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The mRNA Expression of Insulin Receptor Isoforms (IR-A and IR-B) and IGFR-2 in the Bovine Corpus Luteum During the Estrous Cycle, Pregnancy, and Induced Luteolysis

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Isoform A of the human insulin receptor and the IGF-receptor type 2 (IGFR-2) are both receptors for insulin-like growth factor II (IGF II), which plays a major role in luteal development and function in bovine species. The objective of this study was to determine if both insulin receptor isoforms and IGFR-2 were expressed in the bovine corpus luteum (CL) and if they were regulated during the estrous cycle, pregnancy, and induced luteolysis. CL were collected at the slaughterhouse. For induced luteolysis, CL were obtained by transvaginal ovariectomy at 2, 4, 12, 48, and 64 h after prostaglandin (PG) F2α-injection. Real-time RT-PCR was applied to investigate mRNA expression. Two alternatively spliced transcripts encoding the insulin receptor were detected in bovine CL. These two isoforms corresponded to the known isoforms A (IR-A) and B (IR-B) in humans. IR-A mRNA predominated in bovine CL and was significantly down-regulated on d 5–7. IR-B mRNA was significantly up-regulated in the late luteal stage and during early pregnancy. IR-A showed a significant down-regulation at 48 h after PGF2α. IGFR-2 mRNA was significantly up-regulated in mid and late luteal stages (d 8–18). It is proposed that the differential mRNA expression of IR-A and IGFR-2, both binding IGF II, may play a role in the development and function of the bovine corpus luteum.

Key Words: Bovine; corpus luteum; insulin receptor; IR-A; IGFR-2.

Introduction

Seino and Bell *(1)* identified two different isoforms of the insulin receptor in different types of human cells. The same isoforms have been demonstrated in rats *(2)*, rhesus monkeys *(3)*, and sheep *(4)*. Alternative splicing leads to the generation of a shorter and a longer isoform. They differ in

their sequence at the C-terminal end of the insulin-binding α-subunit. In the sequence of isoform A (IR-A), exon 11, consisting of 36 base pairs (bp), is missing, whereas isoform B (IR-B) is positive for exon 11. The relative expression of the two isoforms in humans varies markedly in a tissue-specific manner. Liver expresses almost only IR-B. Muscle, isolated adipocytes, and cultured fibroblasts express preferentially IR-B, but also IR-A. Placenta expresses both types equally, and buffy coat leukocytes and Epstein–Barr virus-transformed lymphocytes express only IR-A *(5)*. Benecke et al. *(6)* found a similar distribution of the protein isoforms in the human: predominance of IR-B in the liver, predominance of IR-A in leukocytes, and similar amounts of both variants in placenta, skeletal muscle, and adipose tissue. Steady-state binding of insulin has a significantly higher affinity to IR-A than to IR-B *(7,8)*. The ability of insulin-like growth factor I (IGF-I) to compete for insulin binding is significantly higher for IR-A than for IR-B *(9)*. IR-A but not IR-B binds insulin-like growth factor II (IGF-II) with a similar affinity to insulin. Activation of IR-A by insulin leads mainly to metabolic effects, whereas activation by IGF II primarily results in mitogenic effects *(10)*. Until now, there is no information about different isoforms of the insulin receptor in cows.

IGF II is a potent mitogen, which is involved in growth, cell proliferation, and differentiation. It plays an important role in embryonic growth and prenatal development *(11)*. Mitogenic effects of IGF II are mediated through IGF-receptor type 1 (IGFR-1) and IR-A. Mice embryos lacking solely the insulin receptor were only mildly affected, whereas the lack of IGFR-1 led to severe growth-deficiency *(12)*. Concerning embryonic development, the signaling of IGF II through IGFR-1 is more important than signaling through the insulin receptor. There is some evidence that IGF II signaling through the insulin receptor is involved in tumor growth *(13)*. In ovarian and breast cancer, especially the isoform A is mediating this mitogenic effect of IGF II *(10,14,15)*; because extracellular IGF II is internalized and degraded by the IGF-receptor type 2 (IGFR-2), this receptor is able to control the level of circulating IGF II *(16,17)*.

In the bovine corpus luteum (CL), IGF II mRNA expression is significantly up-regulated in the period of early angio-
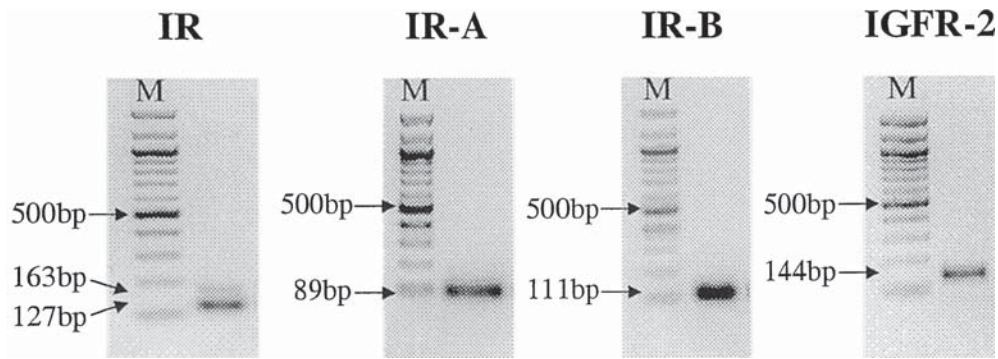
Fig. 1. 1.8% agarose gel with the representative PCR products of IR, IR-A, IR-B, and IGFR-2 of pooled samples in the bovine corpus luteum; the 127 bp product of the IR is notedly stronger expressed than the 163 bp product; bp = base pairs; M = 100 bp marker.

genesis (d 1–4), when compared with d 8–12 of the estrous cycle and pregnancy *(18,19)*. In an in vitro Micro-Dialysis-System (MDS) Sauerwein et al. *(20)* showed that IGF II can stimulate progesterone release in bovine CL, particularly in the late luteal phase. The specific binding of IGF II to the insulin receptor is markedly higher (1.9–4.9-fold) than for IGF I or insulin, and the receptor specificity does not change during luteal development *(20)*. As staining for IGF II peptide is restricted to the perivascular fibroblasts and pericytes of blood vessels and capillaries, Amselgruber et al. *(21)* presumed a role for IGF II in coordinating angiogenic processes during the development of the corpus luteum. IGF I and IGFR-1 mRNA expression does not change significantly during the estrous cycle and pregnancy, except in the early angiogenesis phase, where both are markedly up-regulated *(18, 19)*. The mRNA expression for IGFR-2 has been identified in bovine tissues, such as liver and ileum *(22)*, but not yet in the corpora lutea of this species.

Considering that IGF II is more strongly expressed than IGF I in the bovine CL *(19)*, an important role for IGF II and its receptors in the estrous cycle and pregnancy can be assumed. The objective of this study was to determine whether both IR isoforms (IR-A, IR-B) and IGFR-2 were expressed in the bovine corpus luteum (CL), and whether there were regulatory changes during the estrous cycle, pregnancy and induced luteolysis.

Results

Identification of the Insulin Receptor
Isoform A (IR-A) in Bovine Corpus Luteum Tissue

Using 1.8% high-resolution agarose gel electrophoresis (Fig. 1), there are two PCR products amplified by specific insulin receptor primers (IR). The longer PCR product (IR-B) corresponds to the known bovine sequence (Acc. No. AJ320235) consisting of 163 bp. The shorter PCR product (IR-A) contains 127 bp, which after sequencing was also shown to be 100% homologous to the known bovine sequence, but with a gap of exactly 36 bp in the middle of the sequence

(Fig. 2). The LightCycler PCR product of the IR primer pair showed a specific melting temperature of 85.71ºC, which is 1.29ºC lower than the known specific melting temperature for IR in bovine liver and ileum [22]. As can be seen on the agarose gel, the smaller isoform (127 bp) is markedly more strongly expressed in the bovine corpus luteum than the 163 bp isoform. Moreover, the earliest crossing point (CP) for IR-A is at 22.78 amplification cycles, whereas the earliest CP for IR-B is at 25.25 cycles. This means an approximately fourfold higher expression level of IR-A than of IR-B. The new bovine IR-A sequence has been deposited in the EMBL database (Acc. No. AJ488553).

Expression Data During Estrous Cycle and Pregnancy

The investigated transcripts showed high real-time PCR efficiencies between 1.72 (ubiquitin), 1.77 (IR), 1.84 (IGFR-2), 1.85 (IR-B), and 1.96 (IR-A). The mean coefficients of variation (CV%) on CP base were very low with values between 0.67% and 0.88% (ubiquitin: 0.68%, IR: 0.78%, IR-A: 0.88%, IR-B: 0.68%, IGFR-2: 0.67%). Expression data during the estrous cycle and pregnancy are shown as differences of the CP in comparison to expression data on d 1–2, which is used as a control (Fig. 3).

The CP for IR-A range from 22.78 to 24.90. IR-A mRNA expression is significantly down-regulated by a factor of 1.5 at d 5–7. During the late stage of the estrous cycle (d 13–18 and >18), there is a tendency for mRNA up-regulation. The same can be found during pregnancy with a tendency for up-regulation, reaching a maximum in late pregnancy by a factor of 1.4. The CP for IR-B ranges from 25.44 to 27.52. IR-B mRNA expression shows similar characteristics to IR-A mRNA. Significant up-regulation occurs in the late luteal phase (d 13–18) and is maximally 1.6-fold in early pregnancy. The CP for IGFR-2 ranges from 33.03 to 36.19. IGFR-2 mRNA expression is significantly and maximally (factor 2.1) up-regulated on d 8–12 in the mature CL. This up-regulation decreases on d 13–18, but is still significant. During pregnancy there is a clear tendency for IGFR-2 mRNA down-regulation by a factor of 1.3–1.4.

```
IR-A    1 tcctcaaggagctggaggagtcctcgttcaggaaaacgtttgaagattac 50
          ||||||||||||||||||||||||||||||||||||||||||||||||||
IR-B    1 tcctcaaggagctggaggagtcctcgttcaggaaaacgtttgaagattac 50


IR-A   51 ctgcacaacgtggttttcatccccc.......................... 74
          |||||||||||||||||||||||||
IR-B   51 ctgcacaacgtggttttcatccccagaaaatcatcttcaggccctgttgc 100


IR-A   75 ..........aggccttcgaggaaacgcagagcccttggtgacgtgggga 114
                    |||||||||||||||||||||||||||||||||||||||
IR-B  101 cgaggacgctaggccttcgaggaaacgcagagcccttggtgacgtgggga 150


IR-A  115 atgtgacagcagc 127     (Acc.No. AJ488553)
          |||||||||||||
IR-B  151 atgtgacagcagc 163     (Acc.No. AJ320235)
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Fig. 2. Sequence comparison from the newly sequenced isoform A (IR-A) of the bovine insulin receptor (127 bp) and the known bovine sequence (163 bp), isoform B (IR-B); the visible gap consists of exactly 36 bp, the rest of the sequence shows a 100% homology. The sequences can be found in the EMBL under the indicated accession numbers (Acc.No.).

Expression Data During Luteolysis

The mean coefficients of variation (CV%) were very low, with values between 0.30% and 1.57% (ubiquitin: 1.57%, IR: 0.56%, IR-A: 0.59%, IR-B: 0.51%, IGFR-2: 0.30%). Expression data during induced luteolysis are shown in comparison to expression data of the control group before PGF2α-administration (Fig. 4).

The CP for IR-A ranges from 23.47 to 25.99. IR-A mRNA expression decreases during luteolysis with a maximal and significant 2.1-fold down-regulation 48 h after PGF2α-induced luteolysis. At 64 h after PGF2α, there is still a tendency for down-regulation. The CP for IR-B, ranges from 25.25 to 27.75. IR-B mRNA expression shows more or less the same course with a maximal down-regulation by a factor of 1.6 at 48 h, which is not significant. The CP for IGFR-2 ranges from 31.79 to 34.40. For IGFR-2 mRNA expression, there is a clear tendency ($p < 0.1$) for down-regulation, maximal at 12 h, by a factor of 1.8. From 12 h to 64 h, there is a slight increase in the mRNA expression of IGFR-2, but it does not reach the level of the control group.

Discussion

The mRNA expression of the insulin receptors and IGFR-2 was investigated in the bovine CL during the estrous cycle, pregnancy and PGF2α-induced luteolysis. With a multiple species primer pair for the insulin receptor, annealing in exon 10 and 12 of the human sequence, two discrete PCR products were detected, differing by exactly 36 bp. Alternative splicing of the human insulin receptor results in the occurrence of an exon 11 "negative" and an exon 11 "positive" isoform varying in 36 bp *(1)*. The same was shown for the sheep *(4)*. The new sequence for IR-A shows a 99.2% homology to the ovine IR-A sequence (Acc. No. Y16093). We conclude, that alternative splicing of exon 11 is also the reason for the two different isoforms of the insulin receptor in the cow, and that the smaller and the longer isoform correspond to isoform A and B, respectively, in the human or sheep.

IR-A is mainly expressed in fetal tissue and several cancers (e.g., ovarian and breast cancer), and activation by IGF II primarily leads to mitogenic effects *(10)*. There is growing evidence that IGF II is an important regulator in follicular growth *(23)* and luteal development *(24)*. It is strongly expressed in the bovine corpus luteum *(19,24)* (IGF II transcripts are expressed at 44-fold higher concentrations compared to IGF I mRNA [T.P. Neuvians et al., manuscript accepted]). The rapid growth and angiogenesis in the developing CL is comparable to processes during intensive tumor growth. The mRNA expression of IR-A is about fourfold greater than IR-B mRNA in the bovine CL. Although many publications mention an augmented IR-A expression only in the context of malignant tumor growth *(10,14,15)*, we can presume a physiological role for this receptor in normal luteal growth and function also. Signaling of IGF II through IR-A in the bovine CL may partly account for its enormous tumor-like growth.
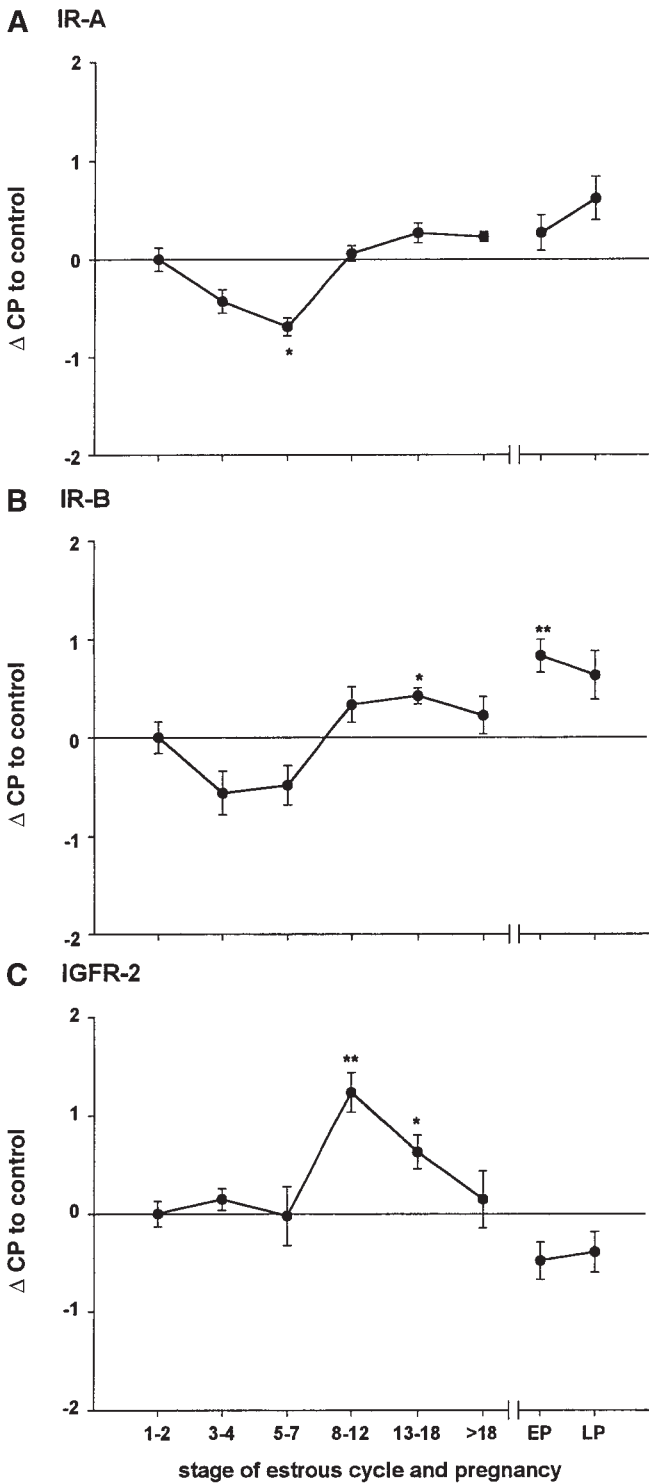
Fig. 3. Expression data (mRNA) for (**A**) IR-A , (**B**) IR-B, and (**C**) IGFR-2 during estrous cycle and pregnancy (EP = early pregnancy, <4 mo; LP = late pregnancy, >4 mo) in bovine corpus luteum; data are shown as mean of crossing point difference (ΔCP) ± SEM between the control group (Co = d 1–2) and the following stages of estrous cycle (n = 4/stage). The CP indicates the PCR-cycle number of maximal acceleration of fluorescence increase; a positive ΔCP means an earlier fluorescence increase and therefore a higher mRNA concentration. Significances are indicated in relation to the control group; * = $p < 0.05$, ** = $p < 0.01$.
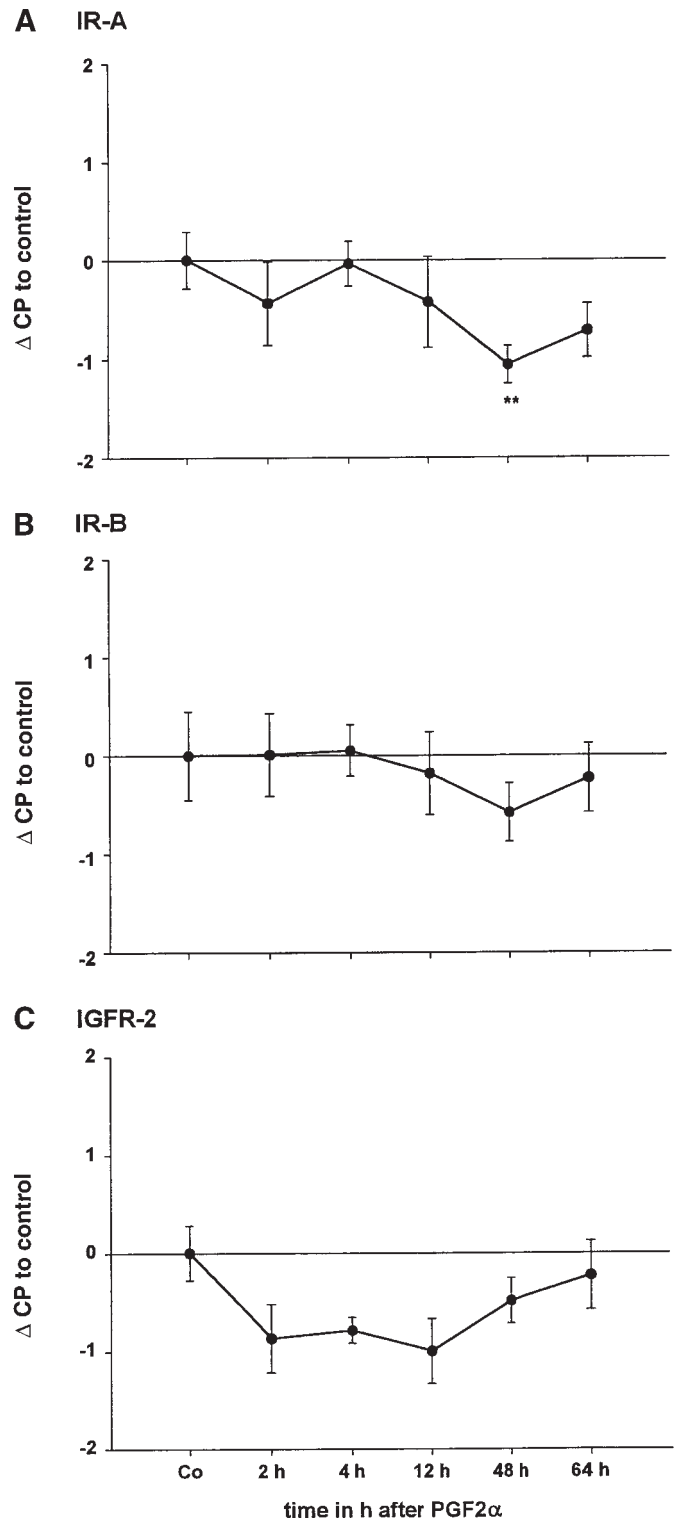
Fig. 4. Expression data (mRNA) for (**A**) IR-A , (**B**) IR-B, and (**C**) IGFR-2 in bovine corpus luteum before (control group, Co) and after PGF2α-induced luteolysis on d 8–12; data are shown as mean of crossing point difference (ΔCP) ± SEM between the control group and the following times in hours after PGF2α-administration (n = 4–5/stage). The CP indicates the PCR-cycle number of maximal acceleration of fluorescence increase; a positive ΔCP means an earlier fluorescence increase and therefore a higher mRNA concentration. Significances are indicated in relation to the control group; ** = $p < 0.01$.

IR-A mRNA expression is significantly down-regulated at the end of angiogenesis (d 5–7). This decrease of IR-A transcripts might be important for preventing overstimulation of luteal growth. Whereas IGFR-1 mRNA expression is still up-regulated on d 3–4 (19), IR-A mRNA already decreases at this time of the estrous cycle. During pregnancy, IGFR-1 mRNA is significantly down-regulated, when compared to d 1–4 (19), but IR-A mRNA is rather up-regulated. This suggests a supporting task for the IR-A ligands (IGF II and insulin) not only for luteal growth, but also for luteal maintenance and pregnancy. The tendency for IR-A up-regulation during the late luteal phase could be a reason for the particular ability of IGF II to stimulate luteal progesterone secretion in this cycle stage, as observed by Sauerwein et al. (20). The mRNA expression of IR-B during the estrous cycle and pregnancy shows a similar course as that for IR-A, but the down-regulation at the end of angiogenesis (d 5–7) is not significant. IR-B mRNA is significantly up-regulated in the mature CL (d 13–18) and in early pregnancy. The anabolic support of insulin for growth and progesterone production of the CL seems to be most important during these cycle stages. The differential expression of the two receptor isoforms IR-A and IR-B during different stages of the estrous cycle and pregnancy may be responsible for the fine-tuning of metabolic and mitogenic effects of their ligands IGF II and insulin on the CL.

During luteolysis IR-A and IR-B mRNA expression data show more or less the same tendency. Both are down-regulated, maximally at 48 h after induced luteolysis by a factor of 2.1 (IR-A) and 1.6 (IR-B), respectively. Insulin and IGF II, the ligands of IR-A and IR-B, support the growth and function of the CL. With the onset of luteolysis, this support is no longer required. Their receptors IR-A and IR-B may be down-regulated not to interfere with the process of regression and degradation of the CL. On the other hand, the mRNA expression of IR-A and IR-B is slightly up-regulated at 64 h after PGF2α, when compared to the preceding stages. As the tissue composition of the CL is changing very rapidly during luteolysis, IR-A and IR-B may be involved in the remodeling of the tissue. It is also possible that the receptor mRNA is produced by different cells during different stages of luteolysis. The localization of the receptors and their mRNA production is thus an interesting aspect for further research.

IGF II binds to IGFR-1, competing with IGF I, and to IR-A, competing with insulin. Differential expression of the receptor mRNAs may facilitate a fine regulation of the similar acting ligands IGF I, IGF II, and insulin, and their effects on the CL.

IGF II mRNA is strongly expressed in the bovine CL during early angiogenesis (d 1–4) and decreases in the following stages and pregnancy (19). In contrast Woad et al. (24), beginning sampling on d 5, found no significant changes in IGF II mRNA expression during the bovine estrous cycle. In the mature CL (d 8–12) and during the late luteal phase (d 13–18), IGFR-2 mRNA is significantly up-regulated. At d 8–12 of the estrous cycle, IGF II mRNA is down-regulated when compared with d 1–4 (19). During luteolysis IGFR-2 mRNA decreases, but slightly increases during structural luteolysis (48 h and 64 h after PGF2α) without reaching the level of the control group. IGF II mRNA expression continuously decreases during luteolysis, which becomes significant at structural luteolysis [T.P. Neuvians et al., manuscript accepted]. In contrast to IGFR-1, IGFR-2 binds only IGF II, not IGF I. IGFR-2 mRNA expression mostly follows the expression of IGF II and seems to keep IGF II on a more or less constant level without a regulative purpose. To be able to clarify the role of IGFR-2 at different luteal stages and its involvement in the control of protein concentrations of IGF II by internalization and degradation, further investigations would be of particular interest. During all stages of the estrous cycle, there is only a very low expression of IGFR-2 mRNA (earliest CP = 31.79), cautioning against over interpretation of such results. In contrast, IR-A and IR-B show high expression levels (earliest CP = 22.78 and 25.25, respectively) compared with IGFR-2. This means approximately a 2^6 to 2^9-fold expression difference. The biological relevance of the regulation of such low and high expression transcripts may be different.

In conclusion, the isoform A of the insulin receptor was newly identified for the bovine. The predominant expression of IR-A in the bovine CL proposes a physiological role in mediating mitogenic effects of IGF II on luteal growth. The differential expression of IR-A and IR-B may regulate the effects of insulin and IGF II on development, maintenance, and function of the CL. Furthermore, it was shown that the bovine CL expresses IGFR-2 mRNA with significant changes during the estrous cycle.

Materials and Methods

Collection of Bovine Corpora Lutea (CL) During Estrous Cycle and Pregnancy

The CL of the cows (mainly German Fleckvieh) were collected at the local slaughterhouse within 10–20 min of slaughter. The stage of the estrous cycle was identified by judging macroscopically ovaries and uterus in consideration of size, color, consistency, connective tissue, and mucus, as previously described (25). The CL were assigned to the following stages: d 1–2, d 3–4, d 5–7, d 8–12, d 13–18, and >18 d, when regression occurs (n = 4/group). Pregnancy was divided into early (<4 months) and late pregnancy (>4 mo) according to the measured crown-rump length of the fetus (n = 4/group). Luteal tissue was frozen in liquid nitrogen immediately after collection and stored at –80ºC until RNA extraction.

Collection of Bovine Corpora Lutea (CL) During Luteolysis

Cows (Holstein Fresians) at the mid-luteal phase (d 8–12) were injected intramuscularly with 500 µg of the PGF2α-

analog cloprostenol (Estrumate®, Intervet, Germany). The CL were collected by transvaginal ovariectomy before (control, d 8–12) and 2, 4, 12, 48, and 64 h ($n = 4$–5/group) after PGF2α injection. All CL were sectioned, immediately frozen in liquid nitrogen, and stored at –80ºC until RNA extraction. All animal experiments were approved by the local ethical review committee.

Total RNA Extraction and Reverse Transcription

Small slices of deep frozen CL were cut and weighed. Total RNA from CL was extracted with peqGOLD TriFast (PeqLab, Erlangen, Germany) according to the manufacturer's instructions. RNA was dissolved in water and spectroscopically quantified at 260 nm. The integrity of RNA was verified by optical density (OD) absorption ratio $OD_{260\,nm}/OD_{280\,nm}$ between 1.8 and 2.0, and by electrophoresis with ethidium bromide staining on a 1% denaturating agarose gel. Constant amounts of 1000 ng of total RNA were reverse-transcribed to cDNA with 200 units of M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions.

Real-Time PCR Quantification

Primers were designed using the EMBL database (ubiquitin: Acc. No. Z18245, IR-A: Acc. No. AJ488553, IR-B: Acc. No. AJ320235) or used according to literature *(22)*: ubiquitin forward 5'-AGATCCAGGATAAGGAAGGCAT-3'; reverse 5'- GCTCCACCTCCAGGGTGAT-3' (198 bp); insulin receptor (IR) forward 5'- TCCTCAAGGAGCTGGAGGAGT-3', reverse 5'-GCTGCTGTCACATTCCCCA-3' (163 bp); IR-A forward 5'-TCCTCAAGGAGCTGGAGGAGT-3', reverse 5'-TTTCCTCGAAGGCCTGGGGAT-3' (89 bp); IR-B forward 5'-TCCTCAAGGAGCTGGAGGAGT-3', reverse 5'-TAGCGTCCTCGGCAACAGG-3' (111 bp); IGFR-2 forward 5'-TACAACTTCCGGTGGTACACCA-3', reverse 5'-CATGGCATACCAGTTTCCTCCA-3' (144 bp). The primer pair for the insulin receptor (IR), amplifying both isoform A and isoform B, is a multiple species primer that spans exon 10 to exon 12 of the human sequence *(22)*. A master-mix of the following reaction components was prepared as follows: 6.4 µL water, 1.2 µL MgCl₂ (25 m*M*), 0.2 µL forward primer (20 µ*M*), 0.2 µL reverse primer (20 µ*M*), and 1.0 µL LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Nine microliters of master-mix were filled in glass capillaries and 1 µL PCR template containing 25 ng reverse-transcribed total RNA was added. To ensure an accurate quantification of the desired product, a high-temperature fluorescence measurement in a fourth segment of the PCR run was performed *(26)*. The elevated temperature for fluorescence acquisition results in melting of unspecific products, e.g., primer dimers, and eliminating nonspecific fluorescence signals. The following general real-time PCR protocol was employed: denaturation for 10 min at 95ºC, 35 (IR and IR-A), 40 (ubiquitin and IR-B), or 50

(IGF-R2) cycles of a four-segment amplification and quantification program, a melting step by slow heating from 60 to 99ºC with a rate of 0.1ºC/s and continuous fluorescence measurement, and a final cooling down to 40ºC. The four-segment amplification and quantification program was carried out as follows: 15 s denaturation at 95ºC, 10 s annealing at 60ºC (ubiquitin and IR-B), 62ºC (IR-A), 64ºC (IGFR-2), and 66ºC (IR), respectively, 20 s elongation at 72ºC, and 5 s fluorescence acquisition at 80ºC (IR-A), 81ºC (IR-B), 83ºC (IR), 86ºC (ubiquitin), and 87ºC (IGFR-2), respectively. Crossing point (CP) values were acquired by using the second derivative maximum method of the LightCycler Software 3.3 (Roche Diagnostics). The CP is the number of PCR cycles when maximal acceleration of the fluorescence increase is reached. The earlier the fluorescence increases, the higher is the concentration of the measured mRNA *(27)*. All CP of the 32 samples ($n = 4$–5/group) per investigated factor were detected in one run to eliminate interassay variance. Real-time PCR efficiencies were acquired by amplification of a standardized dilution series and the given slopes in the LightCycler Software 3.3 (Roche Diagnostics). The corresponding efficiencies (E) were then calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ *(27)*. The specificity of the desired products in bovine CL was documented with a high-resolution gel electrophoresis (Fig. 1) and analysis of the melting temperature, which is product-specific *(22)*. Specific melting temperatures are as follows: 88.48ºC (ubiquitin), 85.71ºC (IR), 83.26ºC (IR-A), 84.23ºC (IR-B), and 89.45ºC (IGFR-2). PCR products of the primer pairs IR, IR-A, and IR-B were purified with the Nucleo Spin Extraction Kit (Macherey-Nagel, Düren, Germany) and sent for commercial sequencing (Medigenomix GmbH, Munich, Germany). The results were compared to the known sequences in the EMBL database.

Statistical Analysis

The statistical significance of differences in mRNA expression of the examined factors was analyzed by the Relative Expression Software Tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR *(28)*. This software calculates an expression ratio in regard to the control group (here: d 1–2 of estrous cycle for the cycle stage, and d 8–12 of estrous cycle, before PGF2α injection, for luteolysis) and is normalized by a reference gene (ubiquitin). The mRNA expression data for ubiquitin showed no significant changes to the control group during any of the investigated stages of the estrous cycle or time points of induced luteolysis. Thus, it was determined to be suitable as a reference gene. REST also indicates coefficients of variation (CV) in % (mentioned under *Results*) and standard deviations based on the CPs of the target gene. The data are shown as the mean difference (Δ) ± SEM of CPs between the control group and the following stages of the estrous cycle, and the following times after PGF2α-induced luteolysis, respectively.

A positive ΔCP means an earlier increase of fluorescence and therefore a higher concentration of the target gene. As the PCR amplification is a process with exponential character, a difference of two CPs signifies approximately a regulation by factor $E^{\Delta CP}$ (with E = efficiency) and is indicated in the text according to the expression ratio calculated by REST.

Acknowledgments

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