Isolation, characterization, and developmental expression of pig intestinal fatty acid–binding proteins

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The goal of this study was to characterize and quantify intestinal fatty acid-binding proteins of the pig. Small intestinal mucosa from 13–19 kg pigs was homogenized and centrifuged to obtain cytosol. Isolation of fatty acid-binding proteins from delipidated cytosol was achieved using molecular sieve, oleic acid affinity, and ion exchange chromatography. Fatty acid-binding protein isolation was monitored using a fatty-acid binding assay in conjunction with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Antisera to rat liver-fatty acid-binding protein cross reacted with an isolated intestinal fatty acid-binding protein of M_r = 13,000, whereas antisera to rat intestine-fatty acid-binding protein was not cross reactive with isolated pig intestinal proteins. These experiments identify a pig intestinal fatty acid-binding protein that exhibits strong immunochemical similarity to rat liver-fatty acid-binding protein. Cytosol prepared from intestinal mucosa of pigs at −4, 2, 4, 7, 15, 22, 28, and 35 d of age was assayed for fatty acid-binding protein activity. Preweaning fatty acid-binding protein activity in cytosol was maximal at 7 days of age when expressed as total jejunal fatty acid binding per kilogram bodyweight, intestinal or mucosal weight or milligram total protein. After weaning (21 d), fatty acid-binding protein activities declined to 28 days, but increased again by 35 days. Total soluble fatty acid-binding protein activity in pig intestine is regulated during postnatal development and this may account in part for the altered intestinal absorption of lipids observed in young pigs at weaning.

Keywords: fatty acid binding protein, small intestine, ontogeny, pig, lipid metabolism

Introduction

Fatty acid-binding proteins (FABPs) are a family of closely related proteins abundant in the liver, heart, kidney, adipose, and epithelial cells lining the small intestine.[1] Putative roles for FABPs include mediating the cellular uptake and intracellular transport of long chain fatty acids.[2] In vitro, FABPs demonstrate high affinity, non-covalent binding of oleate and palmitate. Three unique mammalian FABPs have been isolated and characterized: liver (L-FABP),[3] intestinal (I-FABP),[4] and heart (H-FABP).[5] These FABPs have discrete tissue distributions in the rodent,[6] and are postulated to have distinct roles in the metabolism of long chain fatty acids. The heterogeneity of FABPs in cytosolic fractions of various tissue have made functionality studies difficult, and as a result, isolation of FABPs is necessary as a prelude to understanding their function in other species.

The availability and concentration of digestive enzymes and membrane carriers normally determine the rate of intestinal digestion and absorption of nutrients. The developmental appearance and accumulation of these proteins may be a useful index in predicting the digestibility of various substrates at different neonatal stages. Intestinal absorption of fatty acids in the rat have been postulated to involve a specific low molecu-

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lar weight cytosolic FABP.[4] Therefore, as a first step to elucidating the factors which regulate lipid absorption and metabolism in the young pig, studies to isolate, characterize, and quantify porcine intestinal FABPs were conducted.

Materials and methods

Materials

[1-^{14}C] Oleic acid and ^{125}I-protein A were purchased from ICN Radiochemicals (Irvine, CA). Sephadex G-75 and AH-Sepharose 4B resins were from Pharmacia Fine Chemicals (Piscataway, NJ). Lipidex 1000 was obtained from Packard Instrument Co. (Downers Grove, IL), DE-52 cellulose ion exchange resin from Whatman, Inc. (Clifton, NJ) and nitrocellulose from Schleicher and Schuell Inc. (Keene, NH). Rabbit antisera to rat liver- and intestinal-FABP were generous gifts from Drs. R. Ockner and N. Bass, University of California, San Francisco.

Preparation of cytosol

Pigs (13 to 19 kg) were sacrificed by electrocution and the small intestine excised. The lumen was washed three times with ice cold 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4), mucosa collected and immediately frozen in liquid nitrogen. Frozen mucosa was homogenized in two volumes of 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4) and the homogenate centrifuged for 2.0 h at 105,000 × g at 4° C. The clear supernate (cytosol), exclusive of lipid, was collected and assayed for FABP activity. Protein concentration of cytosol was determined by the Bradford method.[7]

Post-chromatographic fatty acid binding assay

Binding of fatty acid to intestinal FABPs was assayed by a modification of a previously described method.[8] The protein solutions (0.4 ml) in 0.5 ml polypropylene tubes were incubated with 6.0 μm ^{14}C-oleic acid for 10 min at 37° C. Tubes were cooled to 0 to 4° C, 150 μl ice-cold Lipidex 1000 suspension (1:1 vol/vol in 0.154 M KCl/.01 M potassium phosphate buffer, pH 7.4) added, vortexed and incubated 10 min at 4° C. The tubes were centrifuged at 10,000 × g for 3 min at 4° C, and 0.35 ml of the supernate assayed for radioactivity. Protein-free blanks were included in each assay to evaluate nonspecific binding. Each sample was assayed in triplicate and the mean value reported.

FABP isolation

Delipidated cytosol (92 mg) was passed over a preparative G-75 column (2.5 × 100 cm) and eluted with 0.154 M KCl/0.01 M potassium phosphate buffer (pH 7.4). Fractions with FABP activity were pooled and rechromatographed on a Sephadex G-75 column (2.5 × 100 cm). Sephadex G-75 fractions exhibiting FABP activity were applied to an oleic acid coupled AH Sepharose-4B column (1.5 × 20 cm) and washed with 0.154 M KCl/.01 M potassium phosphate (pH 7.4). Retained proteins were eluted with 25% ethanol. Fractions corresponding to A_{280} absorbance peaks were pooled and assayed. Pools, enriched for binding activity, were dialyzed against 20 mM ethanolamine (pH 9.1), applied to a DE-52 cellulose ion exchange column (1.5 × 20 cm) and washed with 20 mM ethanolamine (pH 9.1). Adsorbed proteins were eluted in a linear (0 to 1.0 M) NaCl gradient. Fractions corresponding to A_{280} absorbance peaks were pooled, dialyzed against 0.154 M KCl/.01 M potassium phosphate buffer (pH 7.4) and assayed for FABP activity.

Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Selected protein fractions were monitored by analytical polyacrylamide gel electrophoresis as previously described[9] and silver stained. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose paper and immunoblotting performed using previously described methods.[10]

Cell free translation

Total cellular ribonucleic acid (RNA) was isolated from intestinal mucosa, liver and uterine endometrium using guanidine thiocyanate.[11] Poly (A)$^+$ RNA was isolated[12] and translated in a rabbit reticulocyte-based in-vitro system (Promega, Madison, WI). Aliquots of the reaction mixture were subjected to immunoprecipitation[13] using antibodies specific for rat I- and L-FABP.

Developmental expression of FABP

Small intestinal mucosa (upper 50% of small intestinal length; jejunal region) was obtained from four pigs at the following ages: 4 days prior to birth (110 days gestation) and at 2, 4, 7, 15, 22, 28, and 35 days of age. Four litters were used in the study, with one animal per litter sacrificed at each postnatal time point. Tissue from an additional four fetuses (two litters) was used for the −4 day time point. Cytosol was prepared from the mucosa of each animal and aliquots pooled within each day. Delipidated cytosols were Sephadex G-75 gel filtered (1.6 × 80 cm column), the FABP region collected and assayed for FABP activity (in sextuplet and averaged). The FABP binding assay was repeated on three separate occasions and the mean reported. Jejunal FABP activity was calculated as cpm of ^{14}C-oleic acid bound per: pig, kg bodyweight, g wet small intestine, g wet mucosa, and mg cytosolic protein.

Results

Post chromatographic fatty acid binding assay

A reliable assay for detection and quantitation of FABPs in column chromatographic fractions is essential for isolation of FABPs. A post-chromatographic binding assay[8] was employed to avoid problems with ligand:column interactions, protein:protein competi-

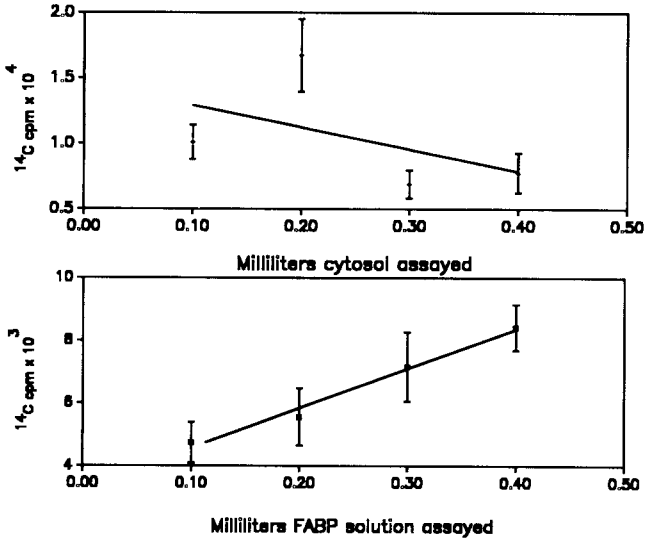

Figure 1 Dose-response curve of ^{14}C-oleic acid binding to cytosol and partially purified FABP. Relative FABP activity was determined at various concentrations of cytosol (top panel) and partially purified FABP preparations (bottom panel). Data points represent mean ± SE for three independent determinations; lines calculated by linear regression analysis.

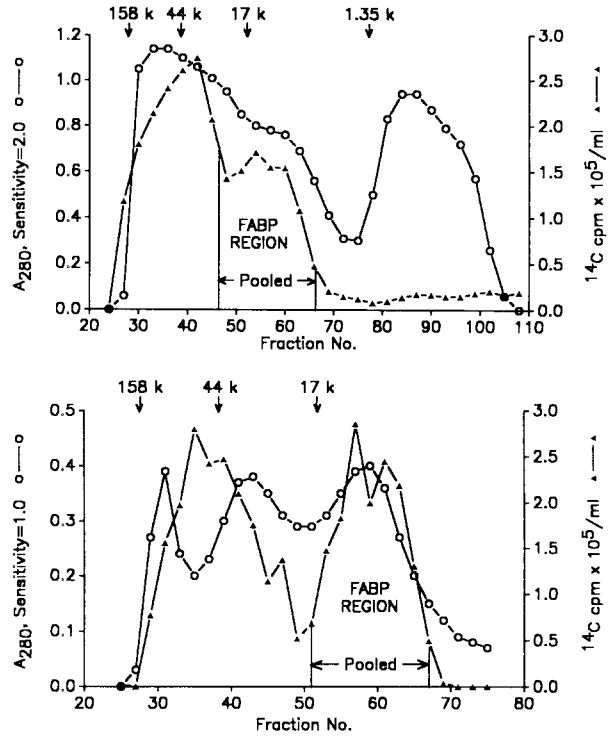

Figure 2 Fatty acid binding to fractionated intestinal mucosa cytosol. Top panel: Delipidated cytosol (92 mg) was chromatographed on a Sephadex G-75 column (2.5 × 100 cm). Elution (5.5 ml/fraction) was performed with 0.154 M/0.01 M potassium phosphate, pH 7.4, at 4° C. Every third fraction was assayed in triplicate for fatty acid binding activity (Materials and methods). Bottom panel: Low molecular weight fractions with FABP activity were pooled (#46-66 from Fig. 2, top) and rechromatographed on a Sephadex G-75 column (2.5 × 100 cm) under the same conditions. Alternating fractions were assayed in triplicate for FABPs.

tion for available ligand, and radioactive contamination of FABP preparation when prechromatographic labeling is used.[14] The assay relies on the temperature-dependent binding of hydrophobic ligands to Lipidex 1000, a 10% (wt/vol) substituted hydroxyalkooxypropyl derivative of Sephadex G-25. At 37° C, Lipidex binds both nonesterified and protein-bound fatty acids while at 4° C, only nonesterified fatty acids are bound by the insoluble material.[8] Oleic acid was used as the radiolabeled ligand since it reportedly binds with the highest affinity to rat FABPs.[2]

Validation studies were conducted to compare assay performance on whole cytosol or partially purified FABP at different concentrations. Examination of the dose response curves revealed that unfractionated cytosol did not produce a linear relationship with increasing cytosol volume (Fig. 1, top panel), probably due to the presence of nonspecific binders. In contrast, a linear response with increasing concentrations of partially purified FABP was observed (Fig. 1, bottom panel). Thus, in order to obtain a quantitative index of FABP activity in cytosol, an initial chromatographic step to separate FABP(s) from other binding species and nonspecific binders is required. Previous work has demonstrated that all forms of FABP may not be detected immunochemically with equal sensitivity.[15] This assay, in contrast, can theoretically monitor total FABP binding activity since it relies on ligand binding and not immunological detection.

Isolation of pig intestinal fatty acid binding proteins

The initial step in the isolation of FABP from delipidated cytosolic proteins of pig intestinal mucosa was gel filtration on Sephadex G-75. Fatty acid binding protein functional activity, as assayed by fatty acid binding, was determined in the column fractions. The binding pattern (Fig. 2, top) revealed a FABP peak corresponding to an approximate molecular weight of 13,200 Da in addition to a large peak of binding activity found in the region where serum albumin elutes. Pooled fractions from the 4,000 to 28,000 Da region were rechromatographed to further resolve the FABP region (Fig. 2, bottom).

These FABP containing fractions were pooled and applied to a ligand affinity column (oleic acid immobilized to Sepharose AH 4-B). Unretained proteins eluted off in Peak 1 (Fig. 3, top), while retained proteins were removed with a 25% ethanol wash (Peak 2). All peaks showed some degree of fatty acid-binding activity, but Peak 2 had the greatest specific activity.

For further purification, ion exchange chromatography was employed. The FABP containing fractions from the affinity chromatography step (Peak 2) were applied to a DE-52 cellulose ion exchange column at pH 9.1 and eluted as described (Materials and methods) (Fig. 3, bottom). Chromatographic regions were pooled, and dialyzed against 0.154 M KCl/.01 M potassium phosphate buffer (pH 7.4), and assayed for binding activity. Most of the FABP activity eluted im-

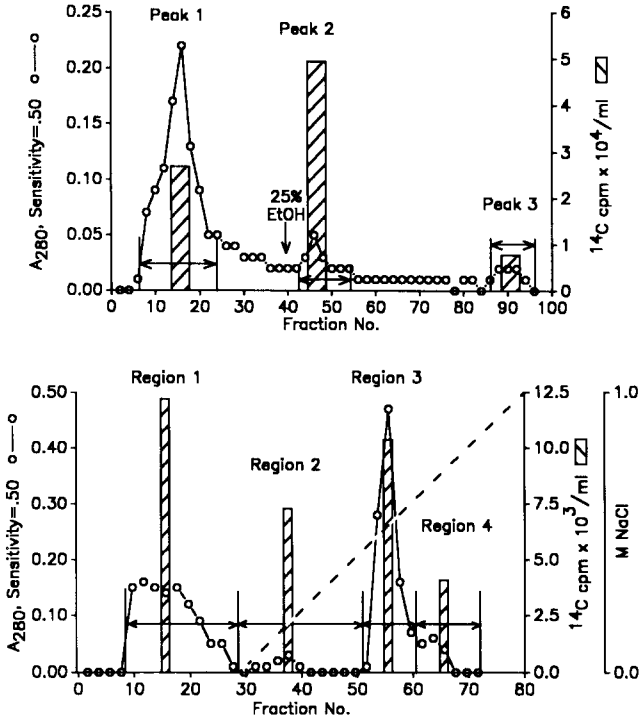

Figure 3 FABP isolation. Top panel: Fractions with FABP activity (#51-67 from Fig. 2, bottom) were pooled and applied to an oleic acid affinity column (1.5 × 20 cm) and washed with 0.154 M KCl/ 0.01 M potassium phosphate, pH 7.4, at 4° C. Retained proteins were eluted with 25% ethanol. Fractions corresponding to A_{280} peaks were pooled and assayed for FABPs. Bottom panel: Fractions #42-56 from Fig. 3, top were dialyzed against 20 mM ethanolamine, pH 9.1, applied to a DE-52 cellulose ion exchange column (1.5 × 20 cm) and washed with 20 mM ethanolamine, pH 9.1. Retained proteins were eluted with a linear (0–1.0 M) NaCl gradient. Fractions corresponding to A_{280} peaks were pooled and assayed for fatty acid binding activity.

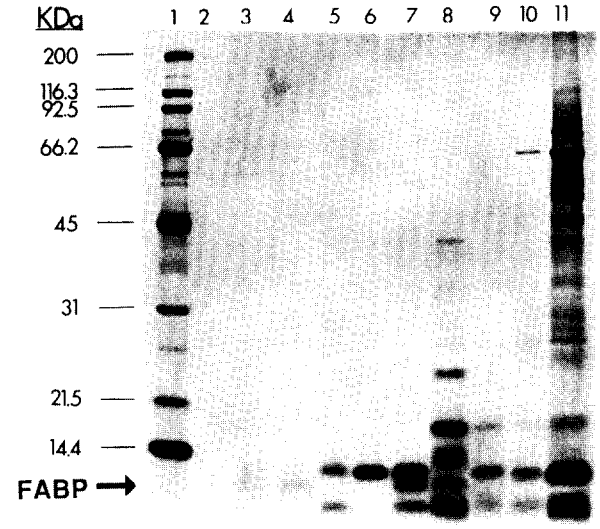

Figure 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of mucosal protein at various stages of isolation. (1) molecular weight markers, (2) Region 4, Fig. 3, bottom (3) Region 3, Fig. 3, bottom (4) Region 2, Fig. 3, bottom (5) Region 1, Fig. 4, bottom (6) Peak 3, Fig. 4, top (7) Peak 2, Fig. 4, top (8) Peak 1, Fig. 4, top (9) #46-67, Fig. 2, bottom (10) #46-66, Fig. 2, top (11) cytosol.

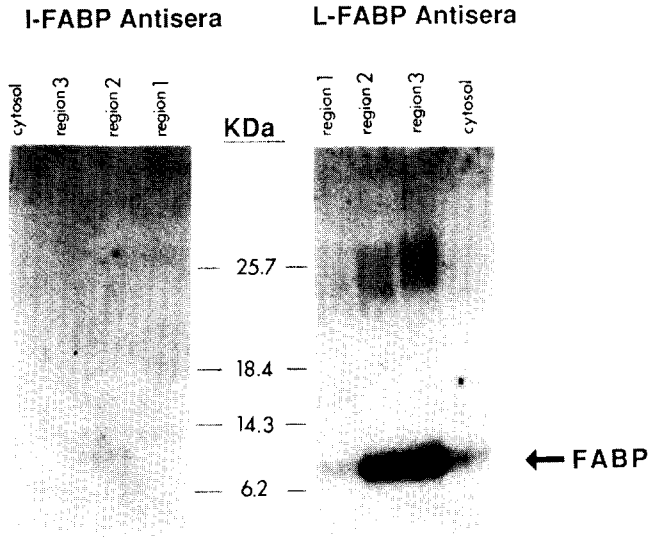

Figure 5 Immunochemical identification of FABP in pig intestine. DE-52 ion exchange fractions were electrophoresed in duplicate 15% polyacrylamide gels and subsequently electroblotted onto nitrocellulose. The nitrocellulose filters were incubated with antiserum specific for rat I- or L-FABP, followed by incubation with ^{125}I-Protein-A of *S. aureus*. After washing, the filter was exposed to X-ray film (autoradiography). Autoradiographic intensity is proportional to the abundance of the antigenic protein.

mediately from the DE-52 cellulose ion exchange column to form Region 1. Region 2 proteins had slight absorption to the column, eluting after the unretained proteins but prior to initiation of the increasing salt gradient. Region 3 proteins were eluted at the beginning of a linear 0–1.0 M NaCl gradient, while Region 4 contained residual proteins that eluted in high ionic strength conditions.

Column fractions from each step in the isolation scheme were subjected to polyacrylamide gel electrophoresis. There were three major protein bands that were retained by the oleic acid affinity column (Fig. 4; lanes 6, 7). Ion exchange chromatography further resolved these three proteins (Fig. 4; lanes 3–5). The relative mobility of the protein in lanes 3 and 4, compared with that of protein standards, indicated a molecular weight of 13,000 Da, a value which agreed closely with that of 13,200 Da as determined by Sephadex G-75 chromatography.

Initial characterization

Initial characterizati' of the FABP(s) was performed using immunoblottin hniques. Unfractionated cytosol, and Regions 1, 2, and 3 from the ion exchange chromatography step were subjected to polyacrylamide gel electrophoresis in duplicate as described in Materials and methods. Figure 5 shows the autoradiograms obtained from immunoblots of identical gels incubated with antisera specific for either rat I-FABP (left) or rat L-FABP (right) and ^{125}I-labeled protein A. Liver-FABP antiserum was highly cross reactive with isolated pig intestinal FABP(s) and unfractionated in-

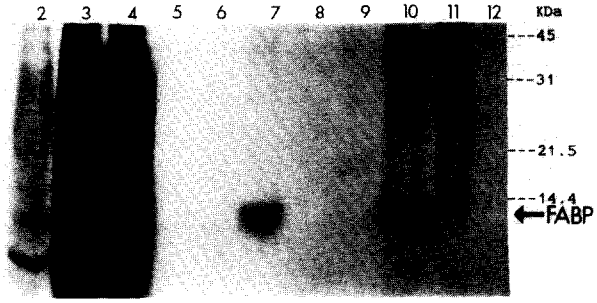

Figure 6 In vitro translation of fatty acid binding protein messenger ribonucleic acids. Polyadenylated RNA purified from pig intestinal mucosa, liver or uterine endometrium was translated in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine. After translation, aliquots of the reaction mixture were subjected to immunoprecipitation (Simmen et al., 1984) using antibodies specific for rat I- and L-FABP. The immunoprecipitates and aliquots of total translation products were analyzed by fluorography of SDS-PAGE gels. Lane (1) no RNA, (2) total translation products from 0.1 μg intestine RNA, (3) total translation products from 1.0 μg intestine RNA, (4) total translation products from 10.0 μg intestine RNA, (5) 1.0 μg intestine RNA, no antiserum immunoprecipitate, (6) no RNA, L-FABP immunoprecipitate, (7) 1.0 μg intestine RNA, L-FABP immunoprecipitate, (8) no RNA, I-FABP immunoprecipitate, (9) 1.0 μg intestine RNA, I-FABP immunoprecipitate, (10) 1.0 μg liver RNA, L-FABP immunoprecipitate, (11) 1.0 μg liver RNA, I-FABP immunoprecipitate, (12) 1.0 μg endometrial RNA, I-FABP and L-FABP immunoprecipitate.

testinal cytosol, while I-FABP antiserum displayed very weak or no cross reactivity with the same pig proteins. Substantial enrichment of the protein with antigenic relatedness to rat L-FABP during the isolation procedures was demonstrated by the immunoblotting procedure. An apparent aggregate of FABP (Region 2 and 3 proteins) was also noted.

In vitro translation of FABP messenger RNAs was conducted to verify the immunological relatedness of proteins. Polyadenylated RNA from several tissue sources was translated in a cell free system as described in Materials and methods. Translated proteins were immunoprecipitated with rat I- and L-FABP antisera and immunoprecipitates and aliquots of the total translation products were analyzed by fluorography of the polyacrylamide gels. Proteins translated from pig intestine and liver polyadenylated RNA were precipitated with the antiserum to rat L-FABP (Fig. 6; lanes 7 and 10), while the I-FABP antisera did not precipitate proteins from any of the translation reactions (lanes 9, 11, 12). Pig uterine endometrial RNA did not translate a protein that cross reacted with either antiserum.

Ontogeny of intestinal FABP activity

Jejunal FABP activity increased during the early neonatal period (days 1 through 21) (Fig. 7, top). At weaning, there was a slight decrease in total FABP activity. By day 35 of age (14 days postweaning), FABP activity exceeded the preweaning levels. The developmental changes in jejunal FABP when adjusted per gram wet intestine and gram mucosa (Fig. 7, middle) do not parallel the trends observed for total FABP activity per animal. By day 7 of postnatal life, adjusted

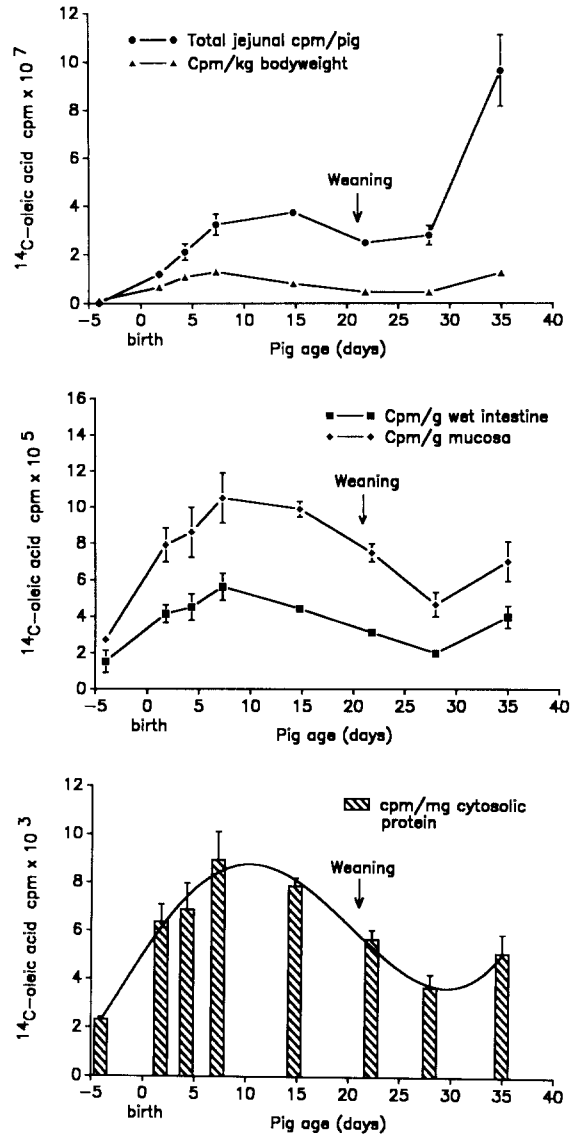

Figure 7 Developmental expression of pig intestinal FABPs. Cytosols were pooled from four pigs at each developmental time point, delipidated with Lipidex and subsequently passaged through a 1.6 × 80 cm Sephadex G-75 gel filtration column. Column fractions containing FABPs were pooled and the post-chromatographic binding assay performed three separate times to obtain an interassay variation (mean ± standard error). Top panel: Data are total cpm ^{14}C-oleic acid bound per pig and total activity adjusted per kg bodyweight. Middle panel: Total FABP activity adjusted per g of small intestine or mucosal scrapings. Bottom panel: Total FABP activity adjusted per mg intestinal mucosa cytosol protein and the response fitted with a 5th degree polynomial regression curve.

jejunal FABP activity was threefold higher than at 110 days of gestation (−4 days), and the highest measured during the perinatal period. In order to determine whether changes in postnatal FABP activity were due solely to fluxes in enterocyte protein concentration, the data were adjusted to constant amount of cytosolic protein. The results (Fig. 7, bottom) demonstrate that FABP activity is preferentially increased during the early neonatal period, with the highest value observed at 7 days.

Discussion

This study reports the presence of fatty acid binding protein activity in the small intestinal mucosa of young pigs. Although identification of FABPs in mammalian tissues is not unique, studies of such proteins in pig intestine have not been previously reported. One of the purified intestinal FABPs has an approximate molecular weight of 13,000 Da and is antigenically related to rat L-FABP but not rat I-FABP. The presence of two distinct, independently regulated FABPs in rat intestine has been postulated to be related to the intestinal uptake of fatty acids from two sources, the lumen and intestinal blood supply.[16] Large quantities of unesterified fatty acids are taken up by the enterocyte from the intestinal lumen and subsequently re-esterified to form chylomicron triglycerides, while fatty acids derived from the intestinal blood supply are used mainly for energy production and phospholipid biosynthesis.[17] Whether the pig also harbors an intestine-specific FABP is unknown. If the pig intestine does express a counterpart to the rat I-FABP, then the two proteins are either immunochemically distinct or the pig I-FABP protein is present in small quantities that make it virtually undetectable by the methodologies used.

Various separation procedures were employed to highly purify pig intestinal FABPs. These efforts resolved proteins in the 10,000 to 20,000 Da range into three protein bands as analyzed by SDS-PAGE. These three protein bands closely resemble the banding patterns of the partially purified rat intestinal proteins.[4] In the rat, the protein with intermediate mobility in a polyacrylamide gel represents an intestine-specific FABP. However, here the corresponding protein reacted with the L-FABP specific antiserum. The high specific activity of the proteins suggest that they may all be authentic FABPs. During immunoblotting studies, FABP aggregates were observed. These aggregates reacted with rat L-FABP antiserum and migrated with a molecular weight of approximately 25,000 Da. This phenomenon has been reported previously[18] for partially purified preparations of rat liver FABP under denaturing conditions in the presence of SDS.

Unlike a number of intestinal adaptive responses that appear to be related to mucosal hyperplasia,[19] quantitative changes in FABP binding activity during the nursing period are at least partially independent of small intestinal mass or mucosal mass and quantity of cytosolic protein. The mechanisms underlying this developmental regulation in the pig remain unclear at the present time. Neonatal rat intestine has been reported to display a fourfold increase in FABP mRNA concentration within 24 hours after birth.[20] A coordinate induction of L- and I-FABP gene expression was observed during this same time period. Parallel increases in liver L-FABP mRNA levels were also noted, but the magnitude of change was much less pronounced.

Postweaning changes in the levels of intestinal FABP activity correlate well with fat digestibility coefficients and dietary fat absorption of weanling pigs.[21] Although this does not imply a causal relationship, it does provide evidence in support of a physiological basis for the poor lipid utilization of weanling pigs relative to older pigs. Fatty acid-binding proteins have been hypothesized to transport long chain fatty acids from the cytoplasmic side of the intestinal brush border to their sites of utilization, and perhaps play a role in promoting esterification of fatty acids[4] and bile acid synthesis.[22] Net fatty acid uptake into cells is highly dependent upon the pool of fatty acid-binding sites available in the cytoplasmic compartment since desorption of fatty acids from cell membranes is rate limiting in the absorption and transport of fatty acids.[23] It is probable that the high concentration of FABPs in rat enterocytes (0.1 to 0.4 mM) is necessary to facilitate desorption of fatty acids from the plasma membranes of these cells. Further studies are required to delineate the factors responsible for the temporal regulation of FABP expression in pig intestine and to clarify the precise relationships of gut FABPs and lipid absorptive capacity. This should in turn lead to a better understanding of the factors that regulate lipid absorption and metabolism in the young pig.

Acknowledgments

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