Insulin-like growth factor and insulin receptors in intestinal mucosa of neonatal calves

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Abstract

Intestinal development is modified by age and nutrition, mediated in part by insulin-like growth factors (IGF-I, IGF-II) and insulin. We have investigated whether expression of IGF-I, IGF-II and insulin receptors (IGF-IR, IGF-IIR and IR; measured by real-time RT-PCR) and binding capacity (B_{max}) of IGF-IR, IGF-IIR and IR in the mucosa of the small and large intestine of neonatal calves are modified by age and different feeding regimes. In experiment 1, pre-term (GrP) and full-term (GrN) calves (after 277 and 290 days of pregnancy respectively) were killed immediately after birth before being fed; a further group of full-term calves were fed for 7 days and killed on day 8 of life (GrC_{1-3}). In experiment 2, full-term calves were killed on day 8 after being fed first-colostrum for 7 days (GrC_{max}), colostrum of the first six milkings for 3 days (GrC_{1-3}) or milk-based formula for 3 days (GrF_{1-3}). Intestinal sites differed with respect to expression levels of IGF-IR (duodenum>jejunum in GrC_{1-3}; ileum>colon, duodenum≥jejunum in GrF_{1-3}), IGF-IIR (colon>duodenum and ileum in GrN), and IR (lowest in ileum in GrP and CrN; highest in colon in GrC_{1-3} and GrC_{max}). They also differed with respect to B_{max} of IGF-IR (ileum and colon>duodenum and jejunum in GrP; ileum and colon>jejunum in GrN; colon>jejunum in GrC_{1-3}; lowest in jejunum in GrF_{1-3}), IGF-IIR (duodenum and colon>jejunum and ileum in GrP; duodenum>ilem and colon>jejunum in GrN; duodenum, jejunum and

colon>ileum in GrC_{max}, GrC_{1-3}, and GrF_{1-3}) and IR (ileum>duodenum, jejunum and colon in GrC_{max}, GrC_{1-3}, and GrF_{1-3}). There were significant differences between groups in the expression of IGF-IR (GrF_{1-3}>GrC_{max} and GrC_{1-3} in ileum), IGF-IIR (GrN>GrP and GrC_{1-3} in colon; GrN>GrC_{1-3} in jejunum and total intestine), and IR (GrC_{max}>GrF_{1-3} in colon) and in the B_{max} of IGF-IR (GrP>GrN in colon; GrC_{max}>GrF_{1-3} in jejunum), IGF-IIR (GrN>GrP in duodenum, ileum and total intestine; GrN>GrC_{1-3} in duodenum, ileum, colon and total intestine) and IR (GrN>GrP in total intestine; GrC_{1-3}>GrN in ileum and total intestine). In addition, B_{max} values of IGF-IR, IGF-IIR and IR were correlated with villus circumference, villus height/crypt depth and proliferation rate of crypt cells at various intestinal sites. There were marked differences in B_{max} of IGF-IR, IGF-IIR and IR dependent on mRNA levels, indicating that differences in B_{max} were the consequence of differences in posttranslational control and of receptor turnover rates.

In conclusion IGF-IR, IGF-IIR and IR expressions and B_{max} in intestinal mucosa were different at different intestinal sites and were variably affected by age, but not significantly affected by differences in nutrition. Receptor densities were selectively associated with intestinal mucosa growth.

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Introduction

In calves, the first 2–3 postnatal weeks are well known to be characterized by high morbidity and mortality rates. Diseases especially of the gastrointestinal (GI) tract (GIT), such as severe diarrhea, are frequent and are often followed by fatal systemic diseases. Adaptations of the GIT and of its

digestive functions are critical due to the change from primarily parenteral nutrition during the fetal stage to exclusively enteral nutrition. Insufficient morphological and functional adaptations of the GIT are considered to be of central etiological importance for GI diseases (Guilloteau *et al.* 1997). This is especially true for pre-term calves (S Bittrich, H M Hammon and J W Blum, unpublished

observations). Better knowledge of factors that stimulate functional development of the GIT for adequate adaptation to the postnatal period is expected to reduce digestive disorders and to improve digestive efficiency, health status, well-being and growth performance.

Morphology and function of the GIT in the postnatal period are influenced by endogenous and exogenous factors. Among exogenous factors nutritional and non-nutritional (bioactive) factors that are ingested with colostrum play a major role, as has been shown in calves by several authors (Guilloteau *et al.* 1997, Bühler *et al.* 1998, Blättler *et al.* 2001, Blum & Baumrucker 2002, Blum *et al.* 2002). Colostrum at the onset of lactation contains the greatest amount of bioactive substances, including growth factors and hormones to which the newborn calf – and primarily the GIT – is exposed (Blum & Baumrucker 2002).

The somatotropic axis of neonatal calves is basically functioning, although it is not yet fully mature (Hammon & Blum 1997). During the postnatal period insulin-like growth factor (IGF)-I becomes more important than IGF–II (Breier *et al.* 2000, Butler & LeRoith 2001). The somatotropic axis and especially IGFs, besides insulin, is involved in GIT development and especially in proliferation and maturation of enterocytes (Laburthe *et al.* 1988, Schober *et al.* 1990, Odle *et al.* 1996, MacDonald 1999, Menard *et al.* 1999). It has been demonstrated that IGF-II and insulin are involved in the mechanisms governing the differentiation of intestinal epithelium while IGF-I is mostly associated with crypt cell proliferation (Jehle *et al.* 1999). In newborn calves mRNAs for IGF-I and IGF-II, for receptors of growth hormone, IGF-I (IGF-IR), IGF-II (IGF-IIR) and insulin (IR), and for IGF binding proteins 1–3 are found in the ileum (Pfaffl *et al.* 2002). Based on ligand binding assays, receptors for IGF-I, IGF-II and insulin are present in the GIT of neonatal calves (Baumrucker *et al.* 1994, Hammon & Blum 2002). Therefore, IGFs and insulin ingested especially with colostrum (Odle *et al.* 1996, Xu *et al.* 1994, Blum & Baumrucker 2002), IGFs produced in the GI wall, and IGFs and insulin circulating in blood plasma can affect GI morphology and function after binding to their respective mucosal receptors (Lund 1994, Fholenhag *et al.* 1997, Jehle *et al.* 1999, Simmons *et al.* 1999, Blum & Baumrucker 2002). The effects of IGFs and insulin depend, at least in part, on receptor number and affinity, i.e. intestinal IGF-IR, IGF-IIR and IR may be involved in GIT development in pre-term calves. Because calves are born relatively mature compared with other species such as rats, mice and humans, differences with other species with respect to receptor numbers and thus of GIT responses to ingested food components can be expected, i.e. specific studies on IGF-IR, IGF-IIR and IR in calves are justified and needed.

This study was conducted to investigate binding capacity (B_{max}) and mRNA levels of IGF-IR, IGF-IIR and IR in the mucosa of duodenum, jejunum, ileum and colon in calves born 14 days pre-term and in calves born at normal term (after 290 days of pregnancy), in full-term calves on day 8 of life after being fed for 7 days, and in full-term 8-day-old calves fed different amounts of colostrum or only a formula (and thus different amounts of bioactive substances such as IGFs and insulin) for 7 days. We have tested the following hypotheses: (1) that mRNA levels and B_{max} of IGF-IR, IGF-IIR and IR are specifically regulated and associated; (2) that IGF-IR, IGF-IIR and IR concentrations at the mRNA and protein level immediately after birth in pre-term calves differ from those of calves born at full-term; (3) that there are variations in IGF-IR, IGF-IIR and IR at the mRNA level in pre-term and full-term neonatal calves at different intestinal sites; (4) that IGF-IR, IGF-IIR and IR at the protein and mRNA level change within the first days of life in full-term neonatal calves; (5) that differences in intake of bioactive substances such as IGFs and insulin (as a consequence of differences in amounts of ingested colostrum or by ingesting only a formula) modify IGF-IR, IGF-IIR and IR mRNA levels and B_{max} of duodenum, jejunum, ileum and colon; and (6) that IGF-IR, IGF-IIR and IR correlate with histomorphometrical traits and with proliferation rates of intestinal epithelium of duodenum, jejunum, ileum and colon.

Materials and Methods

Animals, husbandry, feeding and experimental design

The experiments were performed according to the Swiss law on Animal Protection and the experimental procedures were approved by the Committee for Animal Experimentation of the Canton of Freiburg (Granges-Paccot, Switzerland), supervised by the Swiss Federal Veterinary Administration (Berne, Switzerland).

Experiment 1 Single-born calves ($n=19$; seven Red Holstein, six Simmenthal × Red Holstein, five Holstein Friesian and one Brown Swiss), originating from the Research Station, were assigned to three groups. Pre-term calves, born on day 277 of gestation after cows were injected with 500 µg prostaglandin $F_{2\alpha}$ (Estrumate; Essex Pharma GmbH, Friesoythe, Germany) and 5 mg Flumethason (Flumilar; Veterinaria AG, Zürich, Switzerland), were killed immediately after birth before being fed, i.e. on day 1 of life (GrP, $n=6$). Full-term calves born after the normal duration of pregnancy (290 ± 2 days) were either killed immediately after birth (GrN, $n=6$) or on day 8 of life (GrC$_{1–3}$, $n=7$).

Calves of the GrC$_{1–3}$ group were held on straw in individual pens for 7 days. Colostrum for GrC$_{1–3}$ calves was from cows from the Research Station milked twice daily and was separately stored at -20 °C to make pools of milkings 1 to 6 for days 1, 2 and 3. Before feeding, colostrum was warmed to 40 °C and then immediately

Table 1 Feeding plan for neonatal calves

Age (days)	Fed amounts (g/kg BW/day)	Group		
		GrC_{max}	GrC_{1-3}	GrF_{1-3}
1	60	Colostrum 1st milking	Colostrum 1st +2nd milking	Formula 1
2	80	Colostrum 1st milking	Colostrum 3rd +4th milking	Formula 2
3	100	Colostrum 1st milking	Colostrum 5th +6th milking	Formula 3
4	100	Colostrum 1st milking +MR (v/v=3:1)	MR	MR
5	100	Colostrum 1st milking +MR (v/v=1:1)	MR	MR
6	100	Colostrum 1st milking +MR (v/v=1:3)	MR	MR
7	100	Colostrum 1st milking +MR (v/v=1:3)	MR	MR

Calves of GrC_{max} were fed first-colostrum up to day 7 of life, calves of GrC_{1-3} were fed colostrum from milkings 1 to 6 during the first 3 days of life and then a milk replacer (MR; whose nutrient composition was similar to that of mature milk) and calves of GrF_{1-3} were fed a milk-based formula during the first 3 days of life and then a milk replacer. Calves were fed twice daily. The formula fed on days 1, 2 and 3 was milk-based and contained comparable amounts of nutrients as colostrum of day 1 (milking 1), day 2 (milking 3) and day 3 (milking 5) respectively, but contained only traces of growth factors and hormones such as IGF-I and insulin.

fed. From day 4 onwards calves were fed a milk replacer (MR) diluted with water (100 g/l water) up to day 7, mimicking usual husbandry conditions. The contents of colostrum from milkings 1 to 6 and the MR, together with the feeding plans have been published elsewhere (Blättler *et al.* 2001, Rauprich *et al.* 2000*a*,*b*). Calves were fed twice daily by bottle, beginning $2 \cdot 7 \pm 0 \cdot 7$ h after birth. The ensuing feedings were at 8, 24, 32, 48, 56, and 72 h after the first feeding. From day 4 to day 7 calves were fed daily at 0800 and 1600 h. Starting on day 4 calves had free access to water. The MR (UFA–200–Natura; without antibiotics) was purchased from UFA AG (Sursee, Switzerland).

Experiment 2 Male calves ($n=21$; 13 Simmental × Red Holstein, four Brown Swiss and four Holstein Fresian) were raised at the Swiss Federal Station for Animal Production (Posieux, Switzerland). All calves were single born after a normal length of pregnancy (290 days) and normal parturition. They were obtained immediately after birth and held on straw litter in individual pens up to day 7 of life.

Calves were divided into three dietary groups (GrC_{max}, GrC_{1-3} and GrF_{1-3}), each consisting of seven animals. Calves of the GrC_{max} group were fed twice daily during the first 3 days of life on pooled undiluted colostrum that was the first milking after parturition, and then on first-milked colostrum on days 4, 5, 6, and 7 that was diluted with 25, 50, 75, and 75 parts of MR respectively

(Table 1). Calves of the GrC_{1-3} group received colostrum of milkings 1 to 6 (first 3 days of lactation) on the first 3 days of life and then MR (100 g/l water) up to day 7. Calves of the GrF_{1-3} group were fed a milk-based formula during the first 3 days of life and MR (100 g/l water) from day 4 to day 7.

Calves were fed by plastic bottle twice daily. The total amounts of fed colostrum, formula and MR on days 1, 2, and 3–7 were 60 ml/kg body weight (BW), 80 ml/kg BW, and 100 ml/kg BW respectively. The first feeding was 2–4 h after birth, and then at 8, 24, 32, 48, 56, and 72 h after the first feeding. Starting on day 4 calves were fed daily at 0800 and 1600 h and had free access to water.

Colostrum was collected from cows from the Research Station. Colostrum for GrC_{max} calves was a pool of first milking after parturition. Colostrum for GrC_{1-3} calves was obtained before the start of the experiment from cows milked twice daily. Colostrum of milkings 1 to 6 was used to prepare individual pools which were separately stored in plastic bottles at $-20\ °C$. Before feeding, the colostrum was warmed to $40\ °C$ and immediately fed. Calves of the GrF_{1-3} group were fed three different milk-based formulas during the first 3 days of life (for meals on days 1, 2, and 3 respectively), containing only traces of growth factors and hormones such as IGF-I, IGF-II and insulin, but had comparable amounts of nutrients as colostrum on day 1 (from milkings 1 and 2), day 2 (from milkings 3 and 4) and day 3 (from milkings 5 and 6). Milk formulas were produced in co-operation with UFA AG. They consisted

Table 2 Composition of colostrum milkings, formula and milk replacer fed to calves during the first 7 days of life

	Day 1		Day 2		Day 3		Days 4–7
	Colostrum[1]	Formula[2]	Colostrum	Formula	Colostrum	Formula	Milk replacer[3]
Parameters							
Dry matter (g/kg)	215	210	158	130	154	139	95
Gross energy (MJ/kg)	5·4	5·4	3·9	3·2	3·8	3·6	1·7
Crude protein (g/kg)	109	123	58	53	49	46	21
Crude fat (g/kg)	60	56	48	37	50	49	22
Nitrogen-free extracts (g/kg)	33	22	43	33	47	41	45[4]
Crude ashes (g/kg)	14	12	9	5	9	4	7
IGF-I (µg/kg)	292	8	103	16	71	11	5
Insulin (µg/kg)	20·0	2·7	2·6	1·1	1·9	1·7	0·2

[1]Colostrum pools were prepared from 8 cows and the data are the sums of milkings 1 and 2, 3 and 4, and 5 and 6 on days 1, 2 and 3 respectively.
[2]The formula contained (per kg) calcium caseinate (86, 208 and 282 g on days 1, 2 and 3 respectively), lactalbumin (576, 245 and 80 g on days 1, 2 and 3 respectively), milk fat (267, 262 and 353 g double cream on days 1, 2 and 3 respectively), lactose (57, 271 and 271 g on days 1, 2 and 3 respectively), and a mineral premix (14 g on days 1, 2 and 3 respectively). The mineral prefix (per kg) contained calcium (186 g), magnesium (224 g), sodium (31 g), phosphorus (92 g), chlorine (48 g), iron (12 g), manganese (8·1 mg), copper (1·6 mg), zinc (7·8 mg), iodine (0·03 mg), cobalt (0·02 mg) and selenium (0·02 mg).
[3]The milk replacer contained (per kg) skim milk powder (550 g), whey (40 g), corn-derived products (dextrose, glucose, oat cream, starch; 172 g), tallow (145 g), lard (44 g/kg), lecithin (as emulgator; 19 g), calcium (12 g), phosphorus (7·5 g), magnesium (1·6 g), sodium (4·9 g), zinc (80 mg), manganese (60 mg), iron (20 mg), copper (8 mg), iodine (3 mg), selenium (0·5 mg), cobalt (0·5 mg), vitamin A (26·25 µmol retinol equivalent/kg), cholecalciferol (195 nmol/kg), vitamin E (360 µmol α-tocopherol/kg), thiamine (57 µmol/kg), riboflavin (21 µmol/kg), vitamin B-6 (59 µmol/kg) and vitamin B-12 (37 nmol/kg).
[4]Nitrogen-free extracts in milk replacer contained (per kg) 710 g lactose (i.e. 336 g lactose/kg milk replacer on a dry matter basis).

of calcium caseinate (a gift from Emmi Milch AG, Lucerne, Switzerland), lactoalbumin (a gift from Emmi Milch AG), lactose (UFA AG), milk fat in the form of commercially available dairy double cream provided by the Agricultural Institute of the Canton of Fribourg, Grangeneuve/Posieux, Switzerland, and a vitamin and mineral premix (Provini S.A., Cossonay–Gare, Switzerland). The milk replacer (UFA-200 Natura; containing no antibiotics) was purchased from UFA AG and was prepared as a 100 g/l solution. Composition of colostrum milkings, formula and milk replacer are presented in Table 2.

To protect against infections calves were injected s.c. with 20 ml of an immunoglobulin (IgG) formulation (Gammaserin, 100 g IgG/l; E Gräub AG, Switzerland). In addition, from day 2 of life they received chicken egg–derived immunoglobulin containing high titers against rotavirus and pathogenic *E. coli* type K99 (Lohmann Animal Health, Cuxhaven, Germany) with each meal. The amounts per meal were as follows: 5 g on day 2, 4 g on day 3, 3 g on day 4, 2 g on day 5, and 1 g on days 6 and 7.

Intestinal tissue sampling

Calves were slaughtered on day 8 of life or directly after birth (GrN and GrP), the abdominal cavity was opened and the GIT was immediately removed. Sections with a length of 15–20 cm that originated from the middle parts of different regions of the intestine - duodenum, jejunum, ileum and colon - were opened and washed with ice-cold saline. The mucosa was gently scraped, and was put into ice-cold buffer (50 mM Tris–HCl; 6 mM $MgCl_2$, 1 mM

EGTA; pH=7·4) for radioligand binding assays, or into TRIzol Reagent (Gibco BRL, Basle, Switzerland) before freezing in liquid nitrogen, and was stored at −80 °C until analyzed for mRNA of IGF-IR, IGF-IIR and IR.

Radioligand binding assays

The materials used and the binding assay procedures have recently been described in detail (Hammon & Blum 2002). Immediately after slaughter, the intestinal mucosa was homogenized using an Ultra-Turrax homogenizer (T25, Janke and Kunkel, Staufen, Germany) four times at low speed (8000 revs/min). The homogenate was then centrifuged at 800 **g** for 10 min, the supernatant was centrifuged at 10 000 **g** for 10 min, and then at 100 000 **g** for 1 h as described. The pellet was suspended in ice-cold buffer with a motor-driven Glass-Teflon homogenizer and the obtained membrane suspension was stored at −80 °C until analyzed. For binding studies the protein concentration of mucosal membrane suspensions was measured using a kit (BCA Protein Assay Reagent; Pierce, Rockford, IL, USA). The linearity of protein concentration was tested for each receptor at different protein concentrations. The final protein concentration of the membrane suspension was adjusted to 100, 50 and 200 µg protein/ml for [^{125}I]IGF–I, [^{125}I]IGF–II, and [^{125}I]insulin binding studies respectively, to perform binding studies in a linear range. Receptors were defined based on differences in ligand binding affinities in competitive binding assays and based on ligand blots. For the quantification of binding of [^{125}I]IGF–I, [^{125}I]IGF–II and [^{125}I]insulin the radiolabeled ligand (0·35 ng) was incubated with increasing concentrations of the unlabeled ligand IGF-I

$(10^{-12}$ to 10^{-7} M), IGF-II $(10^{-11}$ to 10^{-7} M), and insulin $(10^{-12}$ to 10^{-6} M) respectively, and B_{max} and binding affinity (evaluated by determination of the 50% inhibition (IC_{50}) of binding of the radioactive label by the unlabeled ligand) was calculated. In contrast to the previous study (Hammon & Blum 2002), IR were best fitted to a model with one binding site. The human/bovine IGF-I was donated by Novartis AG (formerly Ciba Geigy AG), St Aubin, Switzerland, IGF-II was purchased from GroPep, Adelaide, Australia, and bovine insulin was purchased from Sigma, St Louis, MO, USA. IGF-I, IGF-II and insulin were iodinated with the Chloramine T method.

Determination of receptor mRNAs

Total RNA extraction from mucosa of duodenum, jejunum, ileum and colon was performed using TRIzol Reagent (Gibco BRL) and was resuspended in RNase-free water treated with diethyl pyrocarbonate (DEPC, Sigma-Aldrich Vertriebs GmbH, Deisenhofen, Germany). RNA integrity and purity were tested by measurement of optical density and by electrophoresis using ethidium bromide staining. Total RNA was then reverse transcribed into cDNA using random hexamer primers (Amersham-Pharmacia Biotech) as described (Pfaffl *et al.* 2002). Materials used and the procedures followed in RT-PCR assays were recently described in detail (Pfaffl *et al.* 2002). The primers for IGF-IR (forward primer: TTA AAA TGG CCA GAA CCT GAG; reverse primer: ATT ATA ACC AAG CCT CCC AC), IGF-IIR (forward primer: TAC AAC TTC CGG TGG TAC ACC A; reverse primer: CAT GGC ATA CCA GTT TCC TCC A) and IR (forward primer: TCC TCA AGG AGC TGG AGG AGT; reverse primer: GCT GCT GTC ACA TTC CCC A) were generated by Microsyth GmbH (Balgach, Switzerland). RT-PCR quantification was performed with the LightCycle System (Roche Molecular Biochemicals, Rotkreuz, Switzerland) using software package 3·3 (Roche Molecular Biochemicals). Absolute quantification was based on external recombinant DNA standards (Pfaffl *et al.* 2002) and values were expressed on a molar basis.

Histomorphometrical analyses and cell proliferation of intestinal epithelia

For the histomorphometrical analyses hematoxylin and eosin-stained slides were available. Analyses were conducted with a light microscope connected to a video-based, computer-linked system, as described by Bühler *et al.* (1998) and Blättler *et al.* (2001).

Cell proliferation was based on counting cells which incorporate 5-bromo-2′-deoxyuridine that was intravenously injected 1 h before euthanasia (Blättler *et al.*

2001). 5-Bromo-2′-deoxyuridine-labeled (mitotic) crypt cells were calculated relative to unlabeled crypt cells and served as an indicator of cell proliferation rate.

Data evaluation and statistics

For data analysis, the IC_{50} of radiolabeled binding by the unlabeled ligand and B_{max} were calculated by weighted least squares curve fitting using the GraphPad computer program (GraphPad Software Inc., San Diego, CA, USA). The results from real-time RT-PCR quantification on Light Cycler are expressed in fmol mRNAs per 1 mg total RNA. The B_{max}, IC_{50}, mRNAs values, and $B_{max}/$ mRNA ratios are given as means ± S.E.M. Group differences of B_{max}, IC_{50}, and mRNAs were evaluated using the RANDOM and REPEATED methods of the MIXED procedure with an inter-animal random effect of differences between the animals and a correlation structure within animals (SAS 1995). Age (experiment 1), feeding (experiment 2), and gut segments were used as fixed effects within animals. Differences $(P<0·05)$ were evaluated by least significant difference (LSD) (*t*-test). The $B_{max}/$mRNA ratios were analyzed by one-way ANOVA with repeated measurements using receptor type as a variable (SAS 1995). Differences $(P<0·05)$ were evaluated by LSD (*t*-test). The COR procedure of the SAS program was used (SAS 1995) for calculations of correlations (IGF-I, IGF-II and insulin receptor numbers with respective mRNA concentrations in intestinal mucosa) and for calculations of B_{max} with intestinal histomorphometrical traits and with proliferation rates of intestinal epithelia.

Results

Experiment 1: concentrations of mRNA and maximal binding capacities of IGF-IR, IGF-IIR and IR in the mucosa of duodenum, jejunum, ileum and colon in pre-term calves on day 1 and in full-term calves on day 1 and day 7 of life

Expression of IGF-IR (Table 3) was significantly different between intestinal sites (duodenum>jejunum in GrC_{1-3}), but there were no significant group differences. Expression of IGF-IIR differed significantly between intestinal sites (colon>duodenum and ileum in GrN) and between groups (GrN>GrP in colon; GrN>GrC_{1-3} in jejunum, colon and total intestine). Although total RNA concentration was higher in GrC_{1-3} than in GrN, IGF-IIR mRNA concentrations per g wet tissue were still significantly higher in GrN than in GrC_{1-3} (data not shown). Expression of IR differed significantly between intestinal sites (in GrP and GrN it was lowest in ileum; in GrC_{1-3} it was highest in colon), but there were no significant group differences.

B_{max} of IGF-IR (Table 4) differed significantly between intestinal sites (ileum and colon>duodenum and jejunum

Table 3 Concentrations of mRNA (fmol/mg total RNA) for IGF-I, IGF-II and insulin receptors in intestinal mucosa of pre-term calves (GrP), full-term calves (GrN), and 8-day-old calves (GrC_{1-3}) (experiment 1). Results are expressed as means and pooled S.E.M.

	Group			Pooled S.E.M.	Group differences (*P* values)	
	GrP	GrN	GrC_{1-3}		GrP vs GrN	GrC_{1-3} vs GrN
IGF-IR						
Duodenum	3·25	2·96	2·15[A]	0·76	NS	NS
Jejunum	2·56	2·02	0·84[B]	0·53	NS	NS
Ileum	1·88	2·07	1·56[AB]	0·55	NS	NS
Colon	2·66	3·12	1·69[AB]	0·63	NS	NS
Total intestine	2·59	2·55	1·53	0·30	NS	NS
IGF-IIR						
Duodenum	0·085	0·063[B]	0·022	0·013	NS	NS
Jejunum	0·077	0·1[AB]	0·035	0·02	NS	0·05
Ileum	0·05	0·074[B]	0·028	0·01	NS	NS
Colon	0·072	0·15[A]	0·027	0·03	0·01	0·001
Total intestine	0·071	0·1	0·028	0·009	NS	0·01
IR						
Duodenum	16·9[A]	11·2[AB]	9·6[AB]	3·3	NS	NS
Jejunum	15·3[A]	12·8[A]	5·8[B]	3·5	NS	NS
Ileum	7·2[B]	5·8[B]	8·2[B]	1·8	NS	NS
Colon	14·6[A]	14·2[A]	15·3[A]	4·5	NS	NS
Total intestine	13·5	11·5	9·7	1·8	NS	NS

Calves of GrP (*n*=6) were born 2 weeks pre-term (after 277 days of pregnancy) and those of GrN (*n*=6) were born full-term (after 290 days of pregnancy) and were killed immediately after birth. Calves of GrC_{1-3} (*n*=7) were born full-term, fed colostrum derived from milkings 1 to 6 for 3 days and then milk replacer (whose nutrient content was similar to that of mature milk) up to day 7 and were killed on day 8.
[A,B]Within a column means of IGF-IR, IGF-IIR and IR with different capital superscript letters are significantly different (*P*<0·05). NS, not significant (*P*>0·05).

in GrP, ileum and colon>jejunum in GrN; colon>jejunum in GrC_{1-3}) and between groups (GrP>GrN in colon). B_{max} of IGF-IIR differed significantly between intestinal sites (duodenum and colon>jejunum and ileum in GrP; duodenum>ileum and colon>jejunum in GrN; duodenum, jejunum, and colon>ileum in GrC_{1-3}) and between groups (GrN>GrP in duodenum, ileum, and total intestine; GrN>GrC_{1-3} in duodenum, ileum, colon, and total intestine) and there was also a significant group × gut interaction. B_{max} of IR differed significantly between intestinal sites (ileum>duodenum, jejunum and colon in GrC_{1-3}) and between groups (GrN>GrP in total intestine; GrC_{1-3}>GrN in ileum and total intestine) and there was a significant group × gut interaction.

Experiment 2: concentrations of mRNA and maximal binding capacities of IGF-IR, IGF-IIR and IR in the mucosa of duodenum, jejunum, ileum and colon of 8-day-old calves fed different amounts of colostrum or a milk-based formula

Expression of IGF-IR (Table 5) was significantly different between intestinal sites (ileum>colon, duodenum≥jejunum in GrF_{1-3}) and there were significant group differences (GrF_{1-3}>GrC_{max} and GrC_{1-3} in ileum). In addition, there was a significant group × gut interaction. Expression of IGF-IIR in intestinal mucosa did not differ significantly between different intestinal segments, but IGF-IIR expression in duodenum tended to be higher (*P*<0·1) in GrC_{max} than in GrC_{1-3}. Expression of IR was significantly different between intestinal sites (colon>duodenum, jejunum, ileum in GrC_{max}; colon>jejunum and ileum in GrC_{1-3}) and between groups (GrC_{max}>GrF_{1-3} in colon). There was also a significant group × gut segment interaction.

B_{max} of IGF-IR (Table 6) was significantly different between intestinal sites (colon>jejunum in GrC_{1-3} and duodenum, ileum, and colon>jejunum in GrF_{1-3}) and between groups (GrC_{max}>GrF_{1-3} in jejunum). There was a significant group × gut interaction. B_{max} of IGF-IIR was significantly different between intestinal sites (duodenum, jejunum and colon>ileum in all three groups), but there were no significant group differences. B_{max} of IR was significantly different between intestinal sites (ileum>duodenum, jejunum and colon in all three groups) and tended to be different (though not significantly, *P*<0·1) between groups (GrC_{max}>GrF_{1-3} in jejunum and total intestine; GrC_{max}>GrC_{1-3} in colon).

Table 4 Binding capacities (B_{max}; fmol/mg protein) of IGF-I, IGF-II and insulin receptors in intestinal mucosa of pre-term calves (GrP), full-term calves (GrN), and 8-day-old calves (GrC$_{1-3}$) (experiment 1). Results are expressed as means and pooled S.E.M.

	Group				Group differences (P values)	
	GrP	GrN	GrC$_{1-3}$	Pooled S.E.M.	GrP vs GrN	GrC$_{1-3}$ vs GrN
IGF-IR						
Duodenum	7·2[B]	7·9[AB]	9·3[AB]	1·28	NS	NS
Jejunum	6·3[B]	5·1[B]	7·6[B]	1·11	NS	NS
Ileum	12·2[A]	11·3[A]	10·7[AB]	1·89	NS	NS
Colon	15·3[A]	9·8[A]	12·0[A]	2·38	0·05	NS
Total intestine	10·2	8·5	9·7	0·98	NS	NS
IGF-IIR						
Duodenum	176[A]	232[A]	56[A]	21·3	0·05	0·001
Jejunum	60[B]	76[C]	63[A]	12·7	NS	NS
Ileum	66[B]	139[B]	19[B]	11·7	0·01	0·001
Colon	166[A]	160[B]	63[A]	21·3	NS	0·001
Total intestine	119	151	51	12·9	0·05	0·001
IR						
Duodenum	1·7	3·6	5·0[B]	0·53	NS	NS
Jejunum	0·9	2·4	4·1[B]	0·57	NS	NS
Ileum	2·4	5·8	21·6[A]	1·80	NS	0·001
Colon	3·0	3·8	3·1[B]	0·61	NS	NS
Total intestine	1·9	3·8	8·5	0·85	0·05	0·001

[A,B,C] Within a column means of IGF-IR, IGF-IIR and IR with different capital superscript letters are significantly different ($P<0.05$). NS, not significant ($P>0.05$).
For further details see footnote to Table 3.

Comparisons between IGF-I, IGF-II and insulin receptors and relationships between intestinal mucosal mRNAs and binding capacities of IGF-I, IGF-II and insulin receptors

In this section analyses were performed based on pooled data of all groups and all intestinal sites of both experiments. Concentrations of IR mRNA were the highest ($P<0.001$), and concentrations of IGF-IR mRNAs were higher ($P<0.001$) than mRNA levels of IGF-IIR (means for all groups: 2.1 ± 0.1, 0.052 ± 0.005 and 11.5 ± 0.9 fmol/mg total RNA for IGF-IR, IGF-IIR and IR respectively). B_{max} values were higher ($P<0.001$) for IGF-IIR than for IGF-IR and IR (means for all groups: 9.6 ± 0.4, 76.8 ± 5.7 and 6.9 ± 0.7 fmol/mg protein for IGF-IR, IGF-IIR, and IR respectively). Ratios of B_{max} (fmol/mg protein)/mRNA (fmol/mg total RNA) were higher ($P<0.001$) for IGF-IIR than for IGF-IR and IR (Fig. 1). Considering all three receptor types there was a negative correlation between B_{max} and mRNA ($R = -0.72$; $P<0.001$; Fig. 1). The B_{max} values of IGF-IR in colon were negatively correlated with IGF-IR mRNA (-0.44; $P<0.05$), whereas B_{max} values of IGF-IIR were positively correlated with the IGF-IIR mRNA concentration in total intestine ($r=0.4$; $P<0.001$) and especially in jejunum ($r=0.49$; $P<0.01$), ileum ($r=0.57$; $P<0.001$), and colon ($r=0.47$; $P<0.01$). The B_{max} values of IR were negatively correlated with IR mRNA levels in duodenum ($r= -0.41$; $P<0.05$).

Correlations between IGF-I, IGF-II and insulin binding capacities with intestinal epithelial histomorphometric and proliferative measurements

For IGF-IR there was a positive correlation between B_{max} and crypt cell proliferation rates in duodenum ($r=0.5$; $P<0.01$), but negative correlations between B_{max} and villus circumference ($r= -0.28$; $P<0.01$) and villus height/crypt depth ratios ($r= -0.22$; $P<0.01$) in total small intestine. For IGF-IIR, B_{max} correlated positively with villus circumference in duodenum ($r=0.36$; $P<0.05$), jejunum ($r=0.34$; $P<0.05$), and ileum ($r=0.68$; $P<0.001$) and correlated positively with villus height/crypt depth ratios in duodenum ($r=0.72$; $P<0.001$), jejunum ($r=0.37$; $P<0.05$), and ileum ($r=0.7$; $P<0.001$).

B_{max} of IGF-IIR correlated negatively with crypt depth in duodenum ($r= -0.66$; $P<0.01$), jejunum ($r= -0.4$; $P<0.05$), ileum ($r= -0.61$; $P<0.01$), and colon ($r= -0.72$; $P<0.001$) and correlated negatively with the crypt cell proliferation rates in duodenum ($r= -0.42$; $P<0.05$), jejunum ($r= -0.45$; $P<0.05$), and ileum ($r= -0.34$; $P<0.1$).

In ileum, B_{max} values of IR correlated negatively with villus circumference ($r= -0.45$; $P<0.01$) and with villus height/crypt depth ratios ($r= -0.6$; $P<0.001$) and correlated positively with crypt depth ($r=0.53$; $P<0.01$) and crypt cell proliferation rates ($r=0.37$; $P<0.05$).

Table 5 Concentrations of mRNA (fmol/mg total RNA) for IGF-I, IGF-II and insulin receptors in intestinal mucosa of differently fed calves (experiment 2). Results are expressed as means and pooled S.E.M.

	Group				Group differences (P values)		
	GrC_{max}	GrC_{1-3}	GrF_{1-3}	Pooled S.E.M.	GrC_{max} vs GrC_{1-3}	GrC_{max} vs GrF_{1-3}	GrC_{1-3} vs GrF_{1-3}
IGF-IR							
Duodenum	1·62	2·15[A]	2·03[B]	0·38	NS	NS	NS
Jejunum	1·38	0·84[B]	0·90[C]	0·26	NS	NS	NS
Ileum	1·71	1·56[AB]	3·03[A]	0·32	NS	0·05	0·05
Colon	1·72	1·69[AB]	1·98[BC]	0·48	NS	NS	NS
Total intestine	1·62	1·53	2·07	0·21	NS	NS	NS
IGF-IIR							
Duodenum	0·050	0·022	0·030	0·012	NS	NS	NS
Jejunum	0·023	0·035	0·018	0·005	NS	NS	NS
Ileum	0·040	0·028	0·018	0·005	NS	NS	NS
Colon	0·044	0·027	0·029	0·008	NS	NS	NS
Total intestine	0·04	0·029	0·024	0·005	NS	NS	NS
IR							
Duodenum	7·7[B]	9·6[AB]	14·4	2·3	NS	NS	NS
Jejunum	5·3[B]	5·8[B]	8·8	2·4	NS	NS	NS
Ileum	6·9[B]	8·2[B]	13·9	2·5	NS	NS	NS
Colon	21·2[A]	15·3[A]	10·4	5·5	NS	0·05	NS
Total intestine	10·2	9·7	12·1	1·9	NS	NS	NS

Calves of GrC_{max} were fed colostrum of first milking during 7 days, calves of GrC_{1-3} were fed colostrum for 3 days and those of GrF_{1-3} a milk-based formula (with similar nutrient contents as colostrum) for 3 days and then a milk replacer (whose nutrient contents were similar to that in mature milk) up to day 7. Calves were killed on day 8.
[A,B,C]Within a column means of IGF-IR, IGF-IIR and IR with different capital superscript letters are significantly different (P<0·05). NS, not significant (P>0·05).

Discussion

Comparisons and associations between mRNA levels and B_{max} of IGF-I, IGF-II and insulin receptors

That overall expression levels of IGF-IR and IR mRNA were higher than those of IGF-IIR confirms our previous data in the ileum (Pfaffl *et al.* 2002). In agreement with other studies (Young *et al.* 1990, Heinz-Erian *et al.* 1991, MacDonald 1999, Hammon & Blum 2002), binding capacities of IGF-IIR were consistently higher than those of IGF-IR and IR. As B_{max} values were highest and mRNA expression values were lowest for IGF-IIR, the ratios of B_{max}/mRNA were much higher in IGF-IIR than in IGF-IR and IR.

Whereas correlations between IGF-IR B_{max} and mRNA were low, there was an overall negative correlation between IR mRNA and B_{max}, but an overall positive correlation between IGF-IIR B_{max} and mRNA. Obviously, differences in B_{max} of IGF-IR, IGF-IIR and IR were the consequence of differences in posttranslational control and of receptor turnover rates.

Because affinities of binding for IGF-I, IGF-II and insulin were not different between intestinal sites and at different postnatal stages (data not shown), differences in B_{max} were the result of different receptor numbers.

IGF-IR, IGF-IIR and IR concentrations at the mRNA and protein level at different intestinal sites

Studies on site-specific and on postnatal variations of intestinal IGF-IR, IGF-IIR and IR have been reported in pigs, rabbits and rats (Schober *et al.* 1990, Young *et al.* 1990, Nowak *et al.* 1996, Staley *et al.* 1998). Differences in IGF and insulin receptor numbers at different intestinal sites as found in our calves have been reported in rats (Heinz-Erian *et al.* 1991, Buts *et al.* 1997, Fernandez-Moreno *et al.* 1997) and are consistent with our previous studies in calves (Baumrucker *et al.* 1994, Hammon & Blum 2002). However, differences in B_{max} in intestinal sections were not accompanied by corresponding differences in mRNA expression.

To the best of our knowledge this is the first study that demonstrates differences in intestinal IGF-IR, IGF-IIR and IR expressions at different intestinal sites in calves. Based on our data, receptor-mediated effects of IGF-I, IGF-II, and insulin are, therefore, likely to be region-specific.

Table 6 Binding capacities (B_{max}; fmol/mg protein) of IGF-I, IGF-II and insulin receptors in intestinal mucosa of differently fed calves (experiment 2). Results are expressed as means and pooled S.E.M.

	Group				Group differences (P values)		
	GrC_{max}	GrC_{1-3}	GrF_{1-3}	Pooled S.E.M.	GrC_{max} vs GrC_{1-3}	GrC_{max} vs GrF_{1-3}	GrC_{1-3} vs GrF_{1-3}
IGF-IR							
Duodenum	11·1	9·3[AB]	9·0[A]	1·5	NS	NS	NS
Jejunum	10·3	7·6[B]	5·0[B]	1	NS	0·01	NS
Ileum	11	10·7[AB]	11·7[A]	1·3	NS	NS	NS
Colon	13·2	12·0[A]	9·8[A]	1·9	NS	NS	NS
Total intestine	11·2	9·7	8·7	0·8	NS	NS	NS
IGF-IIR							
Duodenum	63·5[A]	56·1[A]	48·9[A]	12·8	NS	NS	NS
Jejunum	52·9[A]	63·4[A]	39·2[AB]	13·4	NS	NS	NS
Ileum	21·4[B]	18·8[B]	23·3[B]	2·8	NS	NS	NS
Colon	66·8[A]	63·9[A]	53·4[A]	12	NS	NS	NS
Total intestine	50·6	50·6	41·0	6·1	NS	NS	NS
IR							
Duodenum	6·8[B]	5·0[B]	4·3[B]	0·7	NS	NS	NS
Jejunum	6·6[B]	4·1[B]	1·8[B]	0·9	NS	NS	NS
Ileum	20·9[A]	21·6[A]	20·3[A]	2·7	NS	NS	NS
Colon	7·6[B]	3·1[B]	4·8[B]	1·3	NS	NS	NS
Total intestine	10·7	8·5	7·8	1·6	NS	NS	NS

[A,B]Within a column means of IGF-IR, IGF-IIR and IR with different capital superscript letters are significantly different ($P<0.05$). NS, not significant.
For further details see footnote to Table 3.

Additional investigations are needed to localize receptors within the intestinal wall. In species other than calves IGF-IIR are mainly concentrated in the intestinal epithelial cell layer, whereas IGF-IR are mostly present at the serosal surface (MacDonald 1999) and there is a high density of IGF-IR, IGF-IIR and IR in crypt cells (Laburthe *et al.* 1988, Buts *et al.* 1997, Jehle *et al.* 1999, MacDonald 1999).

IGF-IR, IGF-IIR and IR concentrations at the mRNA and protein level immediately after birth in pre-term as compared with full-term calves

There were significant differences between GrP and GrN groups with respect to receptor expression only for the IGF-II receptor, whereas binding capacities differed with respect to all three receptor types. This supports the view that posttranslational regulation of IGF-I, IGF-II and insulin receptors is more important than regulation at the expression level. However, significant differences of B_{max} were not seen at all intestinal sites, indicating that the regulation was site-specific. The fact that for IGF-IR the only significant differences in B_{max} between pre-term and full-term newborn calves were seen in the colon was surprising because IGF-I is a ubiquitous growth factor, as

shown in calves (Cordano *et al.* 1998) and has widespread effects (Kimble *et al.* 1999).

Changes of IGF-IR, IGF-IIR and IR at the protein and mRNA level change within the first days of life in full-term neonatal calves

This study is probably the first to demonstrate changes of intestinal IGF-IIR mRNA levels in calves during the first week of life. In pigs, ontogenetic changes of IGF-IIR as well as of IGF-IR expression have been described in tissues other than the intestine (Peng *et al.* 1996). Furthermore, there were age-dependent changes in B_{max} of IGF-IIR and IR in calves from 2 weeks before normal term up to day 8 of life. While B_{max} of IGF-IIR decreased after birth, B_{max} of IR increased, whereas B_{max} values of IGF-IR (except in the colon) were not significantly different during the first week of life. Thus, these receptors exhibited ontogenetic differences. Because IGF-IR and IR numbers in piglets and rabbits were highest at birth and then declined for 3–6 days (Schober *et al.* 1990, Nowak *et al.* 1996), there are obviously species differences with respect to the ontogenetic behavior of B_{max} of IGF-IR and IR. The general decrease in B_{max} of IGF-IIR possibly reflected a decreasing postnatal role of IGF-II as shown in
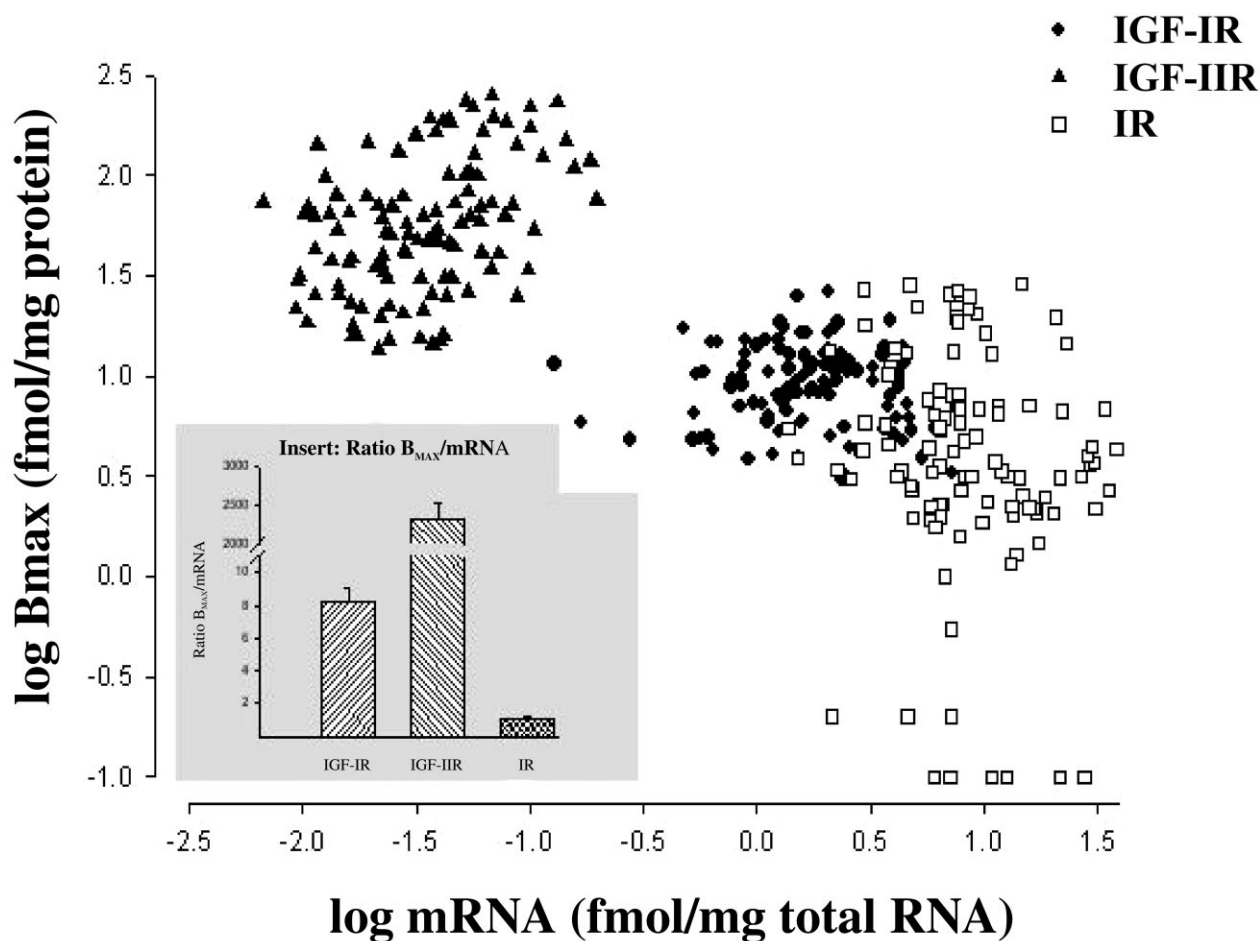
Figure 1 Binding capacities (B_{max}) for insulin-like growth factor-I (IGF-IR), insulin-like growth factor-II (IGF-IIR) and insulin (IR) receptors associated with mRNA levels of IGF-IR, IGF-IIR and IR. Insert: ratios of B_{max}/mRNA for IGF-IR, IGF-IIR and IR in intestinal tissues.

rats (Nissley *et al.* 1993, Breier *et al.* 2000). The overall rise with age of B_{max} of IR in the small intestine, which was especially marked in the ileum from day 1 to day 8 of life, suggests involvement of IR in small intestine development (Nowak *et al.* 1996, Odle *et al.* 1996, Buts *et al.* 1997) and, in calves, especially in the ileum (Hammon & Blum 2002).

Feeding effects on IGF-IR, IGF-IIR and IR mRNA levels and B_{max} in full-term neonatal calves

This study could not demonstrate significant effects of differences in feeding during the first 7 days of life on intestinal expressions and on B_{max} of IGF–IR, IGF–IIR and IR. However, the data showed a trend for reduced B_{max} of IGF–IR, IGF–IIR and IR if calves were fed the formula rather than colostrum for 3 or 7 days. At first view this differs from our previous studies in which significant feeding effects on intestinal IGF-I, IGF-II and insulin receptor numbers have been described in neonatal calves

(Baumrucker *et al.* 1994, Hammon & Blum 2002). Because the formula fed in the present study contained much higher amounts of protein and fat than the milk replacer fed in our previous study (Hammon & Blum 2002), one explanation for the discrepancies between the earlier and the present experiments might have been differences in protein and energy intakes and, hence, probably the status of circulating IGF and insulin concentrations and possibly tissue levels of IGF and insulin receptors.

Despite marked differences in the ingestion of bioactive substances, such as IGF-I and insulin (and probably also of many other growth factors and hormones; Blum & Baumrucker 2002), effects on B_{max} of IGF-IR, IGF-IIR and IR were weak. Therefore, the effects of orally ingested IGFs are variable because orally administered IGF-I for 7 days up-regulated intestinal IGF-IR numbers in neonatal calves (Baumrucker *et al.* 1994), whereas IGF-I administration down-regulated IGF-IR numbers in rats (Simmons *et al.* 1995).

Correlations between IGF-IR, IGF-IIR and IR with histomorphometrical traits and with proliferation rates of intestinal epithelium

Data on intestinal histomorphological traits and of proliferation rates of enterocytes in calves investigated in the present study were published elsewhere (Blättler *et al.* 2001) or have been submitted for publication (Bittrich, H M Hammon and J W Blum, unpublished observations). Significant correlations of B_{max} of IGF-IR, IGF-IIR and IR with villus circumference, villus height/crypt depth or with proliferation rates of crypt cells at various intestinal sites indicate that receptor densities were associated with GIT growth. Coefficients of correlation of B_{max} of IGF-IIR with villus circumference, villus height/crypt depth and proliferation rates were particularly high, suggesting that IGF-II and IGF-IIR may be of special importance for some intestinal sites in neonatal calves. Interestingly, IGF-IR and IR were positively correlated, whereas IGF-IIR was negatively correlated with crypt cell proliferation rates. Negative correlations of IGF-IIR with crypt cell proliferation rates, but positive correlations of IGF-IIR with villus circumference and with villus height/crypt depth suggest that crypt cells and mature villus epithelial cells respond differently to IGFs. The IGF-II receptor serves IGF-II turnover and removal and thus may be involved in growth inhibition (Ludwig *et al.* 1996, Wolf *et al.* 1998, Nakae *et al.* 2001, Butler & LeRoith 2001). Based on these facts, the hypothesis may be advanced that effects of IGF-II on crypt cells through IGF-IIR would be reduced while the stimulation of crypt cell proliferation by IGFs through the IGF-IR and IR would be maintained because growth stimulating effects by IGF-I and IGF-II have been shown to be completely mediated through and by IGF-IR, and to a lesser extent through the IR (Ludwig *et al.* 1996, Rother & Accili 2000, Nakae *et al.* 2001). On the other hand, negative effects of IGF-II, mediated by the IGF-II receptor, on the proliferation of mature villus epithelial cells can be expected.

Conclusions

This study provides evidence that there are differences in IGF-IR, IGF-IIR and IR expression and B_{max} at different intestinal sites and that these may be variably affected by age. IGF-IIR numbers and IGF-IIR mRNA levels were predominant in prenatal life and birth and then decreased, whereas B_{max} of IR numbers increased in early postnatal life (especially in ileum). Ontogenetic variations of IGF-IR (except for colon), IGF-IR mRNA and IR mRNA were not pronounced. The data suggest that physiological effects of IGF-I, IGF-II and insulin on gut growth and function are variable in different intestinal regions and during different developmental periods. Marked differences in amounts of ingested colostrum or even the lack of colostrum intake for 7 days barely affect the expression and B_{max} of IGF-IR, IGF-IIR and IR.

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