ELSEVIER

Characterization of IGF-I receptors in the porcine small intestine during postnatal development

Catherine J. Morgan,† Alistair G.P. Coutts,* Morag C. McFadyen,* Timothy P. King,* and Denise Kelly*

**Rowett Research Institute, Bucksburn, Aberdeen, UK, and †Joslin Diabetes Centre, Boston, USA*

Receptors for insulin-like growth factor I were characterized on intestinal membranes of jejunum from newborn, suckling, and weaned pigs. ^{125}I-labelled insulin-like growth factor I (IGF-I) binding was time-dependent, saturable, linearly related to membrane protein, and reversible. Analysis of saturation curve data from membrane homogenates revealed a single class of receptors, with receptor number declining during development. In vitro autoradiography localized receptors to the small intestinal submucosa and mucosa and further immunohistochemical localization identified receptors on the apical (microvillar membrane) and basolateral surfaces of enterocytes. After affinity cross-linking of ^{125}I-labelled IGF-I to microvillar membranes, 135 and 260 kilodalton bands were visualized on polyacrylamide gels under reducing conditions. Parallel incubations with excess unlabeled IGF-I, -II, or insulin demonstrated that these bands correspond to the α subunit and an incompletely reduced dimeric α complex of the IGF-I receptor. Receptor number on microvillar membranes increased dramatically during the suckling period and was negatively correlated with maltase and sucrase activities. The functional activity of receptors on microvillar membranes was demonstrated using an in vitro phosphorylation assay. The expression of functional IGF-I receptors during development on both apical and basolateral membranes suggests a regulatory role for this receptor and its ligands. (J. Nutr. Biochem. 7:339–347, 1996.)

Keywords: IGF-I; receptors; intestine; microvillar; phosphorylation; neonatal

Introduction

Insulin-like growth factors (IGF-I and IGF-II) are single-chain polypeptides with structural homology to proinsulin and to a lesser extent relaxin.[1] Their biological actions include the regulation of proliferation and differentiation and the induction of insulin-like metabolic actions. The type I receptor (IGF-I receptor) mediates the majority of these biological actions and is present in a wide variety of tissues. IGF-I receptors have been localized in the gastrointestinal tract of a number of species[2–5] and vary according to anatomical position and developmental stage. IGFs can act via endocrine, paracrine, or autocrine mechanisms; however, in the intestine there is also potential for an additional luminal route of action. This mechanism assumes greatest significance in the neonatal intestine, as IGFs are supplied in abundance in maternal colostrum and milk.[6,7]

Address reprint requests to Denise Kelly at Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB2 9SB.
Received October 30, 1995; accepted April 4, 1996.

The ability of growth factors to resist degradation and reach the intestine in a biologically active form has been demonstrated in a number of studies.[8–10] In the case of IGF-I, 40% of orally administered ^{125}I IGF-I could be detected in the gastrointestinal tract of suckling rats[11]; the recovered peptide exhibited chromatographic and receptor binding characteristics equivalent to the native material. Similar observations were described by Baumrucker et al. who also demonstrated the transport of significant amounts of orally administered ^{125}I IGF-I into the circulation of calves,[12] and enhanced DNA synthesis in jejunal and ileal intestinal explants following exposure to IGF-I supplemented artificial milk replacer.[13]

For IGFs to exert a biological effect they must interact with specific membrane bound receptors. The presence of an apical population of intestinal IGF-I receptors is a prerequisite for the biological action of luminal peptide. Receptors for IGF-I have been identified on apical epithelial membranes of the human colon but not as yet in the mucosa of the porcine small intestine.

In this study the expression of specific IGF-I receptors in

Nutritional Biochemistry 7:339–347, 1996

655 Avenue of the Americas, New York, NY 10010

0955-2863/96/$15.00
PII S0955-2863(96)00054-X

the mucosa of jejunum during the development of neonatal piglets was investigated, with particular reference to the distribution of receptors in the intestinal epithelium. In addition the functionality of the receptor was demonstrated using an in vitro phosphorylation assay.

Methods and materials

Materials

All reagents were purchased from Sigma unless otherwise indicated.

Experimental animals

Animals were obtained from a commercial pig herd reared in a high health/minimal disease unit at the Rowett Research Institute (Bucksburn, Aberdeen, Scotland, UK) and were a pure-bred cross of Cotswold (Large White X Landrace) sows and sires. Three developmental ages were studied: newborn, 1 to 1.5 kg (unsuckled animals); suckled, 2-week-old piglets 4–5 kg (suckled without access to solid feed); weaned, 4-week-old piglets 6–7 kg (weaned at 3 weeks). The weaned piglets were fed ad libitum (approximately 600 g/day at week four a cereal-based weaner diet comprising (gkg^{-1}) barley (200.0), wheat (199.0), wheat flakes (150.0), oat flakes (100.0), white fish meal (100.0), soybean meal (110.0), supersoy (50.0), whey (50.0), soybean oil (21.0), vitamin, mineral, and trace element mix (20.0) (Norvite, Aberdeen, UK). At least five pigs, from different litters, were sampled for each age group. Animals were fasted for 3 hr before surgery undertaken between 11 a.m. and 1 p.m. A midline laparotomy was performed under anaesthesia induced by halothane and oxygen inhalation. The small intestine was sampled at the mid jejunum (midpoint between the pylorus and the ileocecal valve) and tissues were processed immediately for the preparation of mucosal homogenates and brush border membranes. For autoradiography and immunohistochemistry, 1 cm lengths of tissue were mounted in mounting compound (OCT; Miles Inc., Elkhart, USA) and snap frozen in isopentane cooled with liquid nitrogen.

Membrane preparation

For the preparation of mucosal homogenates, segments of jejunum were opened longitudinally and 300–350 mg of mucosa (wet weight) removed by scraping with a metal spatula. Mucosa was homogenized in 300 μL of 2 mM Tris-HCl, 50 mM mannitol buffer, pH 7.4 containing 0.1 mM phenylmethylsulfonylfluoride (PMSF), 25 μg/mL soybean trypsin inhibitor and 1 μg/mL leupeptin at 4°C with an Ultraturrax homogeniser for 20 sec at medium speed. Samples were centrifuged at 800 g for 5 min at 4°C, the supernatant was removed and stored at −20°C. Microvillar membranes were purified from mucosal membranes using a Mg^{2+} precipitation protocol as described by Kessler et al.[15] The correct orientation of microvillar membranes was confirmed by scanning electron microscopy and the 13-fold enrichment of the microvillar enzyme lactase relative to homogenates was consistent with the purification reported by Kessler et al.[15] For binding assays, the protein content was determined using the bicinchoninic acid method,[16] and the mucosal homogenates and microvillar membranes were diluted with binding assay buffer (see below) to the appropriate protein concentration and homogenized in a glass/teflon homogenizer.

Enzyme assays

Enzyme determinations were carried out on both mucosal homogenate and microvillar membrane preparations. The substrate concentrations and incubation conditions for determination of sucrase (EC 3.2.1.48) and maltase (EC 3.2.1.20) were as described by Kidder and Manners[17] and Kelly et al.[18] The glucose liberated by the action of mucosal enzymes was determined using the glucose-6-phosphate dehydrogenase (EC 1.1.1.49)-hexokinase (EC 2.7.1.1) assay (Boehringer Mannheim, Lewes, UK).

Iodination of IGF-I

Human recombinant IGF-I (Bachem UK Ltd, Saffron Walden, Essex, UK) was iodinated using bovine lactoperoxidase as the oxidizing agent. This protocol is a modification of the method described by Roth (1975).[19] To an aliquot of IGF-I (2.5 μg in 0.1 M acetic acid) 20 μL of 0.05 M sodium phosphate, pH 7.4, was added. The following reagents were then added as listed; 10 μL 0.4 M sodium acetate, pH 5.6, 5 μL $Na^{125}I$ (ICN, Paisley, Scotland), 1 μL bovine lactoperoxidase dissolved in 0.1 M sodium acetate, pH 5.6. The reaction was timed for 30 sec from the addition of 1 μL hydrogen peroxide (diluted 1:15,000) until the final addition of 100 μL 1% potassium iodide solution. The reaction mixture was then transferred to a C18 column (Bond Elut; Varian SPP, Cambridge, UK) and eluted with a series of 2 mL aliquots of 0.1% trifluoroacetic acid (TFA)/acetonitrile (ACN) increasing from 0 to 70% ACN in 10% steps. Following this initial separation of iodinated IGF-I from free iodine, the fractions eluted with 30 to 40% ACN were concentrated in a Speedvac concentrator (Savant Instruments, Life Sciences, Basingstoke, UK) and then separated by reverse-phase high performance liquid chromatography (HPLC) employing a wide-pore C8 column (Aquapore; Bownlee Labs, Applied Biosystems, Santa Clara, USA). The sample was eluted at a flow rate of 1 mL/min, the aqueous phase being 0.1% TFA (sequencing grade, Rathburn Chemicals, Peebles) in double distilled water and ACN (Far UV grade, Fisons, Norlab, Aberdeen, UK) the organic solvent. The chromatograph was developed in a linear gradient of 25 to 75% ACN over 30 min, IGF-I was eluted after 10 min as a discrete absorbance peak at 214 nm, which was collected in BSA-coated tubes and quantified by measuring the area under the peak and by reference to standards. The specific activity of labelled IGF-I was calculated from the number of counts associated with the mass of ^{125}I IGF-I eluted from the column. For binding assays the ^{125}I IGF-I was diluted from a specific activity of approximately 2,000 cpm/fmol to 400 to 500 cpm/fmol with unlabeled IGF-I.

Dissociation of endogenous ligand

Endogenously bound IGF-I was dissociated from its receptor using the high salt wash procedure described by Kelly et al.[20] for the prolactin receptor.

Assessment of proteolytic stability of IGF-I

The stability of IGF-I was determined using a modification of the protocol described by Britton et al.[10] Intestinal homogenates were incubated in the presence of ^{125}I IGF-I for 1 hr at 37°C. The incubation mixture consisted of 150 μg of tissue protein and 100 ng of IGF-I (associated with 50,000 cpm) in a final volume of 150 μL of 0.01 M Tris buffer containing 6.67 mM $CaCl_2$. To terminate the incubation, 150 μL of 0.1 M sodium acetate containing 0.1% soybean trypsin inhibitor was added and the reaction tube placed on ice. For the determination of immunoreactive IGF-I, 20 μL of the quenched incubation solution was added to the 0.5 mL suspension of anti-IGF-I antiserum conjugated to protein A-Sepharose, and the suspension was then incubated at 4°C for 18 hr. Unbound non-immunoreactive IGF-I was eluted with a series of 10 × 0.5 mL aliquots of 0.01 M Tris-HCl pH 7.4 containing 0.5% BSA, 0.05% bacitracin, 1 mM PMSF, 0.001% aprotinin and

0.005% soybean trypsin inhibitor. The eluates were then collected and counted on a gamma counter. The percentage of degraded peptide was determined as (eluted cpm/total cpm) × 100.

Autoradiographical localization

Transverse cryostat sections of 20 μm thickness were cut from frozen jejunal samples and thaw-mounted onto gelatin-coated slides. Sections were dried at 4°C for 2 hr before being incubated in either 50 pM ^{125}I IGF-I or 50 pM ^{125}I IGF-I in the presence of excess IGF-I (50 nM) to determine total and non-specific binding respectively. All solutions were made up in binding assay buffer, (Buffer 1). For incubations, sections were circled with a water-resistant marker to form a well, into which 200 μL of solution was pipetted. Slides were incubated in humidified chambers at 4°C for 20 hr. To terminate the reaction, the incubation medium was removed and slides washed at 4°C with 5 × 3 min washes of wash buffer 2 (buffer 2 – buffer 1 minus aprotinin and soybean trypsin inhibitor). Sections were air-dried for 2 hr and then fixed in paraformaldehyde vapour at 80°C under reduced pressure. Localization was determined by exposure of the fixed sections to Kodak X-Omat AR film.

Immunohistochemical localization

Transverse 5 μm thick cryostat sections were thaw-mounted onto gelatin-coated slides and air-dried for 1 hr at room temperature. The sections were fixed for 15 min at 4°C in acetone and washed in 0.05 M Tris buffered saline (TBS) pH 7.2. The sections were incubated for 30 min at 37°C with 20 μg/mL BSA-c (Aurion, Wageningen, The Netherlands) in TBS. The sections were incubated for 1.5 hr at 37°C in 1/25 dilution of ascites fluid from clone 17/69, which produces antibodies recognizing the IGF-I receptor.[21] The ascites fluid was diluted in BSA-c TBS. Control sections were incubated in similarly diluted control ascites fluid. All sections were washed in six changes of TBS over a 30 min period prior to incubation for 1 hr at 37°C with a 1/75 dilution of an FITC-conjugated goat anti-mouse IgG (Fc-specific) (Sigma, UK). Sections were then washed in several changes of TBS, mounted in "Vectorshield" antifading mountant (Vector, UK), and examined by incident light fluorescence microscopy on Zeiss Axioscope microscope. Results were recorded on Kodak TMax black and white film.

Receptor binding assays

Assays of receptor binding were carried out on homogenates and purified microvillar membranes from jejunal mucosa. Samples were assayed in triplicate and all procedures were carried out on ice. Each tube contained a final volume of 150 μL, additions being made in the following order; 25 μL binding assay buffer (Buffer 1), 50 μL of either displacing ligand (1 μM) IGF-I or binding assay buffer, 50 μL ^{125}I IGF-I (varying concentration) and 25 μL tissue preparation containing 100 μg protein. Tubes were incubated at 37°C for 1 hr and the reaction terminated by the addition of 1 mL of ice cold Buffer 2. Samples were then centrifuged at 35,000 g for 10 min at 4°C to separate bound and free radioactivity. The supernatant was decanted and tubes drained to remove any excess fluid before being counted in a gamma counter (83% efficiency). Specific binding was calculated as the difference between total and non-specific binding (i.e., binding in the presence of excess cold ligand). Data analysis was carried out using the ENZFITTER computer program.[22]

Competition studies

To determine the specificity of the receptor competition studies were carried out on jejunal mucosa homogenate and microvillar membrane (100 μg protein) in a final volume of 150 μL. Samples were incubated with 0.2 nM ^{125}I IGF-I (50,000 cpm) and increasing concentrations of IGF-I, IGF-II (Bachem UK Ltd, Saffron Walden, Essex, UK), insulin, and EGF (Bachem UK Ltd, Saffron Walden, Essex, UK) ie., Log −5.0 to −11.0 M. The assay was carried out on tissue from three animals and performed on three different occasions.

Affinity labeling of IGF receptors

^{125}I IGF-I was cross-linked to mucosal IGF receptors following previously described methods[5,23] using microvillar membranes (500 μg protein) incubated at 4°C for 24 hr with 0.5 nM ^{125}I IGF-I alone or with −7 and −9 log M concentrations of IGF-I, IGF-II, and insulin. Disuccinimidyl suberate (Pierce Warriner, Chester, UK) was added to give a final concentration of 0.1 mM. Samples were incubated for 20 min at 4°C and the reaction terminated by the addition of 1 mL 10 mM Tris, 1 mM EDTA, pH 7.5. Electrophoresis of membrane proteins was carried out in 7.5% SDS polyacrylamide slab gels, using the molecular weight standards carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase B (97,400), β galactosidase (116,000), myosin (205,000). Dried gels were placed against Kodak X-Omat AR film for 2 weeks at −70°C.

Receptor autophosphorylation

The phosphorylation assay was based on the methods of Carpenter et al.[24] and Hayes and Lockwood[25] with modifications. Receptor preparations (100 μg protein) were incubated with 400 nM IGF-I in a final volume of 50 μL of buffer containing 20 mM Hepes, 5 mM $MnCl_2$, 4 mM NaF, 100 μM Na_3VO_4, 10 mM β-glycerophosphate 1 μg/mL leupeptin, 25 μg/mL trypsin inhibitor, 25 KIU/mL Aprotinin, 0.1 M PMSF, 0.05% bacitracin for 10 min at room temperature and then 15 min on ice. Phosphorylation was initiated by the addition of 20 μM [γ-^{32}P]ATP (20 μCi). The reaction was terminated after 15 min at 4°C using 3X Laemmli buffer. Phosphorylated microvillar membrane proteins were analyzed on 7.5% SDS polyacrylamide gels under reducing conditions. Autoradiograms of dried gels were obtained with X-Omat AR film after exposure for 48 hr at −70°C.

Statistical analysis

The results are presented as arithmetic means with standard errors of the mean (SEM) and were evaluated using a Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's test for multiple comparisons. P values < 0.05 were considered to be statistically significant.

Results

Autoradiographical and immunohistochemical localization

Transverse sections of jejunum were incubated in a solution containing ^{125}I IGF-I with or without an excess of unlabeled IGF-I to determine total and non-specific binding, respectively. Specific binding of ^{125}I IGF-I was evident in the jejunum at all three developmental stages, with a decline in binding between birth and weaning (*Figure 1*). The strongest binding was seen in the muscle and submucosal layers of the intestine and specific binding was also localized to the mucosa. As shown immunohistochemically (*Figure 2a*) IGF-I receptors were present on the epithelium of both the crypt and villus regions. Specific labeling was identified

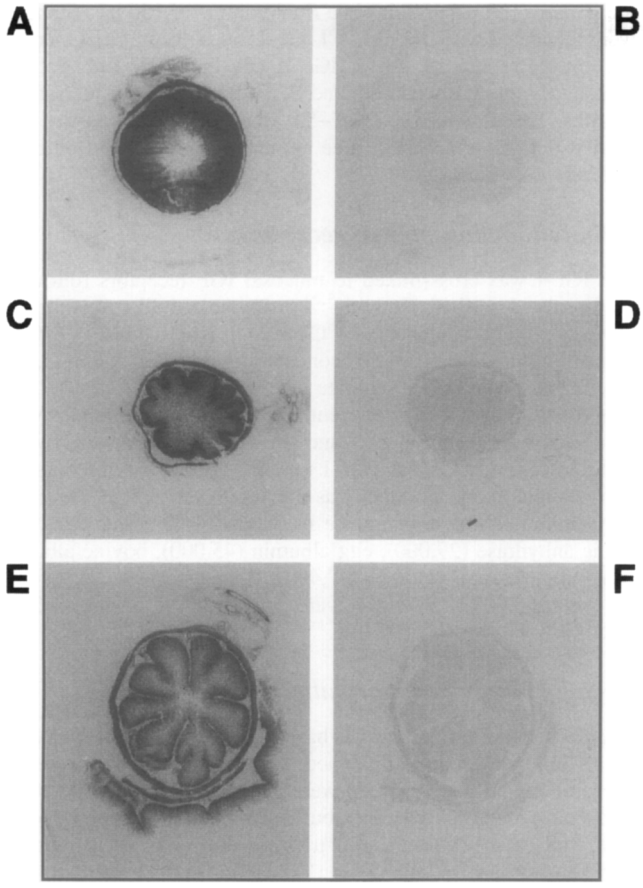

Figure 1 Dry-film autoradiographic images of ^{125}I-labelled insulin-like growth factor (^{125}I IGF-I) binding to transverse jejunal sections from (A) newborn, (C) sucking, and (E) weaned pigs. Non-specific binding (determined in the presence of 50 nM IGF-I) to (B) newborn, (D) sucking, and (F) weaned pigs.

over the apical microvillar membranes, the cytoplasm and basolateral membranes of enterocytes (*Figure 2b, c*). Low levels of non-specific labeling were present on sections incubated with control ascites fluid (anti-glucose oxidase antibody) (*Figure 2d*).

Proteolytic stability of IGF-I

Measurement of immunoreactive IGF-I after incubation with intestinal homogenates from animals at different developmental ages demonstrated minimal degradation of IGF-I. A maximum of 10% of ^{125}I IGF-I was degraded after an incubation period of 60 min at 37°C.

Receptor characterization

Saturable binding of IGF-I to the receptor was reversible, time and temperature-dependent, and increased in a linear fashion with increasing tissue protein concentration. Optimum binding conditions were determined using mucosal homogenates of jejunum from newborn animals. For analysis of time- and temperature-dependence, 100 μg of tissue protein was incubated with 0.5 nM ^{125}I IGF-I at 4°C and 37°C. Saturation binding was achieved at a temperature of 4°C after 18 hr and at 37°C after 45 min (*Figure 3*). The

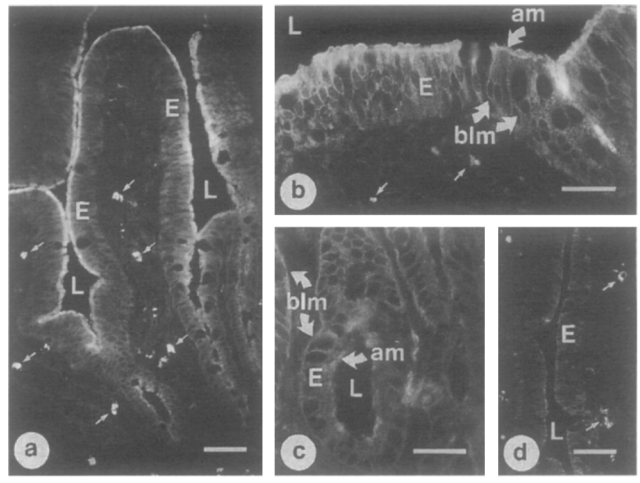

Figure 2 (a) Light micrograph at low magnification showing immunofluorescent labeling of IGF-I receptors on the intestinal villus epithelium (E) of suckled pig jejunum. (b) Higher magnification micrograph illustrating IGF-I receptor localisation to apical (am) and basolateral membranes (blm) and the cytoplasm of enterocytes. (c) Higher magnification micrograph illustrating IGF-I receptor localization to apical and basolateral membranes of crypt epithelial cells (d) labeling of a consecutive section of jejunum incubated with control ascites. (a) and (d) scale bar = 50 μm. (b) and (c) scale bar = 25 μm. (L) indicates the lumen of the gut. Small arrows depict non-specific fluorescence associated with some lamina propria cells.

highest specific binding was obtained at 4°C after 24 hr incubation; however there were no significant differences between the maximum specific binding obtained for the two different temperatures. On the basis of these findings and because 37°C is the most physiologically relevant temperature, all further incubations were carried out for 1 hr at 37°C.

The tissue linearity of specific binding was demonstrated by incubating increasing concentrations of homogenate pro-

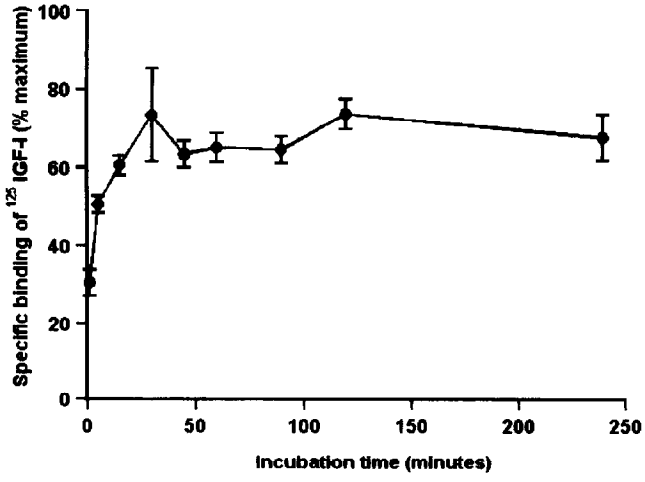

Figure 3 Time course of specific binding of ^{125}I-labeled insulin-like growth factor (^{125}I IGF-I) (0.5 nM) to newborn pig mucosal homogenates (100 μg protein) at 37°C. Data represent means ± SEM of three determinations expressed as a percentage of maximal binding.

tein (0–200 μg) with 0.5 nM ^{125}I IGF-I. Specific binding increased linearly to a tissue protein content of 200 μg/150 μL (*Figure 4*).

To determine the affinity and number of receptors present in jejunal mucosa at different stages of development, homogenates and microvillar membranes from newborn, suckled, and weaned animals were incubated with increasing concentrations of ^{125}I IGF-I (0.1 to 15 nM). The saturation curves and Scatchard plots of homogenates (*Figure 5*) show a developmental decline in receptor number, with newborn animals having significantly higher numbers of receptors than weaned animals ($P < 0.01$), but receptor affinity remaining constant at approximately 1 nM (*Table 1*).

Kd values for IGF-I binding to microvillar membranes were similar to those for intestinal homogenates, although more variable (*Table 2*), whereas Bmax values for microvillar membranes were greater than those for homogenates. The Bmax values for the suckled group were highly variable ranging from 25 to 191 fmol/mg protein and hence the mean for the group was associated with a very large standard deviation. The suckled pigs divided very clearly into two populations one with high receptor number (a) and the other with significantly lower Bmax values (b). The receptor level detected in this high expressing group (a) was approximately threefold higher than either the newborn or weaned groups (*Table 2*) ($P < 0.001$). Receptor levels in group (b) were comparable to or lower than those in weaned piglets. Receptor levels in the two suckling groups were negatively correlated ($P < 0.05$) with sucrase and maltase activities, which were 30.9 ± 10.7 and 114.3 ± 19.3 μmol/min/g protein, respectively in group (a) and 66.5 ± 10.9 and 185.9 ± 10.1 μmol/min/g protein, respectively in group (b).

Receptor occupancy

To assess the potential complication of receptor occupancy by endogenous ligand, a high salt wash procedure was used to dissociate endogenous IGFs from the IGF-I receptor. Using this protocol, 85% of bound ligand could be dissociated from receptors. Prewashed membranes were able to rebind ligand, the affinity of receptors was unaffected by the washing procedure. Comparison of binding to microvillar membranes from newborn and weaned animals, with or without the prewash protocol, demonstrated that prewashing did not significantly increase the number of receptors binding IGF-I. Similarly for suckled pigs, where the potential for receptor occupancy is perhaps greatest, values were 60.4 ± 12.6 fmol/mg protein for unwashed membranes versus 72.8 ± 20.5 fmol/mg protein for washed membranes.

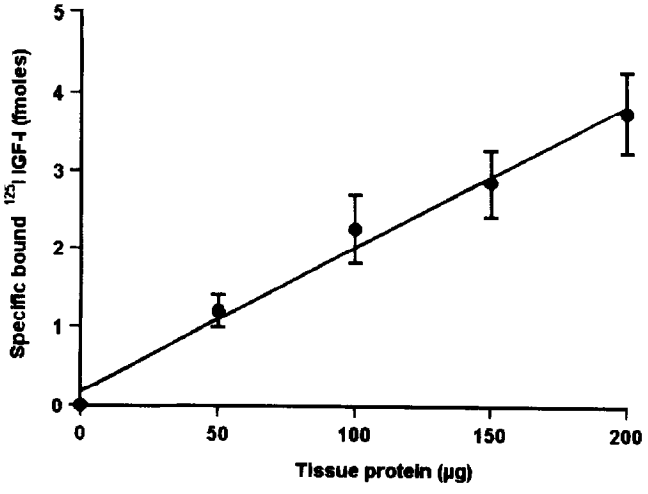

Figure 4 Linear relationship of specific binding of ^{125}I-labeled insulin-like growth factor (^{125}I IGF-I) (0.5 nM) to various concentrations of newborn pig membrane protein at 37°C. Linear regression was performed on the data, which represent means ± SEM of four determinations.

Competition Studies

Both IGF-I and IGF-II inhibited binding of ^{125}I IGF-I to jejunal mucosal homogenates and microvillar membranes, with 50% inhibition of binding occurring at concentrations of 1.05 ± 0.30 nM and 3.36 ± 0.28 nM respectively (*Figure 6*). EGF did not displace ^{125}I IGF-I binding and insulin was only effective at approximately 500 nM. These results confirm the specificity of IGF-I binding. Interference by binding proteins is unlikely since high concentrations of insulin almost completely displaced ^{125}I IGF-I binding.

Affinity labeling of IGF receptors

^{125}I IGF-I was covalently cross-linked to microvillar membrane IGF-I receptors from newborn and suckled pigs and the receptor type identified on SDS-PAGE. Labeled protein complexes were detected at molecular weights of 135,000 and 260,000 (*Figure 7*). The lower of these bands has a molecular mass compatible with the α subunit of the type 1 receptor and the higher to either the monomeric type II receptor or to an unreduced complex of IGF-I receptor subunits. To clarify this, membranes were co-incubated with excess IGF-I, IGF-II, and insulin. Excess IGF-I at −7 log M concentrations resulted in complete inhibition of binding to both complexes. The relative ability of IGF-I, IGF-II, and insulin (at high concentrations) to displace ^{125}IGF-I in both newborn and suckled animals would indicate that binding is predominantly to the IGF-I receptor.

Receptor phosphorylation

IGF-I was found to stimulate the phosphorylation of a number of microvillar membrane proteins in both newborn and suckled pigs (*Figure 8*). In particular, a 97 kD band was phosphorylated that would be consistent with autophosphorylation of the β subunit of the IGF-I receptor. The 97 kD band in the newborn appeared as a doublet, as previously reported for the IGF-I receptor in fetal muscle.[26]

Discussion

Specific binding of IGF-I was detected throughout the postnatal period in the jejunum of piglets. Using in vitro autoradiography it was demonstrated that this binding was localized to various layers of the intestine, with distinct IGF-I binding particularly evident in the muscularis propria and mucosa of the intestine at all three stages of development. These results are consistent with findings reported for the rat[27,28] and the rabbit[3] in which binding was localized to the

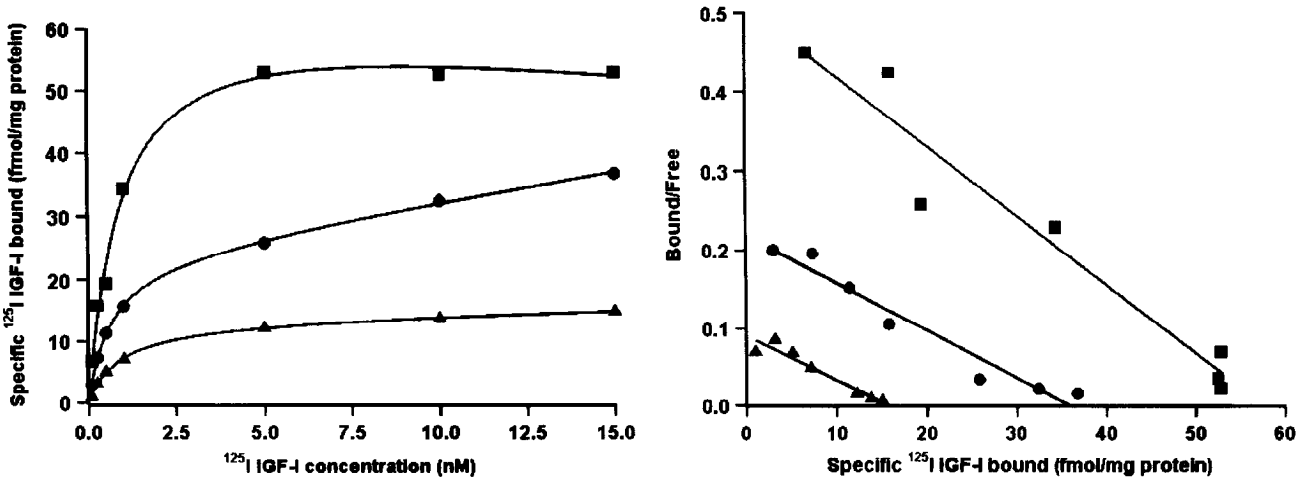

Figure 5 (a) Typical saturation curve showing specific binding of ^{125}I-labeled insulin-like growth factor (^{125}I IGF-I) to ■ newborn, ● suckled and ▲ weaned mucosal homogenates (100 μg protein) with increasing concentrations of ^{125}I IGF-I. Data represents means of triplicate determinations. (b) Scatchard transformation of saturation curve data for ■ newborn, ● suckled, and ▲ weaned pigs showing a single class of binding site.

mucosa and particularly the muscularis mucosal layer. The localization of IGF-I receptors within the epithelium of the intestinal mucosa was further confirmed by immunohistochemistry using a monoclonal antibody against the human IGF-I receptor. Receptors were demonstrated in both the crypt and villus compartments of the intestinal epithelium. This is similar to the distribution of IGF-I receptors described for the rat.[29] The presence of IGF-I receptors in differentiated villus cells suggests a possible role of IGF-I during cellular maturation, similar to that shown for muscle cells where the growth factor induces differentiation of L6 myoblasts.[30] Alternatively, its role may relate to the metabolic function of the cells, since IGF-I receptors have also been shown to mediate insulin-like metabolic effects.[1] IGF-I receptor labeling was also detected in the proliferative crypt compartment of the pig intestine. Similar labeling has also been demonstrated in the rat[2,28] and is consistent with the mitogenic properties of IGF-I.[1]

Immunohistochemistry revealed IGF-I receptors on both apical and basolateral membranes of the intestinal epithelium in the jejunum of the piglet. This bilateral distribution of receptors has not been described previously in the piglet small intestine, although it has been reported in the human proximal colon[14] and the suckling rat jejunum.[27] In the present study apical IGF-I receptors have been identified using several techniques. The characterization was carried out using receptor binding assays, competition binding studies and affinity cross-linking of ^{125}I IGF-I to receptors in purified preparations of microvillar membranes and the functionality was demonstrated using an in vitro receptor phosphorylation assay. Specific apical receptors for IGF-I were identified throughout postnatal development and Scatchard analysis of saturation binding data indicated binding to a single high affinity receptor site. Affinity cross-linking identified two high molecular weight species; a 135 kD species that may correspond to the α subunit of the IGF-I receptor and a 260 kD species, which is likely to correspond to an incompletely reduced dimer of IGF-I receptor α subunits.[23,31] The latter is unlikely to be the monomeric IGF-II receptor, because insulin, at high concentrations was able to displace ^{125}I IGF-I binding.[1] These findings are also supported by the results of competition studies in which the near complete displacement of ^{125}I IGF-I binding could be achieved with insulin at concentrations at 1,000-fold excess. This further confirms that binding was to the IGF-I receptor rather than the IGF-II receptor. The results of both cross-linking and competition studies also suggest that IGF binding proteins were not present at significant levels in the mucosa of the jejunum.

Table 1 Receptor affinity (Kd) and capacity (Bmax) measured in homogenates of jejunal mucosa from newborn, suckled and weaned animals. Means and SEM are shown (n = five to six animals). **denotes significant differences between newborn and weaned animals at the level of $P < 0.01$.

	Newborn	Suckled	Weaned
Kd (nM)	0.96 ± 0.07	1.00 ± 0.11	0.90 ± 0.11
Bmax (fmol/mg protein)	49.9 ± 8.60	28.1 ± 7.41	12.8 ± 1.39**

The number (density) of IGF-I receptors in the piglet intestine declined during postnatal development. Bmax values were lower in the weaned animal than in the unsuckled newborn animal, while receptor affinity remained unchanged at ~1 nM during this period. This is consistent with the developmental decline in IGF-I binding[32,33] and overall steady state receptor mRNA levels[34] in various other tissues. The rate of this decrease has also been shown to be tissue-specific.[34] An altered pattern of developmental decline was observed in microvillar membranes compared to tissue homogenates. In microvillar membranes, although receptor number was decreased in weaned animals compared to newborn animals, a transient increase was observed in suckled animals. A similar pattern of IGF-I receptor expression has been reported previously in crude plasma membranes of suckled rat jejunum.[27] The expression of IGF-II receptors in the muscle and intestine of the rat shows a similar developmental pattern, with a small increase post-

Table 2 IGF-I receptor affinity (Kd) and capacity (Bmax) measured in microvillar membranes prepared from jejunal mucosa of newborn, suckled-a, suckled-b, and weaned animals. Means and SEM are given (n = 5 to 6 animals). *** denotes significant differences between all other unmarked groups at the level of $P < 0.001$

	Newborn	Suckled (a)	Suckled (b)	Weaned
Kd (nM)	1.04 ± 0.43	0.73 ± 0.34	0.67 ± 0.28	0.65 ± 0.08
Bmax (fmol/mg protein)	67.8 ± 7.23	130.1 ± 32.9***	42.0 ± 9.75	48.7 ± 7.31

natally at days 5 and 10 respectively.[35] In the present investigation a striking feature of the suckled group was the diversity of receptor levels; approximately 50% of suckled piglets expressed high receptor numbers, an increase of approximately 3-fold compared to either the newborn or weaned animals. In contrast, in the suckled group expressing low receptor density, the levels frequently fell below that of the other experimental groups. The dichotomy in apical receptor densities within the suckling group seems most likely to reflect dynamic regulation of the IGF-I receptor during this period.

Several factors could contribute to this variation in IGF-I receptor levels in suckling pigs. Firstly, they may have been influenced by differences in energy/nutritional status between the two groups of suckling piglets. IGF-I plasma levels are strongly correlated with the nutritional status of the mature pig[36] and IGF-I receptor numbers are negatively regulated by the ambient concentration of its ligand.[34] In rats, IGF-I receptor number doubled in the intestinal epithelium after decreased food intake, which results in lowered serum IGF-I levels.[37] In the current study, there was no control of food intake in suckling piglets but the similar liveweight gains of the two subgroups of suckling animals suggests that there were no nutritional differences. Moreover, IGF-I plasma concentrations in artificially reared neonatal piglets did not change in response to altered nutrition.[38] This suggests that variation in the nutritional status of suckled animals is unlikely to explain the variation in IGF-I receptor levels observed. Alternatively, IGF-I present in milk could regulate IGF-I receptor expression in the intestinal epithelium. Concentrations of IGF-I and IGF-II in milk decrease during lactation,[6,7] however levels are still at physiologically relevant concentrations and milk intake generally increases during the postnatal period.[39] Thus the overall quantity of IGF-I ingested is likely to remain relatively high over the postnatal period. This exposure of apical IGF-I receptors to physiological concentrations of IGF-I could lead to receptor occupancy, internalization or down-regulation. The possibility that the lower level of receptor expression in 50% of the suckled animals was attributable to receptor occupancy is unlikely because a high salt wash used to displace bound ligand failed to alter the measured values of Bmax in either the high and low expressing suckled groups. However, the possibility of internalization or down-regulation cannot be discounted, and, perhaps significantly, internalization is supported by the immunochemical labeling of the cytoplasm of enterocytes. The transient nature of the increase in IGF-I receptor number may represent a third factor contributing to receptor variation observed in suckling animals. This transient phenomenon has been reported in other tissues. In muscle cells, for example, IGF-I receptor numbers increase during the early stages of differentiation, subsequent to which they decrease to low levels associated with the differentiated cell.[40] The patterns of receptor expression observed in the current study, may suggest that a similar mechanism is operating in the small intestine. Hence, if receptor expression is correlated with dif-

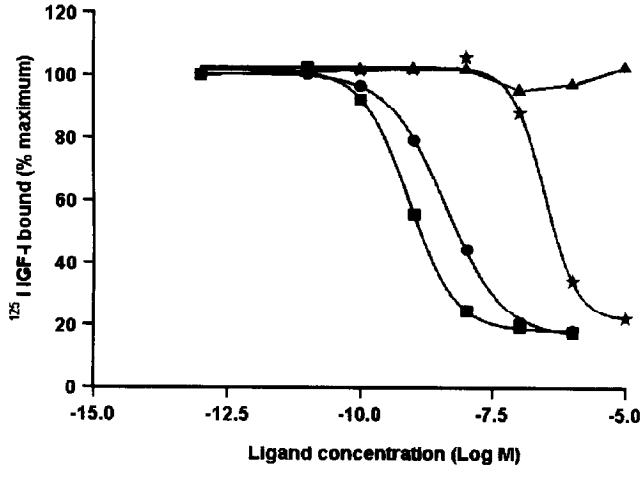

Figure 6 Typical displacement curves for specific binding of ^{125}I-labeled insulin-like growth factor (^{125}I IGF-I) to mucosal homogenates from newborn pigs. Displacement is illustrated for unlabeled IGF-I ■, insulin-like growth factor II ●, insulin *, and epidermal growth factor ▲. Data represents means of triplicate determinations.

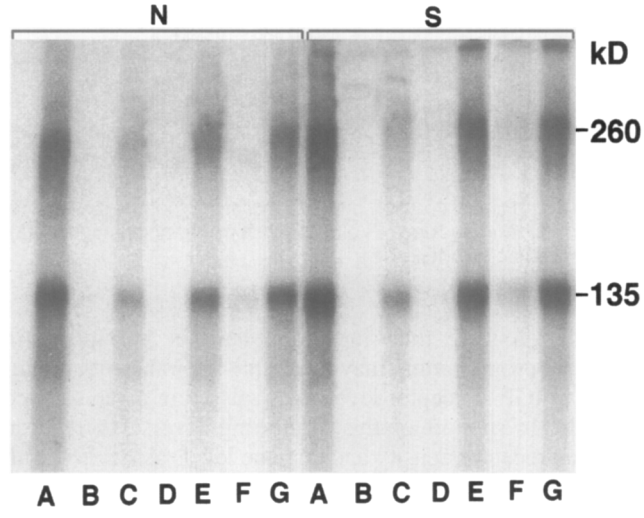

Figure 7 Affinity crosslinking of ^{125}I-labeled insulin-like growth factor (^{125}I IGF-I) to its receptor in microvillar membranes from newborn (N) and suckled (S) pigs. In lanes (A) crosslinking was performed in the absence of unlabeled ligand, and in (B) with Log −7 M IGF-I, (C) with Log −9 IGF-I, (D) with Log −7 IGF-II, (E) with Log −9 IGF-II, (F) with Log −5 M insulin, and (G) with Log −7 M insulin.

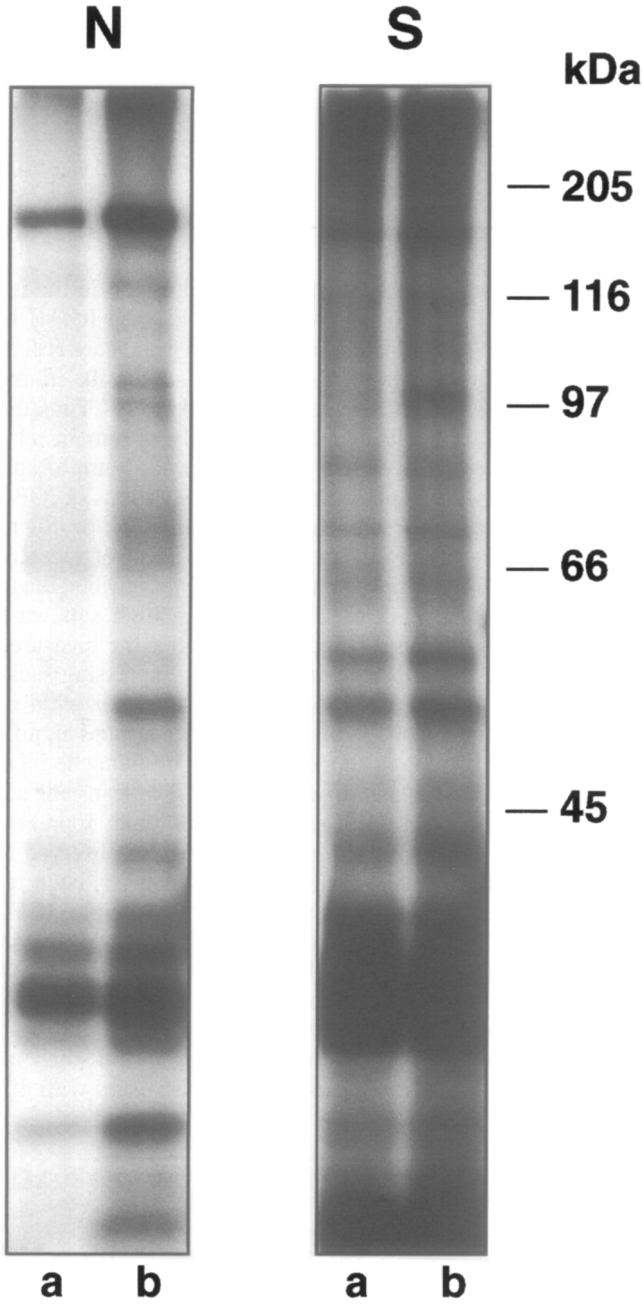

Figure 8 ^{32}P incorporation into microvillar membrane proteins of newborn (N) and suckled (S) pigs in response to no stimulation (a), and stimulation with IGF-I (b).

ferentiation state then individual animal variability in intestinal programming may mean that the transient upregulation in IGF-I receptor levels was missed in some suckled animals. In support of this, a striking inverse correlation between receptor density and enzyme levels associated with the mature differentiated epithelium was noted in suckling animals. However, irrespective of the explanation for the differences between the two suckled groups, it remains that receptor density was significantly upregulated during suckling and attained levels not seen in either the newborn or weaned animals.

Recent work on intestinal enzyme and receptor characteristics of both artifically reared pigs and conventionally-reared pigs revealed dissimilar patterns of expression.[41] In particular, artifically reared pigs expressed significantly lower levels of brush border enzymes and IGF-I receptors. The upregulation of the apical IGF-I receptor characteristic of the conventional animal was not observed in the artifically reared group by 24 days postpartum, raising the possibility that factors present in sow's milk may contribute to the upregulation of the IGF-I receptor. Milk contains a variety of hormones and growth factors that are known to regulate the IGF-I/binding protein/receptor system either directly or indirectly. These include hormones such as prostaglandins, prolactin and growth hormone and growth factors such as bFGF, EGF and PDGF.[1,42,43] Furthermore, in vitro studies using the Caco-2 intestinal cell line have demonstrated that 7- to 14-day sow's milk contains factors that will upregulate the expression of both IGF-I receptor protein and its mRNA.[41]

In conclusion, apical receptors may be involved directly in the mediation of IGF-I and IGF-II effects in the developing intestine. Compatible with this hypothesis are the findings that orally administered IGF-I and IGF-II promotes gut growth and increases the expression of a number of brushborder enzymes in the jejunum of suckling rats and pigs.[27,41,44] The transfer of colostrum/milk-derived IGF-I to the circulation by mechanisms such as IGF-I receptor-mediated endocytosis and transcytosis has also been postulated.[12] However, the consensus viewpoint is that IGF-I is poorly absorbed[11,45] contributing less than 0.05% to the circulating IGF-I pool of a neonatal pig[46] and therefore colostral/milk IGF-I is likely to function locally within the intestine.

Acknowledgments

The authors would like to thank Dr. Maria Soos, Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Cambridge for the monoclonal antibody against the human IGF-I receptor.

This research was supported by the Scottish Office Agriculture and Fisheries Department.

References

1 Jones, J.I. and Clemmons, D.R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocr. Rev.* **16,** 3–34

2 Laburthe, M., Rouyer-Fessard, C., and Gammeltoft, S. (1988). Receptors for insulin-like growth factors I and II in rat gastrointestinal epithelium. *Am. J. Physiol.* **254,** G457–G462

3 Termanini, B., Nardi, R.V., Finan, T.M., Parikh, I., and Korman, L.Y. (1990). Insulin-like growth factor I receptors in rabbit gastrointestinal tract: Characterisation and autoradiographic localization. *Gastroenterology* **99,** 51–60

4 Rouyer-Fessard, C., Gammeltoft, S., and Laburthe, M. (1990). Expression of two types of receptor for insulin-like growth factors in human colonic epithelium. *Gastroenterology* **98,** 703–707

5 Schober, D.A., Simmen, F.A., Hadsell, D.L. and Baumrucker, C.R. (1990). Perinatal expression of type I IGF receptors in porcine small intestine. *Endocrinology* **126,** 1125–1132

6 Simmen, F.A., Simmen, R.C., and Reinhart, G. (1988). Maternal and neonatal somatomedin C/insulin-like growth factor-I (IGF-I) and IGF binding proteins during early lactation in the pig. *Dev. Biol.* **130,** 16–27

7 Donovan, S.M., Klis McNeil, L., Jimenez-Flores, R., and Odle, J.

(1994). Insulin-like growth factors and insulin-like growth factor binding proteins in porcine serum and milk throughout lactation. *Pediatr. Res.* **36,** 159–168

8 Asplund, J.M., Grummer, R.H., and Phillips, P.H. (1962). Absorption of colostral gamma-globulins and insulin by the newborn pig. *J. Anim. Sci.* **21,** 412–413

9 Aloe, L., Calissano, P., and Levi-Montalcini, R. (1982). Effects of oral administration of nerve growth factor and its antiserum on sympathetic ganglia of neonatal mice. *Dev. Brain Res.* **4,** 31–34

10 Britton, J.R., George-Nascimento, C., and Koldovsky, O. (1988). Luminal hydrolysis of recombinant human epidermal growth factor in the rat gastrointestinal tract: segmental and developmental differences. *Life Sci.***43,** 1339–1347

11 Philipps, A.F., Rao, R., Anderson, G.G., McCracken, D.M., Lake, M., and Koldovsky, O. Fate of insulin-like growth factors I and II administered orogastrically to suckling rats *Pediatr. Res.* **37,** 586–592.

12 Baumrucker, C.R., Hadsell, D.L., Skaar, T.C., Campbell, P.G., and Blum, J.W. (1992). Insulin-like growth factors (IGFs) and IGF binding proteins in mammary secretions: origins and implications in neonatal physiology. In *Mechanisms regulating lactation and infant nutrient utilization.* p 285–308, (Picciano, M.F. and Lonnerdal, B., eds), Wiley-Liss, New York, NY USA

13 Baumrucker, C.R. and Blum, J.W. (1993). Secretion of insulin-like growth factors in milk and their effect on the neonate. *Livestock Prod. Sci.* **35,** 49–72

14 Pillion, D.J., Haskell, J.F., Atchison, J.A., Ganapathy, V., and Leibach, F.H. (1989). Receptors for IGF-I, but not for IGF-II, on proximal colon epithelial cell apical membranes. *Am. J. Physiol.* **257,** E27–E34

15 Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., and Semenza, G. (1978). A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. *Biochim. Biophys. Acta* **506,** 136–154

16 Smith, P.K., Krohn, R.I., Gartner, F.H., Provenzano, M.D., and Fujimoto, E.K. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150,** 76–85

17 Kidder, D.E. and Manners, M.J. (1980). The level and distribution of carbohydrases in the small intestinal mucosa of pigs from 3 weeks of age to maturity. *Br. J. Nutr.* **43,** 141–153

18 Kelly D., Smyth, J.A., and McCracken, K.J. (1991). Digestive development of the early-weaned pig. *Br. J. Nutr.* **65,** 169–180

19 Roth, J. (1975). Methods for assessing immunologic and biologic properties of iodinated peptide hormones. *Methods Enzymol.* **37,** 223–233

20 Kelly, P.A., Leblanc, G., and Djiane, J. (1979). Estimation of total prolactin-binding sites after in vitro desaturation. *Endocrinology* **104,** 1631–1638

21 Soos, M.A., Field, C.E., Lammers, R., Ullrich, A., Roth, R.A., Andersen, A.S., Kjeldsen, T., and Siddle, K. (1992). A panel of monoclonal antibodies for the type I insulin-like growth factor receptor. *J. Biol. Chem.* **267,** 12955–12963

22 Leatherbarrow, R.J. (1987). 'Enzfitter. A non-linear regression data analysis program, Elsevier, Amsterdam, the Netherlands

23 Massague, J. and Czech, M.P. (1985). Affinity cross-linking of receptors for insulin and the insulin-like growth factors I and II. *Methods Enzymol.* **109,** 179–187

24 Carpenter, G., King, L., Jr., and Cohen, S. (1979). Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* **254,** 4884–4891

25 Hayes, G.R. and Lockwood, D.H. (1987) Role of insulin receptor phosphorylation in the insulinomimetic effects of hydrogen peroxide. *Proc. Natl. Acad. Sci. (USA)* **84,** 8115–8119

26 Alexandrides, T.K. and Smith, R.J. (1989). A novel fetal insulin-like growth factor (IGF) I receptor. Mechanism for increased IGF I- and insulin-stimulated tyrosine kinase activity in fetal muscle. *J. Biol. Chem.* **264,** 12922–12930

27 Young, G.P., Taranto, T.M., Jonas, H.A., Cox, A.J., Hogg, A., and Werther, G.A. (1990). Insulin-like growth factors and the developing and mature rat small intestine: receptors and biological actions. *Digestion* **46**(suppl 2), 240–252

28 Heinz-Erian, P., Kessler, U., Funk, B., Gais, P., and Kiess, W. (1991). Identification and in situ localization of the insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor in the rat gastrointestinal tract: comparison with the IGF-I receptor. *Endocrinology* **129,** 1769–1778

29 Ryan, J. and Costigan, D.C. (1993). Determination of the histological distribution of insulin-like growth factor I receptors in the rat gut. *Gut* **34,** 1693–1697

30 Allen, R.E. and Boxhorn, L.K. (1989). Regulation of skeletal muscle satellite cell proliferation and differentiation by transforming growth factor-beta, insulin-like growth factor I and fibroblast growth factor. *J. Cell Physiol.* **138,** 311–315

31 Kasuga, M., Van Obberghen, E., Nissley, S.P., and Rechler, M.M. (1981). Demonstration of two types of insulin-like growth factor receptors by affinity cross-linking. *J. Biol. Chem.* **256,** 5305–5308

32 Bassas, L., DePablo, F., Lesniak, M.A., and Roth, J. (1985). Ontogeny of receptors for insulin-like peptides in chick embryo tissues: early dominance of insulin-like growth factor over insulin receptors in brain. *Endocrinology* **117,** 2321–2329

33 Alexandrides, T., Moses, A.C., and Smith, R.J. (1989). Developmental expression of receptors for insulin, insulin-like growth factor I (IGF-I), and IGF-II in rat skeletal muscle. *Endocrinology* **124,** 1064–1076

34 Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z., Roberts, C.T., and LeRoith, D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proc. Natl. Acad. Sci. (USA)* **86,** 7451–7455

35 Sklaar, M.M., Kiess, W., Thomas, C.L., and Nissley, S.P. (1989). Developmental expression of the tissue insulin-like growth factor II/mannose 6-phosphate receptor in the rat. *J. Biol. Chem.* **264,** 16733–16738

36 Morovat, A., Burton, K.A., and Dauncey, M.J. (1994). Short-term regulation of plasma IGF-I concentration by food intake in young growing pigs. *Horm. Metab. Res.* **26,** 265–269

37 Grey, V., Rouyer-Fessard, C., Gammeltoft, S., Bourque, M., Morin, C., and Laburthe, M. (1991). Insulin-like growth factor II/mannose-6-phosphate receptors are transiently increased in the rat distal intestinal epithelium after resection. *Mol. Cell. Endocrinol.* **75,** 221–227.

38 Dauncey, M.J., Burton, K.A., and Tivey, D.R., (1994). Nutritional modulation of insulin-like growth factor-I expression in early postnatal piglets. *Pediatr. Res.* **36,** 77–84.

39 Hartman, P.E. and Holmes, M.A. (1985) Sow lactation. In *Manipulating pig production; Proceedings-Australian Pig Science ASSO* **2,** 72–97

40 Rosenthal, S.M., Brunetti, A., Brown, E.J., Mamula, P.W. and Goldfine, I.D. (1991) Regulation of insulin-like growth factor (IGF) I receptor expression during muscle cell differentiation. *J. Clin. Invest.* **87,** 1212–1219

41 Morgan, C.J. (1995). Role of insulin-like growth factors and type I receptors in the developing porcine intestine. PhD thesis, University of Aberdeen, Scotland.

42 Rosenthal, S.M., Brown, E.J., Brunetti, A. and Goldfine, I.D. (1991). Fibroblast growth factor inhibits insulin-like growth factor-II (IGF-II) gene expression and increases IGF-I receptor abundance in BC3H-1 muscle cells. *Mol. Endocrinol.* **5,** 678–684

43 Hakeda, Y., Haradas, S., Matsumoto, T., Tezuka, K., Higashinp, K., Kodama, H., Hashimito-Goto, T., Ogata, E., and Kumegawa, M. (1991). Prostaglandin $F_{2\alpha}$ stimulates proliferation of clonal osteoblastic MC3t3-E1 cells by up-regulation of insulin-like growth factor I receptors. *J. Biol. Chem.* **266,** 21044–21050

44 Kelly, D., Morgan, C.J., Coutts, A.G.P., McFadyen, M., and King, T.P. (1994) The effect of orally administered IGF-1 on intestinal development of artificially-reared piglets. In *Proceedings of American Gastroenterological Association Symposium* on "Peptide growth factors in the GI tract." June 25–29, 1994 Vail, Colorado. (Abstract)

45 Burrin, D.G., Davis, T.A., Ebner, S., Schoknecht, P.A., Fiorotto, M.L., Reeds, P.J., and McAvoy, S. (1995). Nutrient-independent and nutrient-dependent factors stimulate protein synthesis in colostrum-fed newborn pigs. *Pediatr. Res.* **37,** 593–599

46 Donovan, S.M., Chao, C.J., Zilstra, R.T., and Odle, J. (1995). Absorption of orally administered iodinated insulin-like growth factor-I (IGF-I) by the neonatal piglet. *Faseb J.* **9,** A730.