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PERIPHERAL CIRCULATING INSULIN-LIKE GROWTH FACTOR-I AND -II IN CATTLE

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Interrelationships between circulating concentrations of the insulin-like growth factors (IGF-I and IGF-II) were investigated in 235 blood samples taken from 145 healthy beef or dairy calves, bulls and cows of different breeds and ages. Autoradiography of Western ligand blots indicated different IGF binding protein (IGFBP) profiles between sera from different categories of cattle. Each IGF radioimmunoassay was validated by determining the effects of IGFBPs, ligand and contraligand, as well as serial dilution and comparison with results obtained after molecular sieve chromatography in acid. In female cattle mean values for IGF-I varied from 5.1 nmol/l in postparturient Holstein cows to 18.5–20.5 nmol/l in growing beef heifers, while mean IGF-II concentrations ranged from 30.0 nmol/l in the cows to 14.7–15.7 nmol/l in the beef heifer calves. In male cattle mean serum IGF-I ranged widely from 8.2 nmol/l in 1-day-old Holstein calves to 67.4 nmol/l in 16-month-old Simmental-type bulls. Mean IGF-II concentrations decreased from 22.9 nmol/l in 1-day-old Holstein bull calves to 11.9 nmol/l in 12-month-old beef bulls. Thus, total molar IGF concentrations were fairly stable in female cattle (24.7–35.1 nmol/l) but extended from 27.3 nmol/l to 81.8 nmol/l in the male cattle. The tendency for a reciprocal relationship between serum concentrations of these growth factors was most obvious in the periparturient cows.

Key words: IGF-I, IGF-II, IGFBPs, radioimmunoassay, cattle

Insulin-like growth factor (IGF)-I (somatomedin-C) and its close relation IGF-II are single-chain polypeptides similar in structure to proinsulin. Besides local paracrine and autocrine actions on cells in various tissues, these mitogenic growth factors are present in the circulation from where they can regulate growth and metabolism of the whole organism (Stewart and Rotwein, 1996; Hossner et al., 1997; Hill et al., 1999). The anabolic effect of these peptides is mediated primarily via the IGF-I cell membrane receptor.

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Circulating IGFs are almost entirely bound to one of the specific binding proteins (IGFBPs) which attenuate or enhance IGF activity by multiple routes. This array probably reflects the endocrine action of the liver on other tissues, such as muscle and ovary (Gutiérrez et al., 1997). Hepatic synthesis of IGF-I and IGFBPs 1–4 can be stimulated or inhibited by other hormones including somatotropin, insulin, cortisol and the thyroid hormones, whereas synthesis of IGF-II appears to be constitutional. Due to the marked positive effect of somatotropin on IGF-I synthesis and secretion, this axis has been much studied in order to increase the growth rate of beef cattle and the milk yield of dairy cows (e.g. Elsasser et al., 1989; McShane et al., 1989; Groenewegen et al., 1990). With a few exceptions (Skaar et al., 1991; Vega et al., 1991; Vicini et al., 1991; McGuire et al., 1992) IGF-II has been ignored, although IGF-II appears to be dominant during the fetal period and synthesis continues throughout life.

Quantitative determination of these peptides must be carried out with care due to the high affinity constants of the IGFBPs for IGF which may interfere with the specific antibody-ligand reaction in the assay. It is necessary to validate test procedures for each category of sample because the relative amounts and properties of the IGFBPs may differ in different circumstances (Bang, 1995).

Considering that assessment of IGF activity in animals is incomplete unless both ligands are determined, this paper presents the results obtained for serum IGF-I and IGF-II as well as the sum and ratio of their molar concentrations in healthy beef and dairy cattle of different ages. The complement of IGFBPs present was also evaluated by ligand blotting and autoradiography.

Materials and methods

Quantitative analysis

a) Purification. Bovine IGF was partially purified by subjecting bull serum (5 ml) to molecular sieve chromatography on a calibrated column of Sephadex G-75 (2.8 × 32 cm) using 1 M acetic acid as the eluent. Fractions (3.1 ml) containing the IGFs or IGFBPs were lyophilised. As required they were redissolved at 1 mg/ml in assay buffer (0.05 M sodium phosphate with 0.005 M EDTA; pH 7.8).

b) Extraction. Aliquots of serum or plasma (0.15 ml) were mixed and allowed to stand with 0.85 ml solution containing ethanol (87.5%) and 2 M HCl (12.5%) for 30 min (Daughaday et al., 1982). After centrifugation for 30 min at 3000 × g the supernatant (0.5 ml) was neutralised with 0.855 M tris-(hydroxymethyl)aminomethane (TRIS) base and kept for at least 2 h at –18 °C (Breier et al., 1991). Following further centrifugation, aliquots of the supernatant were assayed. Blank extracts of 0.15 ml assay buffer were prepared in parallel.

c) IGF-I assay. Human IGF-I (ICN Biomedicals Inc., Aurora, USA) labelled with ^{125}I was used as the tracer and polyclonal rabbit antibodies to human IGF-I (Biogenesis, Poole, UK) as the reagent (Nikolić et al., 1996). Since bovine IGF-I has the same amino acid composition as human IGF-I (Simmen, 1991), recombinant human IGF-I was used as the working standard (0.063–6.25 ng/tube). Cross-reactivity with IGF-II at 50% inhibition of tracer binding was 0.054%. Reproducibility was checked by including the reference preparation (WHO 87/518) in each test. The mean intraassay coefficients of variation (CV) for duplicate samples were routinely from 3% to 6%. Interassay CVs were below 12%.

d) IGF-II assay. The procedure described for human serum (Nikolić et al., 1998*b*) was used, employing recombinant human IGF-II (ICN Biomedicals Inc., Aurora, USA) labelled with ^{125}I as the tracer and as the working standard (0.03–1.00 ng/tube). The mouse monoclonal anti-rat IGF-II (Biogenesis, Poole, UK) was declared to be 100% cross-reactive with h-IGF-II. Under our assay conditions, cross reactivity with IGF-I at 50% inhibition of tracer binding was 0.4%. Inhibition curves using h-IGF-II and purified bovine IGF fractions were compared for parallelism. Reproducibility was monitored with fresh extracts of human serum pools in each test (intraassay CVs 3–6%; interassay CVs < 13%).

e) Assay validation. The effect of the bovine IGFBP fractions on IGF-I and IGF-II determinations was examined. Possible interference from IGFBPs carried through the serum extraction procedure was checked by adding contral ligand, which was not expected to alter the result, or ligand, which should increase the result by 100% of the amount added. Both serum extracts and IGF column fractions were progressively diluted and the relation between obtained and expected values determined. Serum stability was checked by determining IGF-I again after several years storage.

Determination of IGFBP patterns

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of serum samples was performed as described (Hossenlopp et al., 1986; Nikolić et al., 1998*a*). Proteins were electrotransferred to nitrocellulose membranes (0.45 µm, Schleicher and Schuell), followed by autoradiography after incubation with ^{125}I-labelled IGF-I of specific activity approximately 36 MBq/nmol. Protein bands were putatively identified according to the mobility of reference standards: bovine serum albumin (BSA, 66 kD), ovalbumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), chymotrypsin (24 kD), β-lactoglobulin (18 kD) and ribonuclease (14 kD).

Animals and procedure

Group 1 consisted of 44 Holstein-Friesian calves of both sexes born within three weeks in May and reared on a large dairy farm. Blood samples were taken

by jugular puncture soon after birth before intake of colostrum and again at 4.5 months old when the calves were sent to the feedlot.

Group 2 contained calves, mainly of the Limousine and Charolais breeds (19 female, 14 male), kept with their dams and fed forage-based diets. A total of 55 samples was obtained from these beef cattle at 7 days, 6 months and 12 months of age over a period of 18 months in 1995-6. IGF-I was determined shortly after sampling and again in 1999, when IGF-II was measured.

Samples were also taken from 29 young bulls (6 Limousine and 23 Si mmental-type domestic pied) reared under the same conditions (Group 3).

The fourth group consisted of 24 healthy periparturient Holstein cows (parity 2–5) kept tethered in the maternity parlour on another large dairy farm. Blood samples were obtained within the last week of pregnancy and during the first week post partum from each cow 4 to 10 days apart.

All blood samples were allowed to clot spontaneously at room temper ature. The serum was decanted, centrifuged at 2500 × g and preserved at –18 °C until analysis.

Heparinised plasma from Holstein cows in early lactation (Group 5) was also assayed.

Statistical analyses

Single-factor, two-way and two-factor-split-plot analysis of variance (ANOVA) were employed to analyse the results for the groups of cattle where appropriate, including Bartlett's test for non-homogeneity of variance.

Results

IGFBP patterns

The ligand blots (Fig. 1) showed significant differences in serum IGFBP patterns between newborn and 12-month-old cattle. At 7 days and 6 months old two of the calves exhibited predominantly 34 kD IGFBP and to a lesser extent 40–45 kD and 24 kD IGFBPs. Sera of both the male and female 12-month-old animals contained predominantly 40–45 kD IGFBP but the 34 kD band markedly decreased in intensity only in the bull. Samples from the periparturient cow were characterised by very intensive 34 kD bands and faint 40–45 kD and 24 kD bands. A faint IGFBP band (possibly a doublet) with a molecular mass around 30 kD appeared in both prepartal and postpartal sera. The electrophoretic pos i-tions of the 40–45 kD, 34 kD, 30 kD and 24 kD bands, which bound the tracer IGF-I, would be compatible with the sizes of IGFBP-3, -2, -1 and -4 respectively (McGuire et al., 1992).

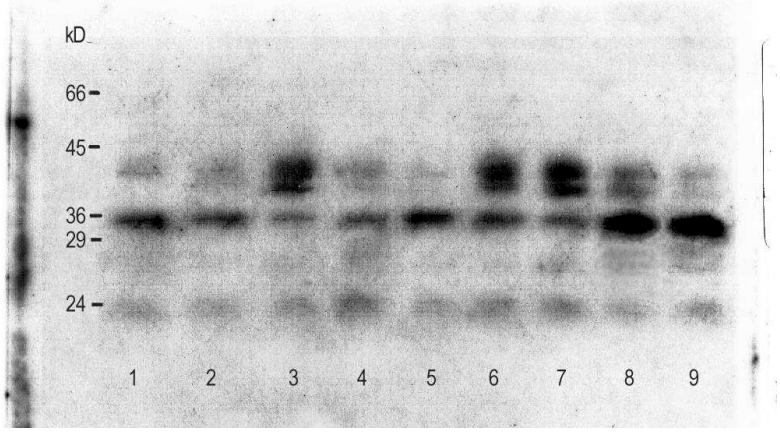

Fig. 1. Autoradiographic patterns of serum IGFBPs after SDS-PAGE and ligand blotting with ^{125}I-IGF-I. Samples in lanes 1–3 were from a male Limousine calf aged 7 days, 6 and 12 months, in lanes 4–5 from a female calf at 7 days and 6 months old, in lanes 6–7 from a female calf at 6 and 12 months old and in lanes 8–9 from a Holstein cow 5 days before and 1 day after calving, respectively

Assay validation

Molecular sieve chromatography of bull serum at a low pH separated the IGF ligands from the IGFBPs. A total of 90% of the IGF-I in the original serum sample was detected in the expected volume of eluate. Dilution curves of these fractions were parallel to reference IGF-I preparations. Additional IGF-I increased the result by 102% ± 4% (n = 7) of the expected amount but additional h-IGF-II had no effect in the IGF-I assay. However, in the IGF-II assay increasing concentrations of the bovine IGF fraction were not parallel to the standard curve. Since no pure preparation of bovine IGF-II was available for reference, one point (0.5 ng/tube) on the curve was taken as the human equivalent and the remaining points were adjusted to follow the dilution profile of IGFBP-free bovine IGF fractions. This standard curve (assay range 0.03–1.2 ng/tube) was used for all determinations of IGF-II in extracts of bovine serum.

When fractions containing the IGFBPs were assayed in the absence of cold ligand, they bound to the tracer, were not sedimented by the precipitation system and therefore appeared as artefact IGF. Values apparently increased by similar amounts in both tests as added IGFBP was increased, indicating that a rival competitive binding system may operate with reversed roles for the tracer and reagent antibodies. In the IGF-I assay the inclusion of h-IGF-II in the incubation mixture in physiological amounts (0.5 ng/tube) completely suppressed the spurious results. IGF-I alone suppressed equivalent amounts of IGFBP completely (101 ± 10% of the expected results) but only partially when IGFBP was in apparent excess. For example, when IGF-I (0.42 ng) was included with IGFBP, which had given a false value of 1 ng/tube, the new result was 0.7 ng or

167% of the expected amount of IGF-I, whereas with 0.5 ng IGF-II/tube the result was 0.03 ng, which was below the first standard. Added ligand (IGF-II) behaved similarly in the IGF-II assay (107 ± 7% of expected values; n = 6) when the amounts added were greater or similar to the false results but inflated values when the IGFBPs remained in excess. However, adding contraligand (IGF-I) only partially suppressed the spurious results.

Having established the effects of separated bovine IGFBPs on the test results, the effects of spiking extracts of serum and plasma from different categories of cattle with ligand and contraligand was investigated (Table 1). The extracts originally provided 0.1–1.1 ng IGF-I and 0.15–0.86 ng IGF-II per tube. Since adding contraligand did not decrease the results obtained, while added ligand increased them by the expected amount within experimental error, it was concluded that there was no significant interference by IGFBPs carried through the extraction procedure.

Table 1

Effect of adding additional IGF-I and IGF-II to the incubation medium during IGF assays [Mean (SEM)]

Category	No.	Expected increment with ligand (%)	No.	Expected value with contraligand (%)
IGF-I assay				
Newborn calf serum	6	94.6 (9.3)	6	102.9 (6.6)
Lactating cow plasma	6	101.9 (8.0)	6	99.9 (8.3)
IGF-II assay				
Newborn calf serum	4	96.5 (7.1)	2	100.1 (6.1)
Newborn calf serum*	4	86.5 (4.6)	–	–
7-day-old calf serum	3	96.9 (7.9)	3	108.3 (7.9)
4-month-old calf serum	5	88.7 (4.8)	2	98.8 (4.4)
6-month-old calf serum	3	87.4 (8.8)	3	92.2 (3.1)
Peripartal cow serum	4	106.5 (5.8)	–	–
Lactating cow plasma	4	86.8 (7.1)		

*Incubation mixture spiked with bovine IGF fraction

The relation between obtained and expected values for IGF-II after progressive dilution of serum extracts from several calves followed the diagonal as for the bovine IGF column fractions (Fig. 2).

IGF-I determined again in representative beef cattle sera containing a wide range of IGF-I concentrations (10–80 nmol/l) after about three years storage at –18 °C showed excellent agreement (Y = 0.96X – 0.15; r = 0.999).

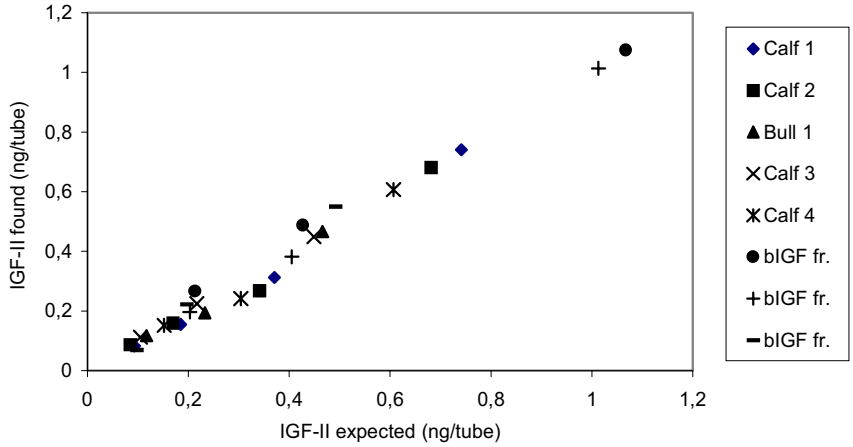

Fig 2. Relation between obtained and expected values for IGF-II after serial dilution of extracts of cattle serum (Calves 1 and 2 – newborn; Calf 3 at 6 months old; Calf 4 at 7 days old) and bovine IGF fractions separated by molecular sieve chromatography in 1M acetic acid

Serum IGF concentrations

Mean IGF-I concentrations in female calves ranged from 8.8 to 20.5 nmol/l, tending to be greater in the suckled beef calves than in the dairy calves (Table 2). Lower values (5.1–10.3 nmol/l) were found in periparturient cows. Highly significant age-related increases were noted in male calves. Very large differences between individual animals were e ncountered at 6 months old.

Compared with the 10-fold difference in mean values for IGF-I found between young beef bulls and periparturient dairy cows, serum IGF-II levels varied within narrow limits. Thus, the greatest mean value found in one group of postpartal dairy cows was just over double the lowest mean value recorded for the group of 12-month-old male beef cattle. Within the groups of cattle a statist i-cally significant difference was detected between Holstein-Friesian calves at 1 day and 4 months of age.

Since IGF-I concentrations increased with age in male calves without an accompanying decline in IGF-II concentrations, there was a significant increase in overall IGF concentrations as well (Table 2). In a similar way, the decline in IGF-II concentrations in Holstein-Friesian calves between 1 day and 4 months of age led to significant decreases in the sum as well as the IGF-II/IGF-I molar r a-tio. Moreover, for the dairy cows, the slight decrease in IGF-I and increase in IGF-II concentrations after calving caused a statistically significant difference in their molar ratio to emerge.

Table 2

Mean circulating concentrations of IGF-I and -II (nmol/l), their molar sum and ratio in groups of cattle of different breeds, ages and sex

Category	Age	Sex	No. of samples	IGF-I	IGF-II	IGF-I + IGF-II	IGF-II / IGF-I
Calves							
1. Holstein-Friesian calves*	< 1 day	female	22	8.8	23.5	32.3	2.96
(n = 44)		male	22	8.2	22.9	31.1	3.24
	4.5 months	female	22	9.3	17.4	26.7	2.31
		male	22	10.1	17.2	27.3	2.37
			SE	0.9	1.4	1.8	0.12
			F-age	1.63	17.61	7.03	6.14
			P	NS	0.0001	0.011	0.017
2a. Beef calves (n = 19)	7 days	female	10	20.5	14.7	35.1	0.81
(14 Limousine,	6 months	female	11	18.5	15.4	33.9	1.06
2 Charolais,	12 months	female	7	18.5	15.7	34.2	1.20
1 Simmental			SE	1.7	0.5	1.6	0.12
2 crossbred)†			F	0.15	0.39	0.05	0.84
			P	NS	NS	NS	NS
2b. Beef calves (n = 14)	7 days	male	10	14.4	12.9	27.3	1.04
(8 Limousine,	6 months	male	9	32.0	14.8	46.8	0.85
4 Charolais,	12 months	male	8	49.8	11.9	61.8	0.25
2 crossbred)†			SE	4.3	0.8	4.3	0.11
			F	9.20	1.00	7.58	6.21
			P	0.0011	NS	0.0019	0.0067
Adults							
3. Young (~ 16 months) bulls (n = 29)			29	67.4	13.4	81.8	0.22
(6 Limousine, 23 Simmental)			SE	3.3	0.7	3.2	0.02
4. Holstein-Friesian cows**	< 1 week prepartum		24	6.3	26.4	32.7	4.95
(n = 24)	< 1 week postpartum		24	5.1	30.0	35.1	7.75
			SE	0.7	1.5	1.9	0.58
			F-time	1.52	2.88	0.85	12.28
			P	NS	NS	NS	0.0019
5a. Holstein-Friesian cows‡	< 1 week postpartum		10	10.3	17.8	28.0	2.14
(n = 10)			SE	1.5	1.4	1.8	0.39
5b Lactating cows‡	2–10 weeks postpartum		5	8.2	16.5	24.7	2.91
(n = 5)			SE	2.6	1.6	2.0	0.89

*Two-factor-split-plot ANOVA. Sex and interaction effects not statistically significant; †Bartlett's test showed non-homogeneity of variance for IGF-I due to great individual differences in bull calves at 6 months old. Mathematical transformation of the data was without effect, so single-factor ANOVA was performed separately for each sex; **Significant cow × time interaction in two-way ANOVA abolished by square root transformation without effect on the statistical significance of the variable time; ‡Heparinised plasma

Discussion

Our results comply with and extend those obtained by other authors whose investigations were usually confined to particular age categories or breeds of cattle. When methodological problems were discussed, the difficulties encountered and solutions found were comparable with ours. Thus, Lee and Henricks (1990) described 'false' displacement curves with IGFBPs but obtained satisfactory comparative values for IGF-I by acid-ethanol extraction of sera. Vega et al. (1991) reported 85% and 88% recoveries for IGF-I and -II, respectively when known amounts were added to samples before acid-ethanol extraction. One ethanol-acetone-acetic acid extraction procedure led to overestimation of low concentrations and underestimation of high concentrations of IGF-I (73%–115%; Gutiérrez et al., 1997) but no such relationship could be deduced for our IGF-I assay. Moreover, since a similar tendency in our IGF-II assay was relatively weak, values obtained were taken as measured and represent h-IGF-II equivalents.

Mean serum concentrations for IGF-I were lowest in puerperal cows and highest in bulls at slaughter, whereas mean values for IGF-II were lowest in 12-month-old bull calves and highest in the cows. The predominance of the putative IGFBP-3 doublet in postpubertal calf serum and the great intensity of the 34 kD band (putative IGFBP-2; Fig. 1) in puerperal cow serum confirm the positive or negative associations observed between IGF-I and IGFBP-3 or IGFBP-2 levels, respectively (Renaville et al., 1993, 1996).

The difference between the sexes found for IGF-I levels in 6- and 12-month-old calves was greater than that observed for Holstein calves kept under similar conditions (Kerr et al., 1991) and similar to that recorded for Angus beef cattle (Bishop et al., 1989). According to Breier et al. (1988), the age-dependent prepubertal rise in serum IGF-I in male calves commences with the appearance of functional somatotrophic receptors in the liver. It is independent of nutritional factors and testosterone status (Renaville et al., 1996). Different timing of these changes may have been responsible for the wide range of IGF-I concentrations in our male calves at 6 months old.

In general, the values obtained for IGF-I in growing cattle were similar to those reported by other authors (Bishop et al., 1989; McShane et al., 1989; Renaville et al., 1993). Concerning IGF-II, Holland et al. (1997) reported mean serum concentrations of 42.8 nmol/l in crossbred beef fetuses at term which was nearly fivefold higher than the IGF-I levels. Mean IGF-II levels in our dairy calves (< 1 day old) were just twofold higher than IGF-I concentrations (Table 2). Our values for puerperal Holstein cows also confirmed those of others (Davis et al., 1987; Vega et al., 1991; Vicini et al., 1991). Higher concentrations were observed in Brahman heifers and mid-lactation cows (Hill et al., 1999; McGuire et al., 1995). This report is the first study of both growth factors in beef and dairy cattle of different ages and sexes.

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