicle development and thereby endogenous oestrogen production, but inhibits ovulation. To investigate the dose-dependent effects on mRNA expression levels, six heifers were fed for 8 weeks with different levels of MGA (0.5, 1.5, 5 mg) daily and two heifers served as controls. The expression of steroid receptor mRNA [androgen receptor (AR), progesterone receptor (PR), oestrogen receptor (ER) ERα and ERβ], insulin-like growth factor-1 (IGF-1) and its receptor were quantified in liver, neck (m. splenius) and shoulder muscularity (m. deltoideus). Plasma concentrations of IGF-1 were quantified by radioimmunoassay. In treated animals the MGA plasma levels were elevated over the complete treatment period, corresponding to the MGA treatment concentrations. IGF-1 concentrations of control animals were at constant levels. Plasma levels for oestradiol (E2) and IGF-1 were increased in the low MGA treatment group. Overdosed MGA decreased progesterone (P4) and E2 levels. To quantify the IGF-1 and all receptor mRNA transcripts, sensitive and reliable real-time reverse transcription–polymerase chain reaction (RT–PCR) quantification methods were developed and validated in the LightCycler. A dose-dependent relationship between increasing MGA concentration and mRNA expression was observed in liver for AR and IGF-1 receptor, and in neck muscularity for IGF-1. ERα in liver and neck muscle showed a trend of increasing expression.

Introduction

Synthetic progestagens are used in humans for medical reasons, e.g. hormonal contraception and hormone replacement therapy, and in farm animals to synchronize oestrus in cattle as well as to inhibit oestrus and ovulation in fattening heifers (Zimbelman and Smith, 1966a; Patterson et al., 1989). Melengestrol acetate (MGA) belongs to the most active synthetic gestagens, and if given parenterally the hormonal activity is 125 times more effective than progesterone (P4) (Lauderdale et al., 1983). In the USA and Canada, MGA is also licensed as a growth-promoting feed additive for feedlot heifers. It is applied in doses of 0.5 mg MGA/day to calm animal behaviour and to improve feed efficiency and growth while suppressing the oestrus. It is speculated that MGA stimulates follicle development, but inhibits ovulation (Kojima et al., 1992, 1995; Custer et al., 1994; Kastelic et al., 1997; Imwalle et al., 1998). The optimal dose to stimulate pre-ovulatory follicle development is 0.42 mg MGA/day (Zimbelman and Smith, 1966b). Its anabolic effects may be mediated via stimulating the ovarian synthesis of endogenous anabolic steroids like oestradiol (E2) (Lauderdale et al., 1983; Henricks et al., 1997; Hageleit et al., 2000) and by suppressing P4 secretion (Hageleit et al., 2000). The biological effects of MGA exceed its primary potency as a progestagen. In cattle, MGA binds with 5.3 times higher affinity to the bovine progesterone receptor (PR) compared with its physiological ligand P4 (Bauer et al., 2000).

Regarding steroid receptor action, it is well known that sex steroids lead to an increased mRNA transcription rate and in consequence to the synthesis of specific proteins (Schütz, 1988; King, 1992). The activated steroid–receptor complex binds to specific DNA segments and acts as a transcriptional factor (Gronemeyer, 1992; Collins, 1994). However, knowledge of the influence of synthetic progestins on the expression pattern and regulation of mRNA, acting at various sex steroid receptors, is missing at this time. A more detailed study on dose-dependent effects of synthetic progestagens on mRNA expression is essential to continue investigations of the regulation and its possible physiological function.

The first goal of this study was to investigate the dose-dependent MGA effects in order to show the coherence of hormonal effects on the expression pattern of various steroid receptors: androgen receptor (AR), PR, oestrogen receptor (ER) ERα and ERβ. In the literature, it is controversially discussed whether the steroid hormones up- or down-regulate their own receptor expression. For the uterus it is proposed that E2-17β can stimulate ERα expression (Jungblut et al., 1976), but excessive oestrogen-active Zeranol treatment will

down-regulate ERα mRNA expression in kidney medulla and jejunum (Pfaffl et al., 2001).

The second goal of this study was to determine the influence of MGA on the expression pattern of insulin-like growth factor-1 (IGF-1) and its corresponding receptor (IGF-1R). The effects of steroid hormones on the somatotropic axis are evident from the sexual dimorphism in growth hormone and IGF-1 secretion. IGF-1 is an important growth regulator in numerous tissues acting in an endocrine as well as in an autocrine and paracrine way (Thissen et al., 1994). In general, males show higher IGF-1 plasma concentrations than females or castrates (Plouzek and Trenkle, 1991; Pfaffl et al., 1998). Also, oestrogen can stimulate IGF-1 gene expression (Rutanen, 2000) and IGF-1 plasma levels (Sauerwein et al., 1992).

To detect and quantify these rare mRNA transcripts, a reverse transcription (RT) followed by a real-time polymerase chain reaction (PCR) on the LightCycler system was used. Real-time RT–PCR with an external calibration curve is a fully quantitative methodology and therefore an absolute comparison of all transcripts within the investigated tissue RNA preparation is possible (Pfaffl and Hageleit, 2001).

Materials and Methods

Animal experiment

Eight cycling Holstein Friesian heifers between 12 and 16 months of age with a mean weight of 320 kg were assigned randomly to four treatment groups (Daxenberger et al., 1999). In each group, two heifers were treated with 0 (negative control), 0.5, 1.5 or 5 mg MGA/day via a feed supplement for 56 days. The feed supplements were prepared with MGA standard (ICN Biochemicals, Eschwege, Germany) in bruised grain.

Sample collection and plasma IGF-1 quantification

For the IGF-1 analyses, plasma samples were collected twice a week by punctuation of the jugular vein. After 56 days of MGA feeding all animals were slaughtered. Immediately after slaughtering and bleeding, liver, neck muscle (m. splenius) and shoulder muscle (m. deltoideus) samples were taken and subsequently frozen in liquid nitrogen. IGF-1 plasma levels were quantified according to a modified radioimmunoassay method published previously (Daxenberger et al., 1998). The sample dilution was 1/100 and the amount of IGF-2 to displace IGF-1 from its complex with IGF-binding proteins was 20 μg/ml plasma.

Total RNA extraction

A sample of 200 mg of frozen tissue was homogenized in 4 M guanidinium thiocyanate buffer to destroy RNase activity (Chirgwin et al., 1979). 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, optical density (OD) was determined 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 the OD_{260}/OD_{280} absorption ratio > 1.9.

Primer design

The primers were derived from bovine and ovine sequences available at GenBank (http://www.ncbi.nlm.nih.gov/Entrez/index.html) or EMBL (http://www.ebi.ac.uk). The primers were designed to produce an amplification product which spanned at least two exons in the highly conserved coding region (CDS) of the appropriate coding sequence of multiple species. Therefore a multiple CDS alignment (clustal alignment in HUSAR® software) of the available mRNAs was carried out at DKFZ (http://genome.dkfz-heidelberg.de/biounit). Primer design and optimization were carried out in the high homology regions of the multiple alignment with regard to primer dimer formation, self-priming formation and primer melting temperature (HUSAR® software at DKFZ). The primer sequences are summarized in Table 1. AR (Malucelli et al., 1996), PR, ERα (Pfaffl et al., 2001) and ERβ were designed in the region of the receptor ligand binding domain to produce 172, 227, 234 and 242 bp amplification products, respectively. IGF-1 primers were designed to produce a 240 bp amplification product in the highly conserved region of exons 3 and 4 (Gilmour et al., 1992) coding for the mature IGF-1 protein (Pfaffl et al., 1998). For IGF-1R, no information about the intron and exon structure is available in the published se-quence databases and primer design was based on multispecies sequence clustal alignment (Plath-Gabler et al., 2001).

RT

Total RNA (1 μg) from the sample preparation was reverse transcribed in 40 μl as follows: M-MLV RT buffer (Promega, Mannheim, Germany) and 300 μM dNTPs (MBI Fermentas) were denatured for 5 min at 65°C in a Mastercycler Gradient

Table 1. Primer sequences: androgen receptor (AR; Malucelli et al., 1996), oestrogen receptor α (ERα; Pfaffl et al., 2001), ERβ, progesterone receptor (PR), insulin-like growth factor-1 (IGF-1; Pfaffl et al., 1998), IGF-1 receptor (IGF-1R; Plath-Gabler et al., 2001)

AR forward primer	5′-CCT GGT TTT CAA TGA GTA CCG CAT G-3′
AR reverse primer	5′-TTG ATT TTT CAG CCC ATC CAC TGG A-3′
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′-CTT CGT GGA GCT CAG CCT GT-3′
ERβ reverse primer	5′-GAG ATA TTC TTT GTG TTG GAG TTT-3′
PR forward primer	5′-GAG AGC TCA TCA AGG CAA TTG G-3′
PR reverse primer	5′-CAC CAT CCC TGC CAA TAT CTT G-3′
IGF-1 forward primer	5′-TCG CAT CTC TTC TAT CTG GCC CTG T-3′
IGF-1 reverse primer	5′-GCA GTA CAT CTC CAG CCT CCT CAG A-3′
IGF-1R forward primer	5′-TTA AAA TGG CCA GAA CCT GAG-3′
IGF-1R reverse primer	5′-ATT ATA ACC AAG CCT CCC AC-3′

(Eppendorf, Hamburg, Germany). The subsequent RT was carried out at 37°C for 60 min by adding 2.5 mM random hexamer primers (Pharmacia), 200 U of M-MLV RNase H-reverse transcriptase (Promega), 12.5 U of RNasin RNase inhibitor (MBI Fermentas). The samples were then heated for 1 min at 99°C to terminate the RT.

Optimization of the specific RT–PCR

Conditions for the RT–PCRs were optimized in a gradient cycler with regard to *Taq* DNA polymerase (Roche Diagnostics, Basel, Switzerland), PCR water, primers (MWG), $MgCl_2$ (Roche) concentrations and various annealing temperatures. Amplification products were separated using 4% high resolution NuSieve agarose (FMC Bio Products, Rockland, ME, USA) gel electrophoresis and analysed with the Image Master system (Pharmacia). The optimized results were transferred to the following LightCycler PCR master mix and protocol.

LightCycler PCR master mix

For LightCycler reactions, a master mix of the following reaction components was prepared to the indicated end concentration: 6.4 μl of water, 1.2 μl of $MgCl_2$ (4 mM), 0.2 μl of forward primer (0.4 μM), 0.2 μl of reverse primer (0.4 μM) and 1.0 μl of LightCycler Fast Start DNA Master SYBR Green I (Roche). An aliquot of 9 μl of LightCycler master mix was added to the LightCycler glass capillaries and 1 μl volume was added as the PCR template. The capillaries were closed, centrifuged and placed into the LightCycler rotor. To improve SYBR Green I quantification, a high temperature fluorescence measurement point was included in the amplification protocol performed (Pfaffl, 2001). This melts the non-specific PCR products at the elevated temperature, e.g. primer dimers, eliminates the non-specific fluorescence signal and ensures accurate quantification of the desired product. The following LightCycler protocols were used: denaturation programme (95°C for 10 min), a four segment amplification and quantification programme repeated 40 times (factor-specific amplification conditions with a single fluorescence measurement at an elevated temperature level are listed in Table 2), melting curve programme (60–99°C with a heating rate of 0.1°C/s and continuous fluorescence measurements) and finally a cooling programme down to 40°C.

Calibration curves

For all quantitative assays an external calibration curve was used, based on a single-stranded DNA (ssDNA) molecule calculation, according to Pfaffl and Hageleit (2001). Individual RT–PCR products from *Bos taurus* were cloned separately in pCR2.0 or pCR4.0 (Invitrogen, Leek, The Netherlands), linearized by a unique restriction digest and dilutions of each plasmid preparation from single copy ssDNA up to 10^{10} ssDNA molecules were used in the calibration curve (Table 3).

Statistical analysis

Expression data (mRNA molecules per 20 ng of total RNA) of AR, ERα, ERβ, IGF-1 and IGF-1R ($n = 8$) were calculated as a function of increasing MGA concentration [c(MGA)] and plotted as linear regression (Table 4):

$$\text{mRNA molecules} = \text{intercept} + [\text{slope} \times \text{c(MGA)}]$$

Statistical comparisons were performed using SIGMA-STAT software version 2.0 for WINDOWS 95 (Jandel Scientific Software, San Rafael, CA, USA). Expression data groups passed the normality test and the constant variance test (Jandel Scientific Software). Data pairs of variables (concentration of MGA and corresponding mRNA molecules) with positive correlation coefficient and $P < 0.05$ tended to increase together, and were regarded as significant. Plasma concentrations of hormones and expression data were analysed using the GLM procedure of Statistical Analysis Systems. Student–Newman–Keuls tests for comparisons between subgroups were calculated. Differences of $P < 0.05$ were regarded as significant.

Results

Confirmation of primer specificity and sequence analysis

For exact length verification, the RT–PCR products were separated using high resolution gel electrophoresis. Amplified real-time RT–PCR products showed a single band at the expected length (Table 3). The specificity of the RT–PCR products was additionally documented using melting curve analysis of LIGHTCYCLER software 3.39 (Roche). Sequence analysis (MWG Biotech, Ebersberg, Germany) of cloned RT–PCR products from *Bos taurus* showed 100 % homology to the published sequences.

Real-time RT–PCR assay validation

All performed real-time assays were product specific, and effective PCR amplification kinetics were shown by high PCR efficiency (Table 3). Assay sensitivities were confirmed by detection limits down to a few molecules. PCR efficiency (E) was calculated from the given slope of the calibration curve in the LIGHTCYCLER software, according to the equation: $E =$

Table 2. Cycling conditions of androgen receptor (AR), oestrogen receptor α (ERα), ERβ, progesterone receptor (PR), insulin-like growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) in a four segment LightCycler real-time reverse transcription–polymerase chain reaction (RT–PCR). The amplification and quantification programme was repeated 40 times with a single fluorescence acquisition point at an elevated temperature (segment IV)

			Temperature profile (°C)					
No.	Segment	Duration (s)	AR	ERα	ERβ	PR	IGF-1	IGF-1R
I	Denaturation	15	95	95	95	95	95	95
II	Product-specific annealing	10	60	64	64	65	63	62
III	Elongation	20	72	72	72	72	72	72
IV	Fluorescence acquisition	3	83	82	87	81	80	82

Table 3. Characteristics and validation parameters of real-time reverse transcription–polymerase chain reaction (RT–PCR) assays. Intra-assay (test precision) and inter-assay variation (test variability) of real-time RT–PCR assays were determined over the complete quantification range. The detection limit, quantification limit and variations were on a molecule basis (m)

	AR	ERα	ERβ	PR	IGF-1	IGF-1R
Product length (bp)	172	234	262	227	240	314
Detection limit (m)	12	2	3	14	16	200
Quantification limit (m)	120	165	11	760	1600	200
Quantification range (m)	120–1.20×10^{10}	165–1.65×10^{9}	11–1.06×10^{10}	760–7.60×10^{9}	1600–1.6×10^{11}	200–1.6×10^{8}
Test linearity (r)	0.998	0.995	0.996	0.998	0.992	0.967
PCR efficiency	1.91	1.81	1.82	1.94	1.93	1.94
Intra-assay variation (%)	31.2 ($n = 3$)	18.7 ($n = 4$)	17.6 ($n = 4$)	5.7 ($n = 4$)	11.8 ($n = 3$)	9.8 ($n = 3$)
Inter-assay variation (%)	24.3 ($n = 7$)	28.6 ($n = 4$)	29.7 ($n = 4$)	25.7 ($n = 4$)	28.2 ($n = 4$)	8.6 ($n = 4$)

AR, androgen receptor; ER, oestrogen receptor; PR, progesterone receptor; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor.

$10^{[-1/\text{slope}]}$ (Rasmussen, 2001). Test linearity was given between 10^2 and 10^{10} molecules, demonstrating a linear quantification over a wide quantification range. Intra- and inter-assay variations of < 31.2% were determined over the entire quantification range (Table 3). The advantage of a high temperature fluorescence acquisition in the fourth segment during the amplification programme results in reliable and sensitive mRNA quantification with high linearity (Pearson correlation coefficient; $r > 0.967$) up to eight orders of magnitude. High temperature fluorescence acquisition melts the non-specific PCR products at elevated temperatures, eliminates the non-specific fluorescence signal derived from primer dimers and ensures an accurate quantification of the desired products.

Expression data

Expression data were derived for AR, ERα, ERβ, IGF-1 and IGF-1R from all investigated tissues. No quantitative RT–PCR product results could be generated from PR. PR quantitative RT–PCR test integrity and reproducibility could be determined in uterus total RNA (Table 3). mRNA expression data for AR, ERα, ERβ, IGF-1 and IGF-1R (molecules/20 ng total RNA) were compared with increasing treatment concentration of MGA. The results are plotted in Table 4 according to the equation: mRNA molecules = intercept + [slope × c(MGA)]. Figure 1 shows the principle of the data evaluation with the example of hepatic AR mRNA expression. Data pairs of variables, treatment concentration of MGA versus corresponding mRNA molecules, with positive Pearson correlation coefficient and P-values under 0.05, tended to increase together and were regarded as significant. A significant ($P < 0.05$) relationship between increasing MGA concentration and expression were observed in liver for AR (Fig. 1) and IGF-1R, and in neck muscularity for IGF-1 (Table 4). ERα mRNA expression in the liver and neck muscles showed a trend of increased expression ($0.05 < P < 0.1$). All remaining tissues and quantified mRNA had no trend of expression change with increasing MGA treatment concentration.

Table 4. Expression data (mRNA molecules/20 ng of total RNA) of the androgen receptor (AR), oestrogen receptor α (ERα), ERβ, insulin-like growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) ($n = 8$) were compared with increasing melengestrol acetate (MGA) concentration [c(MGA)] and plotted as linear regression: mRNA molecules = intercept + [slope × c(MGA)]. Expected expression results from the progesterone receptor (PR) were under the detection limit of the quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR). Significance analysis was performed using SIGMA-STAT software. Data pairs of variables (MGA concentration and corresponding mRNA molecules) with a positive correlation coefficient and $P < 0.05$ tended to increase together, and were regarded as significant

	Linear regression		
	mRNA molecules = intercept + [slope × c(MGA)]	Pearson correlation coefficient	P-value
AR	AR (liver) = 4450.74 + [1138.22 × c(MGA)]	0.806	0.016
	AR (neck) = 1117.67 − [72.98 × c(MGA)]	0.272	0.515
	AR (shoulder) = 1931.57 + [64.20 × c(MGA)]	0.137	0.746
ERα	ERα (liver) = 2048.71 + [552.0 × c(MGA)]	0.704	0.051
	ERα (neck) = 4344.06 + [750.48 × c(MGA)]	0.651	0.081
	ERα (shoulder) = 7865.57 + [872.46 × c(MGA)]	0.533	0.174
ERβ	ERβ (liver) = 2.62 + [0.019 × c(MGA)]	0.018	0.966
	ERβ (neck) = 2.73 + [0.609 × c(MGA)]	0.480	0.229
	ERβ (shoulder) = 4.84 − [0.232 × c(MGA)]	0.130	0.759
PR	Under detection limit	–	–
IGF-1	IGF-1 (liver) = 9243.26 − [22.64 × c(MGA)]	0.008	0.984
	IGF-1 (neck) = 114.67 + [181.42 × c(MGA)]	0.825	0.012
	IGF-1 (shoulder) = 315.16 − [22.08 × c(MGA)]	0.404	0.321
IGF-1R	IGF-1R (liver) = 41.39 + [12.04 × c(MGA)]	0.715	0.046
	IGF-1R (neck) = 292.66 + [19.48 × c(MGA)]	0.268	0.522
	IGF-1R (shoulder) = 519.63 + [36.98 × c(MGA)]	0.216	0.607

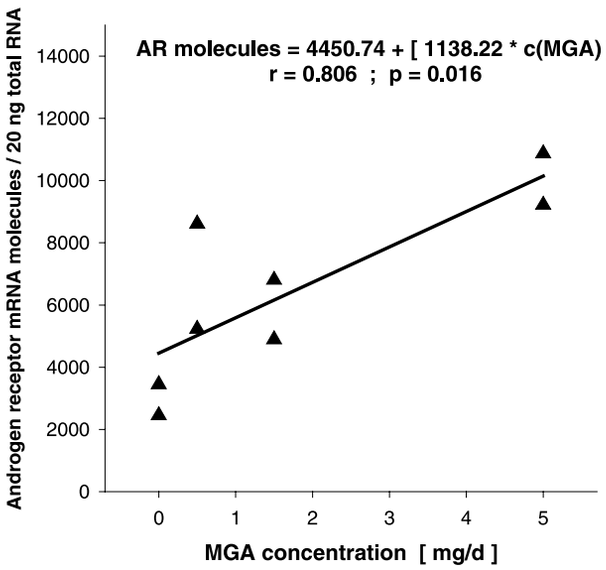

Fig. 1. Relationship between different concentrations of melengestrol acetate (MGA) and androgen receptor (AR) mRNA expression on a molecule basis in 20 ng of total RNA in heifers ($n = 8$).

IGF-1 concentration in plasma

IGF-1 plasma concentrations of control animals and in groups before MGA treatment were constant between 232 and 349 ng/ml. In 0.5 mg MGA/day-treated animals, the plasma levels were significantly elevated in all three treatment periods (374–438 ng/ml). Overdosed MGA treatment scarcely increased plasma IGF-1 to levels of 241–322 ng/ml. IGF-1 plasma data are shown in Table 5. For a better comparison of hormone plasma levels, data for MGA (Daxenberger et al., 1999), E2 and P4 (Hageleit et al., 2000) are also summarized.

Discussion

MGA was chosen as an ideal model in heifers to mimic a pure gestagenic activity. MGA is a highly active synthetic progestagen, has no undesired pharmacological properties to other steroid receptors, has a low endogen clearance rate and exact dosages can be easily applied orally. For the quantification of various mRNA transcripts and IGF-1 protein, very sensitive and reliable assays were applied. IGF-1 and ERα mRNA expression were measured with established quantitative real-time RT–PCR assays (Pfaffl, 2001; Pfaffl et al., 2001). Assays for AR, PR, ERβ and IGF-1R were newly developed and validated on the LightCycler system. IGF-1 in plasma was determined using an established and reliable non-extraction radioimmunoassay, according to a recently published method (Daxenberger et al., 1998).

Plasma hormone concentrations

In treated animals, the MGA plasma levels were elevated over the complete treatment period, corresponding to the elevated MGA treatment concentrations (Daxenberger et al., 1999). Hormone profiles clearly demonstrate that stimulation of follicular E2 synthesis is the main anabolic pathway of MGA in heifers (Hageleit et al., 2000). Plasma P4 levels were suppressed under MGA treatment (Kojima et al., 1995; Hageleit et al., 2000). A trend of co-regulation of IGF-1 and E2 in the 0.5 mg MGA/day group seemed obvious, but elevated IGF-1 and E2 plasma concentrations in this group showed no significant relationship.

Expression profiles

For the exact quantification of AR, ERα (Pfaffl et al., 2001), ERβ, PR, IGF-1 (Pfaffl, 2001) and IGF-1R, quantitative real-

Table 5. Plasma concentration (mean ± standard deviation) of melengestrol acetate (MGA), P4, E2 and insulin-like growth factor-1 (IGF-1) in heifers treated with increasing MGA concentrations: control group, 0.5, 1.5 and 5 mg MGA/day. Plasma data before treatment ($n = 2$) and during MGA treatment were divided into three periods: first ($n = 5$), second ($n = 6$) and third ($n = 6$). Different upper-case letters designate significant differences ($P < 0.05$) between MGA treatment periods. Different lower-case letters designate significant differences ($P < 0.05$) between MGA treatment concentrations

Stage of treatment	Before	First period	Second period	Third period
MGA (pg/ml)				
Control	nd	nd	nd	nd
0.5 mg	nd	38.5 ± 11.9^{Aa}	36.0 ± 7.2^{Aa}	31.8 ± 7.7^{Aa}
1.5 mg	nd	138.2 ± 39.9^{Ab}	130.1 ± 18.9^{Ab}	116.7 ± 19.3^{Ab}
5 mg	nd	375.0 ± 52.3^{Ac}	277.4 ± 66.1^{Bc}	203.4 ± 37.4^{Cc}
P4 (ng/ml)				
Control	0.58 ± 0.60^{a}	1.31 ± 0.72^{Aa}	1.39 ± 1.49^{Aa}	1.25 ± 1.22^{Aa}
0.5 mg	2.81 ± 3.45^{a}	1.31 ± 1.17^{Aa}	0.16 ± 0.03^{Bb}	0.16 ± 0.04^{Bb}
1.5 mg	0.22 ± 0.09^{a}	0.71 ± 0.96^{Aa}	0.15 ± 0.00^{Bb}	0.15 ± 0.00^{Bb}
5 mg	2.91 ± 3.82^{a}	0.75 ± 1.23^{Aa}	0.19 ± 0.06^{Ab}	0.17 ± 0.06^{Ab}
E2 (pg/ml)				
Control	0.66 ± 0.08^{a}	1.08 ± 0.78^{Aa}	2.05 ± 3.05^{Aa}	1.45 ± 1.59^{Aa}
0.5 mg	1.26 ± 0.10^{a}	3.84 ± 2.39^{Ab}	6.68 ± 2.42^{Bb}	7.79 ± 3.44^{Bb}
1.5 mg	2.29 ± 1.49^{a}	1.68 ± 0.49^{Aa}	2.17 ± 1.03^{Aa}	1.82 ± 1.10^{Aa}
5 mg	1.12 ± 0.42^{a}	1.17 ± 0.31^{Aa}	1.39 ± 0.45^{Aa}	1.05 ± 0.38^{Aa}
IGF-1 (ng/ml)				
Control	294.6 ± 76.1^{a}	232.5 ± 56.2^{Aa}	297.9 ± 73.6^{Ba}	280.2 ± 42.0^{Ba}
0.5 mg	349.0 ± 104.4^{a}	389.5 ± 100.8^{Ab}	374.2 ± 118.0^{Ab}	438.2 ± 101.2^{Ab}
1.5 mg	257.8 ± 9.9^{a}	241.5 ± 29.5^{Aa}	286.9 ± 32.8^{Ba}	258.4 ± 35.7^{Aa}
5 mg	321.2 ± 67.9^{a}	310.9 ± 80.7^{Ac}	322.5 ± 68.2^{Aab}	295.2 ± 51.4^{Aa}

nd, not detectable.

time RT–PCR assays were applied. The sensitivity, linearity and reproducibility of the assays allow for absolute and accurate quantification even in tissues, even with low mRNA abundancies down to a few molecules. We have used these assays to compare the expression rates in a dose-dependent MGA treatment in liver, m. splenius and m. deltoideus of heifers. The presence and the amount of the steroid receptor mRNA in the tissue is the first determination of specificity and the magnitude of the response (King, 1992). Except PR, all investigated transcripts could be quantified with high accuracy. The PR mRNA quantities were under the detection limit of the applied quantitative real-time RT–PCR of 14 molecules per LightCycler capillary. The integrity and reproducibility of the PR assay could be confirmed in uterus total RNA. However, MGA effects exceed the responses of the physiological ligand P4 and might have an influence on other members of the steroid receptor family, like AR, ERα and ERβ. AR could be detected and quantified in liver, as found recently (Cho and Lim, 1997; Cohen et al., 1998), and in m. deltoideus and m. splenius. Liver AR mRNA expression was 2–4-fold more abundant than in investigated muscles. Derived expression data on a molecule basis were compared in a linear regression model with increasing treatment MGA concentration. Significant coherence ($r = 0.806$; $P = 0.016$) between increasing MGA concentration and AR mRNA expression was only observed in the liver. This suggests that MGA can induce the expression of related sex steroid receptors, at least the AR in liver. As shown in other studies, AR were present in the liver (Cohen et al., 1998) and muscularity (Sauerwein and Meyer, 1989) and therefore may mediate the action of sex hormones or androgenic steroids.

To detect ERβ transcripts, quantification was performed in 50 ng of total RNA. However, in all three tissues the amount of ERβ mRNA was under the quantification limit of 11 molecules. ERβ mRNA molecules and linear regression, listed in Table 4, were calculated on a level of 20 ng of total RNA. No dose-dependent relationship could be found for both ER subtypes. In monkey breast tissue, conjugated oestrogens and medroxy-P4 acetate could decrease the percentage of ER and PR (Cline et al., 1996). Similar to other studies, the ERα subtype was the major transcript in comparison with ERβ (Byers et al., 1997; Pfaffl et al., 2001). The ERα/ERβ expression ratio was determined to be in the range of $1–2 \times 10^3$, even under MGA treatment.

The hypothesis that steroid hormones can stimulate steroid receptor expression via their own receptor could not be confirmed for the tissues used in this study, because of the missing PR expression results. However, a complementary new hypothesis can be drawn that excessive progestagen will down-regulate endogenous P4 and E2 and therefore the AR expression is up-regulated in liver and ERα expression tends to be up-regulated in liver and neck muscularity. For ERβ no clear coherence between plasma E2 and receptor transcripts could be drawn.

A significant and MGA dose-dependent relationship was also observed for IGF-1 in neck muscles and for IGF-1R in liver. In general, IGF-1 mRNA expression in liver and IGF-1 plasma levels are highly correlated (Pfaffl et al., 1998), stimulated by E2 (Sauerwein et al., 1992; Rutanen, 2000; Englund et al., 2000) and levels are higher in males than in females or castrates (Plouzek and Trenkle, 1991; Pfaffl et al., 1998). Liver IGF-1 is the major source for the circulating IGF-1 (Thissen et al., 1994) with 30–80 times higher expression levels in liver total RNA. IGF-1R is expressed at lower levels in bovine liver than in the muscularity. This might be attributed to relatively higher IGF-1 sensitivities in different muscles and the molecular basis for the sexual dimorphism in muscle growth pattern (Pfaffl et al., 1998). This implies that local differences in IGF-1 and IGF-1R expression, as well as the steroid receptor expression, might be the mediators of the allometric growth of these muscles. In this study, the regulation through MGA was evident in liver for IGF-1R (at low levels) and in neck muscularity for IGF-1. In the literature, expression levels under hormonal treatment are discussed differentially. E2 inhibits IGF-1 and IGF-1R mRNA expression in vascular smooth muscle cells (Scheidegger et al., 2000). Hormonal contraceptive treatment (P4 and ethinyl-E2) elevates serum IGF-1 levels and IGF-1 mRNA in breast tissue (Isaksson et al., 1999), but in the uterus IGF-1 mRNA is down-regulated while IGF-1R and ER expression remain constant (Wang et al., 2000).

Conclusion

The expression results indicate the existence of AR, ERα, ERβ, IGF-1 and IGF-1R in various bovine tissues, their different expression patterns and regulation under progestagen treatment. In view of the data provided for sensitivity, linearity and reproducibility, the RT–PCR assay developed herein allows the absolute and accurate quantification of mRNA molecules with a sufficiently high sensitivity. The presented results, as well as the results derived from Daxenberger et al. (1999) and

Table 6. Synopsis of the knowledge about the anabolic mechanism of melengestrol acetate (MGA) action

Effect	Low MGA (0.5 mg/day)	High MGA (≥ 1.5 mg/day)
Growth	Anabolism	No anabolism
Hypothalamic feedback	Inhibition of positive oestradiol feedback	Negative feedback, less gondotropins
Follicle stimulation	Continuously	Low
Ovulation	Inhibited	Inhibited
Plasma E2	Increased	Low
Plasma P4	Decreased	Decreased
Plasma IGF-1	Increased	Scarcely increased
AR expression (liver)	Dose-dependent increase ($P < 0.05$)	
IGF-1 expression (neck)	Dose-dependent increase ($P < 0.05$)	
IGF-1R expression (liver)	Dose-dependent increase ($P < 0.05$)	
ERα expression (liver and neck)	Tendentially increased ($0.05 < P < 0.1$)	

E2, oestradiol; P4, progesterone; IGF-1, insulin-like growth factor-1; AR, androgen receptor; IGF-1R, IGF-1 receptor; ER, oestrogen receptor.

Hageleit et al. (2000), are summarized in Table 6. MGA could stimulate the follicle and thereby inhibit ovulation. An effect on the plasma hormone profiles and anabolism was evident. Plasma concentrations showed elevated MGA levels in treated animals and, therefore, significantly decreased levels of P4 and E2. IGF-1 concentrations only seemed elevated in the low treatment group. Positive correlations were found between MGA concentration and mRNA expression in the liver for AR and IGF-1R, as well as in neck muscularity for IGF-1. ERα showed a trend of increasing expression. For PR, no quantitative RT–PCR product could be generated in the investigated tissues. Thus, mechanisms other than the direct steroid–receptor mechanism probably regulate IGF-1 and ERβ expression.

Acknowledgements

The study was financially supported by the European Union. The experimental animals were slaughtered at the EU official slaughterhouse of the Bayerische Landesanstalt für Tierzucht at Grub, 85580 Poing, Germany.

References

Bauer, E. R. S., A. Daxenberger, T. Petri, H. Sauerwein, and H. H. D. Meyer, 2000: Characterisation of the affinity of different anabolics and synthetic hormones to the human androgen receptor, human sex hormone binding globulin and to the bovine gestagen receptor. APMIS **108,** 838–846.

Byers, M., G. G. Kuiper, J. A. Gustafsson, and O. K. Park-Sarge, 1997: Estrogen receptor-beta mRNA expression in rat ovary: down-regulation by gonadotropins. Mol. Endocrinol. **11,** 172–182.

Chirgwin, J. M., E. A. Przybyla, J. MacDonald, and W. J. Rutter, 1979: Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. J. Biochem. **18,** 5291–5299.

Cho, H., and I. K. Lim, 1997: Expression of androgen receptor and its implication in hepatoma cells. Cancer Lett. **115,** 135–140.

Cline, J. M., G. Soderqvist, E. von Schoultz, L. Skoog, and B. von Schoultz, 1996: Effects of hormone replacement therapy on the mammary gland of surgically postmenopausal cynomolgus macaques. Am. J. Obstet. Gynecol. **174,** 93–100.

Cohen, C., D. Lawson, and P. B. DeRose, 1998: Sex and androgenic steroid receptor expression in hepatic adenomas. Hum. Pathol. **29,** 1428–1432.

Collins, D. C., 1994: Sex hormone receptor binding, progestin selectivity, and the new oral contraceptives. Am. J. Obstet. Gynecol. **170,** 1508–1513.

Custer, E. E., W. E. Beal, S. J. Wilson, A. W. Mesdows, J. G. Berardinelli, and R. Adair, 1994: Effect of melengestrol acetate (MGA) or progesterone-releasing intravaginal device (PRID) on follicular development, concentrations of estradiol-17β and progesterone, and luteinizing hormone release during an artificially lengthened bovine estrous cycle. J. Anim. Sci. **72,** 1282–1289.

Daxenberger, A., B. H. Breier, and H. Sauerwein, 1998: Increased milk levels of insulin-like growth factor 1 (IGF-1) for the identification of bovine somatotropin (bST) treated cows. Analyst **123,** 2429–2435.

Daxenberger, A., K. Meyer, M. Hageleit, and H. H. D. Meyer, 1999: Detection of melengestrol acetate residues in plasma and tissues of heifers. Vet. Quart. **21,** 154–158.

Englund, K., B. Lindblom, K. Carlstrom, I. Gustavsson, P. Sjoblom, and A. Blanck, 2000: Gene expression and tissue concentrations of IGF-1 in human myometrium and fibroids under different hormonal conditions. Mol. Hum. Reprod. **6,** 9915–9920.

Gilmour, R. S., J. C. Saunders, M. C. Dickson, J. M. Pell, A. L. F. Owden, and M. Silver, 1992: Molecular control of IGF-1 gene expression. In: Buttery, P. J., K. N. Boorman, and D. B. Lindsay (eds), The Control of Fat and Lean Deposition. Butterworth-Heinemann, Oxford.

Gronemeyer, H., 1992: Control of transcription activation by steroid hormone receptors. FASEB J. **6,** 2524–2529.

Hageleit, M., A. Daxenberger, W. D. Kraetzl, A. Kettler, and H. H. D. Meyer, 2000: Dose-dependent effects of melengestrol acetate (MGA) on plasma levels of estradiol, progesterone and luteinizing hormone in cycling heifers and influences on estrogen residues in edible tissues. APMIS **108,** 847–854.

Henricks, D. M., R. T. Brandt, E. C. Titgemeyer, and C. T. Milton, 1997: Serum concentrations of trenbolon-17β and estradiol-17β and performance of heifers treated with trenbolone acetate, melengestrol acetate, or estradiol-17β. J. Anim. Sci. **75,** 2627–2633.

Imwalle, D. B., D. J. Patteron, and K. K. Schillo, 1998: Effects of melengestrol acetate on onset of puberty, follicular growth, and patterns of luteinizing hormone secretion in beef heifers. Biol. Reprod. **58,** 1432–1436.

Isaksson, E., L. Sahlin, G. Soderqvist, E. von Schoultz, B. Masironi, M. Wickman, N. Wilking, B. von Schoultz, and L. Skoog, 1999: Expression of sex steroid receptors and IGF-1 mRNA in breast tissue – effects of hormonal treatment. J. Steroid Biochem. Mol. Biol. **70,** 257–262.

Jungblut, P. W., J. Gaues, A. Hughes, E. Kallweit, W. Sierralta, P. Szendro, and R. K. Wagner, 1976: Activation of transcription-regulating proteins by steroids. J. Steroid Biochem. **7,** 1109–1116.

Kastelic, J. P., D. H. McCartney, W. O. Olsen, A. D. Barth, and R. J. Mapletoft, 1997: Melengestrol acetate and estradiol-17β for synchronizing estrus and improving performance in beef cattle. Can. J. Anim. Sci. **77,** 683–688.

King, R. J., 1992: Effects of steroid hormones and related compounds on gene transcription. Clin. Endocrinol. **36,** 1–14.

Kojima, N., J. R. Chenault, M. E. Wehrman, E. G. Bergfeld, A. S. Cupp, and L. A. Werth, 1995: Melengestrol acetate at greater doses than typically used for estrus synchrony in bovine females does not mimic endogenous progesterone in regulation of secretion of luteinizing hormone and 17β-estradiol. Biol. Reprod. **52,** 455–463.

Kojima, N., T. T. Stumpf, A. S. Cupp, L. A. Werth, M. S. Roberson, and M. W. Wolfe, 1992: Exogenous progesterone and progestins as used in estrus synchrony regimens do not mimic the corpus luteum in regulation of luteinizing hormone and 17β-estradiol in circulation of cows. Biol. Reprod. **47,** 1009–1017.

Lauderdale, J. W., 1983: Use of MGA (melengestrol acetate) in animal production. In: Meissonnier, E., and J. Mitchchell-Vigneron (eds), Anabolics in Animal Production, pp. 193–212. OIE, Paris.

Malucelli, A., H. Sauerwein, M. W. Pfaffl, and H. H. D. Meyer, 1996: Quantification of androgen receptor mRNA in tissues by competitive co-amplification of a template in reverse transcription polymerase chain reaction. J. Steroid Biochem. Mol. Biol. **58,** 563–568.

Patterson, D. J., G. H. Kiracofe, J. S. Stevenson, and L. R. Corah, 1989: Control of the bovine estrous cycle with melengestrol acetate (MGA): a review. J. Anim. Sci. **67,** 1895–1906.

Pfaffl, M. W., 2001: Development and validation of an externally standardised quantitative insulin like growth factor-1 (IGF-1) RT-PCR using LightCycler SYBR® Green I technology. In: Meuer, S., C. Wittwer, and K. Nakagawara (eds), Rapid Cycle Real-time PCR, Methods and Applications, pp. 281–291. Springer, Heidelberg.

Pfaffl, M. W., and M. Hageleit, 2001: Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol. Lett. **23,** 275–282.

Pfaffl, M. W., I. G. Lange, A. Daxenberger, and H. H. D. Meyer, 2001: Tissue specific expression pattern of estrogen receptors (ER): quantification of ERα and ERβ mRNA with real-time RT-PCR. APMIS **109,** 345–355.

Pfaffl, M., H. H. D. Meyer, and H. Sauerwein, 1998: Quantification of the insulin like growth factor-1 (IGF-1) mRNA: development and validation of an internally standardised competitive reverse transcription-polymerase chain reaction. Exp. Clin. Endocrinol. Diabetes **106,** 502–512.

Plath-Gabler, A., C. Gabler, F. Sinowatz, B. Berisha, and D. Schams, 2001: The expression of the IGF family and GH receptor in the bovine mammary gland. J. Endocrinol. **168,** 39–48.

Plouzek, C. A., and A. Trenkle, 1991: Insulin-like growth factor-I concentrations in plasma of intact and castrated male and female cattle at four ages. Domest. Anim. Endocrinol. **8,** 73–79.

Rasmussen, R., 2001: Quantification on the LightCycler. In: Meuer, S., C. Wittwer, and K. Nakagawara (eds), Rapid Cycle Real-time PCR, Methods and Applications, pp. 21–34. Springer, Heidelberg.

Rutanen, E. M., 2000: Insulin-like growth factors and insulin-like growth factor binding proteins in the endometrium. Effect of intrauterine levonorgestrel delivery. Hum. Reprod. **15,** 173–181.

Sauerwein, H., and H. H. D. Meyer, 1989: Androgen and estrogen receptors in bovine skeletal muscle – relation to steroid induced allometric muscle growth. J. Anim. Sci. **67,** 206–212.

Sauerwein, H., H. H. D. Meyer, and D. Schams, 1992: Divergent effects of estrogens on the somatotropic axis in male and female calves. J. Reprod. Dev. **38,** 271–278.

Scheidegger, K. J., B. Cenni, D. Picard, and P. Delafontaine, 2000: Estradiol decreases IGF-1 and IGF-1 receptor expression in rat aortic smooth muscle cells. Mechanisms for its atheroprotective effects. J. Biol. Chem. **275,** 38921–38928.

Schütz, G., 1988: Tenth Adolf Butenandt lecture. Control of gene expression by steroid hormones. Biol. Chem. **369,** 77–86.

Thissen, J. P., J. M. Ketelslegers, and L. E. Underwood, 1994: Nutritional regulation of the insulin-like growth factors. Endocrinol. Rev. **15,** 80–101.

Wang, H. S., T. H. Wang, and Y. K. Soong, 2000: Elevation of insulin-like growth factor-binding protein-1 mRNA expression following hormone replacement therapy. Hum. Reprod. **15,** 50–54.

Zimbelman, R. G., and L. W. Smith, 1966a: Control of ovulation in cattle with melengestrol acetate. I. Effect of dosage and route of administration. J. Reprod. Fert. **11,** 185–191.

Zimbelman, R. G., and L. W. Smith, 1966b: Control of ovulation in cattle with melengestrol acetate. II. Effect of follicular size and activity. J. Reprod. Fert. **11,** 193–201.