Early Weaning Reduces Small Intestinal Alkaline Phosphatase Expression in Pigs

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Abstract
Expression of the small intestinal alkaline phosphatase (IAP) is enterocyte differentiation dependent and plays essential roles in the detoxification of pathogenic bacterial lipopolysaccharide endotoxin, maintenance of luminal pH, organic phosphate digestion, and fat absorption. This study was conducted to examine the effect of early weaning on adaptive changes in IAP digestive capacity ($V_{\text{cap}}$) and IAP gene expression compared with suckling counterparts in pigs at ages 10–22 d. Weaning decreased ($P<0.05$) IAP enzyme affinity by 26% and IAP maximal enzyme activity by 22%, primarily in the jejunal region, with the jejunum expressing 84–86% of the whole gut mucosal IAP $V_{\text{cap}}$ [mol/(kg body weight-d)]. The majority (98%) of the jejunal mucosal IAP maximal activity was associated with the apical membrane and the remaining (2%) existed as the intracellular soluble IAP. Weaning reduced the abundance of the 60-kDa IAP protein associated with the proximal jejunal apical membrane by 64% ($P<0.05$). Furthermore, weaning reduced ($P<0.05$) the relative abundance of the proximal jejunal IAP mRNA by 58% and this was in association with decreases ($P<0.05$) in the abundances of cytoplasmic (27%) and nuclear (29%) origins of IAP caudal-associated homeobox transcription factor 1. In conclusion, early weaning decreased small intestinal IAP $V_{\text{cap}}$, IAP catalytic affinity, and IAP gene expression, and this may in part contribute to the susceptibility of early-weaned piglets to increased occurrence of enteric diseases and growth-check. J. Nutr. 140: 461–468, 2010.

Introduction
Weaning is a critical stage of postnatal growth and gut development in mammals, including rodents (1,2), pigs (3), and humans (4,5). The early weaning transition is commonly associated with a growth-check (6), increased occurrence of enteric diseases and diarrhea (7), and decreased digestive capability (3) in nursery pig management. Abrupt weaning is commonly associated with a period of low feed intake, contributing to alterations in the gut mucosal structure and functions in weaning piglets (8,9). In addition to diets, social and stress factors due to separation from sows and housing in new environments collectively contribute to changes of the gut mucosa (9,10). However, weaning-associated gut mucosal atrophy involves variable expression patterns of digestive enzymes and other functional proteins in the small intestine, such as decreases in lactate activity (8), as well as increases in maltase and sucrase activities (11), increases in both anti- and proinflammatory cytokine gene expression (12,13), and increases in citrulline and polyamine biosynthesis activities (14,15). In newborn pigs, gut mucosa possesses both fetal and adult types of enterocytes and during normal suckling, the complete replacement of fetal enterocytes occurs at 19 d of age (16,17). Thus, weaning not only depletes fetal enterocytes but also provides an opportunity to the host for growing a new phenotype of enterocytes to reprogram and adapt gut functions. Furthermore, changes in individual gut mucosal genes are uniquely regulated and cellular and molecular events associated with these changes in the gut during the weaning of pigs are yet to be elucidated.

The expression of the small intestinal alkaline phosphatase (IAP)4 is enterocyte differentiation dependent and IAP is regarded as a key marker enzyme when considering changes in the primary digestive and absorptive functions of the small intestine (18). Physiological functions of IAP include hydrolysis of monophosphate esters (19), transcellular solute transport (20), and participation in the absorption of fat (21). More recently, IAP activity has been shown to increase bicarbonate secretion into the gut, thereby allowing for the neutralization of the acidic gastric digesta entering the small bowel while providing an optimal condition for IAP activity (22). Also, the

4 Abbreviations used: cdx1, caudal-associated homeobox transcription factor 1; $K_m$, enzyme affinity; IAP, intestinal alkaline phosphatase; PMSF, phenylmethylsulfonyl fluoride; SU, suckling group; $V_{\text{cap}}$, digestive capacity; $V_{\text{max}}$, maximal enzyme activity; WN, early weaning group.
gut mucosal defense role of IAP as an endogenous detoxification factor against luminal pathogenic bacterial lipopolysaccharide endotoxin is well recognized (23,24) and this is maintained by enteral nutrition (25). Thus, understanding changes in gut IAP digestive capacity \( (V_{\text{cap}}) \) as well as cellular and molecular events regulating IAP gene expression during weaning will improve our knowledge in gut physiology and allow strategies for improving nursery nutrition and growth.

Expression of enterocytic IAP is cell differentiation dependent along the crypt-villus axis (18,26) and is regulated at the transcriptional level via several well-clarified transcriptional factors, including the caudal-associated homeobox transcriptional factor (cdx1) that binds to the promoter region of the IAP gene (27). Hepatocyte nuclear factor 4α (28), the gut-enriched Kruppel-like factor (29), and the zinc finger binding protein-89 (30) are among other elucidated transcription factors present in the IAP gene transcription complex. Transcriptional regulation of the IAP gene is subjected to further modulations by nutritional (25), inflammatory (31), and hormonal factors (32). Intracellular biosynthesis of IAP protein occurs in the rough endoplasmic reticulum as high-mannose polypeptides (33), which is likely limited by IAP mRNA availability and affected by luminal nutrient availability and hormonal factors (34). Major events necessary for the biosynthesis and further processing of IAP protein include its posttranslational glycosylation in the Golgi apparatus (33), sorting for its trafficking via distinct vesicular carriers for anchoring on the apical membrane (35,36) or for its degradation (37) via lysosome or ubiquitin-proteasome pathways (34,38). Although mature IAP can be assembled into surfactant-like particles and secreted into the lamina propria, lymphatic capillaries, serum, and the small intestinal lumen in particular response to fat intake (21), this enzyme protein is found predominantly on the apical membrane surface attached through a covalent phosphatidylglycerol linkage (39). Weaning triggers dramatic nutritional, hormonal, and inflammatory changes; thus, IAP gene expression is likely affected at multiple levels by weaning in pigs.

Although changes in IAP activity were reported in pigs weaned at 4–6 wk old (40) and during postnatal development (41), major cellular and molecular events associated with IAP gene expression have not been examined in early-weaned pigs. In neonatal pigs, small intestinal epithelial life span has been measured (16,26) and weaning-associated gut mucosal changes occur in 2 phases, i.e. an acute period immediately after weaning and followed by an adaptive phase after 5 d of weaning (42). Therefore, the major objectives of this study were to examine adaptive changes in IAP \( V_{\text{cap}} \) and IAP gene expression associated with weaning, including: 1) IAP activity kinetics, i.e. its maximal enzyme specific activity \( (V_{\text{max}}) \) and enzyme affinity \( (K_m) \), and IAP \( V_{\text{cap}} \) along the small intestinal longitudinal axis; 2) distribution of jejunal IAP protein abundance between the intracellular pool and the apical membrane; and 3) jejunal IAP mRNA abundance in association with the IAP transcriptional factor cdx1 protein abundance in early weaning group (WN) of piglets compared with their corresponding suckling group (SU).

Materials and Methods

The following animal handling procedures were approved by the Animal Care Committee at the University of Guelph. The piglets used in this study were cared for in accordance with the guidelines established by the Canadian Council of Animal Care (43).

Piglets and treatment groups. A total of 24 Yorkshire piglets, with a body weight (mean ± SE) of 3.23 ± 0.13 kg randomly taken from 12 different sows at 10 d of age were obtained from the Arkell Research Station at the University of Guelph and used in this study. The 12 piglets of the WN group, including 6 barrows and 6 females, were randomly taken from 6 different sow litters, moved off-site, and weaned on a corn and soybean meal-based weaning diet for 12 d according to standard swine industry early weaning practices (6). The diet was formulated to meet all nutrient requirements for the pigs according to the NRC (44) requirements. The weaning diet contained (g/kg diet, on an as-fed basis): corn meal (160), soybean meal (360), lactose (150), dextrose (81), corn oil (8.0), calcium carbonate (10.5), dicalcium phosphate (15.8), iodized salt (5.0), and an antibiotic mixture (1.0) that provided 0.044 g lincomycin/kg diet. Vitamin premix was included in the weaning diet to provide the following (mg/kg diet): retinyl palmitate (13.2), cholecalciferol (0.84), all-rac-α-tocopheryl acetate (96.0), menadione (3.0), riboflavin (5.25), niacin, (22.5), d-pantothenic acid (15.0), vitamin B-12 (0.026), thiamine (1.5), choline chloride (750.0), pyridoxine (2.25), d-biotin (0.08), and folic acid (0.45). The mineral premix was included in the weaning diet to provide the following (mg/kg diet): FeSO₄·H₂O (152), ZnCO₃ (95.9), MnSO₄·H₂O (6.2), CuSO₄·H₂O (11.8), KI (0.6), and Na₂SeO₃ (0.3). The other set of 12 piglets, including 6 barrows and 6 females, were randomly obtained from another 6 different sow litters and were allowed to continue suckling with their sows as the suckling treatment group for 12 d. The timing for tissue sample collection in this study was based on our previous report on the small intestinal epithelial life span in young pigs (26) and the expected adaptive changes in gut mucosa of weaning pigs (42).

Sample collection and processing. Piglets were sedated and maintained under anesthesia by inhalation of anesthetic isoflurane via a facial mask for the collection of the small intestinal samples (45). The abdomen was opened and the entire small intestine distal to the ligament of Treitz was removed and was immediately flushed with ice-cold saline containing 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The segment of small intestine proximal to the ligament of Treitz was designated as the duodenum, with the stomach being removed. The mesentery-free small intestinal segment proximal to the ileo-cecal ligament was designated as the ileum. The remainder of the small intestinal segment was divided into 2 equal portions as the proximal and the distal jejunal. All of the 4 intestinal segments were weighed and sampled from their middle regions and flash-frozen in liquid nitrogen (45). The frozen samples were subsequently pulverized under liquid nitrogen using mortar and pestle and stored at −80°C. For analyzing mucosal morphology, histology samples (15-cm segments) were taken from the middle portion of the proximal and the distal jejunal segments and stored in 80-mL vials containing 7.5 mmol/L phosphate-buffered formalin. At the end of tissue sampling, piglets were killed by an intracardiac injection of sodium pentobarbital (50 mg/kg body weight) (46).

Gut mucosal morphology measurements. The collected proximal and distal jejunal segments were further divided into short sections, placed in histology cassettes, and stored in the formalin. Slides were prepared by the Department of Histology at the Ontario Veterinary College at the University of Guelph. Each slide contained 9 transverse sections from each pig and was stained with hematoxylin and eosin. Measurements were obtained using a Leica DMR model light microscope (Leica Microscopy and Scientific Instruments Group) with a magnification of 5.0×. Morphological images were obtained with a Cool Cam Spot camera (Diagnostic Instruments) and were recorded by using the Open Lab (version 2.0) imaging software program (University of Warwick Science Park). Villus height, crypt depth, and smooth muscle thickness from 4 resulting cross-sections were measured from each slide for each pig. Measurements were taken only from well-oriented groups of 5 villi within each cross-section and a total of 13 villi from each cross-section were used for crypt and villous measurements (26).

Intestinal tissue homogenization and fractionation. Specifically, ~1.3 g of pulverized and frozen intestinal tissue samples was thawed in an ice-cold homogenizing buffer (50 mmol/L Tris-n-mannitol and 0.1 mmol/L PMSF at pH 7.4) at a ratio of 20 mL homogenizing buffer/g frozen intestinal tissue sample and homogenized using a polytron homogenizer.
The resulting homogenate samples were measured for their total volumes and sampled for analyses of protein content and enzyme activity kinetics for calculating intestinal segmental and whole gut mucosal IAP $V_{cap}$ as well as for Western blot analyses of IAP protein abundances.

The proximal jejunal homogenate was further partitioned to obtain the intracellular fraction and the apical membrane by Mg$^{2+}$ precipitation and differential centrifugation at 4°C according to our previous procedures (47). The remaining homogenate was centrifuged at 2400 × g for 20 min. The supernatant was mixed with 1 mol/L MgCl2 to contain a final concentration of 10 mmol/L MgCl2, stirred for 15 min, and then centrifuged at 2400 × g for 15 min. The top foamy layer was discarded and the resultant supernatant was centrifuged at 19,000 × g for 30 min to pellet the crude apical membrane. The resulting supernatant samples, regarded as the intracellular fraction, were removed, measured for their total volumes, and sampled for the analyses of their protein content and enzyme activity kinetics for calculating the proximal jejunal intracellular IAP $V_{cap}$ as well as for Western blot analysis of the IAP protein abundance.

The crude apical membrane pellets were resuspended in a suitable volume of buffer (300 mmol/L D-mannitol at pH 7.4) and centrifuged at 39,000 × g for 30 min to generate the final apical membrane pellets. These pellets were resuspended in the same buffer to yield the apical membrane suspension samples before measuring their total volumes and were sampled for the analyses of their protein content and enzyme activity kinetics for calculating proximal jejunal intracellular IAP $V_{cap}$ as well as for Western blot analysis of the IAP protein abundance.

To measure nuclear cdx1 protein abundance by Western blot analysis, the proximal jejunal nuclear protein was extracted using the CellLytic NuCLEAR extraction kit (Sigma-Aldrich). Approximately 100 mg of pulverized proximal jejunal tissue sample was homogenized in 1 mL of lysis buffer (50 mmol/L Tris-HCL, 10 mmol/L MgCl2, 15 mmol/L CaCl2, and 1.5 mol/L sucrose, pH 7.5) with 0.01% of the protease inhibitor cocktail (Sigma-Aldrich) by using a handheld polytron homogenizer. The homogenate was centrifuged at 11,000 × g for 20 min and the supernatant, i.e., the cytoplasmic, was removed. The crude nuclei pellet was resuspended in 150 µL of the provided extraction buffer with protease inhibitors, shaken for 30 min at 4°C, and centrifuged at 20,000 × g for 5 min according to the kit’s instructions. The supernatant, i.e., the nuclear protein fraction, was then removed, aliquoted, and snap-frozen in liquid nitrogen for later use in immunoblot procedures.

**Enzyme activity kinetic analyses.** Samples of the small intestinal homogenate and the partitioned proximal jejunal intracellular fraction and the apical membrane were analyzed for their protein contents using a commercial kit (Bio-Rad) and bovine serum albumin (fraction V) as the protein standard. Enzyme activities for IAP were conducted according to our previously established procedures (41). Potassium fluoride (2.0 mmol/L) was used in all IAP activity assays to inhibit acid phosphatase activity (41). Kinetics of IAP-specific activities in the proximal jejunal tissue homogenate and the partitioned intracellular fraction and apical membrane samples were carried out at 37°C for 10 min in a final volume of 1 mL suspension containing 10 µg of sample protein, 2.0 mmol/L KF, 4.0 mmol/L MgCl2, and 0–10.0 mmol/L P-nitrophenyl phosphate at pH 10.5 (41).

**Immunoblot analyses.** The proximal jejunal homogenate (100 µL), intracellular fraction (400 µL) and apical membrane (100 µL) samples were diluted (1:3) and solubilized in a resuspension buffer containing 25 mmol/L HEPES, 150 mmol/L NaCl, 0.20 mmol/L PMSF, 1% Triton X-100, and 0.4 g/L each of aprotinin, leupeptin, pepstatin A, N-tosyl-l-phenylalanine chloromethyl ketone, and N-α-tosyl-l-lysine ketone at pH 7.4. Immunoblot analyses of the target IAP and cdx1 proteins and the housekeeping protein β-actin were conducted as previously reported (48). Solubilized sample suspensions were boiled for 5 min and 30 µg of sample proteins were loaded and separated using 10.0% SDS-PAGE. Proteins were then transferred onto polyvinylidenedifluoride membranes (Bio-Rad) using a semidy transfer apparatus (Bio-Rad). Immunoblot for IAP was performed using a rabbit anti-human IAP (60 kDa) polyclonal antibody (GeneFex) under reducing conditions of SDS-PAGE (1:15,000 dilution in 6% skim milk powder), with a secondary rabbit anti-human IgG antibody (Bio-Rad), conjugated with horseradish peroxidase, and diluted to 1:20,000 in 6% skim milk powder. Immunoblot for cdx1 was performed using a mouse-anti-human cdx1 (23 kDa) monoclonal antibody diluted to 1:20,000 in 6% skim milk powder (Novus Biologicals). A mouse-anti-human β-actin (44 kDa) monoclonal antibody was diluted to 1:20,000 in 6% skim milk powder with a secondary mouse anti-human IgG antibody (Bio-Rad). Blots were developed using the chemiluminescence kit CPS1 (Sigma-Aldrich). Photographs of the membranes were taken using the Kodak Image Station 440 and densitometry was performed with Scion imaging software (Scion).

**Design of oligonucleotide primers.** Primers for amplification of target and housekeeping genes were developed using Primer 3 (49) software and were designed to overlap at least 2 exon boundaries. IAP primers were designed based on pig expressed sequence tag (Z84038) (50). Primer sequences were as follows: IAP (estimated product size, 105 bp): forward primer, 5'-CTAAAGGGGCGATGATGTTG-3’; and reverse primer, 5’-CACCTGTCGTGTCACAGTTG-3’; and β-actin (AY550069 (51); estimated product size, 150): forward primer, 5’-GGAAGTGGAGAGGATAAGC-3’ and reverse primer, 5’-ATCTGCTGGAAGGTTCCAAG-3’.

**RNA preparation and real time RT-PCR.** Total RNA was isolated from the proximal jejunal tissue samples using TRIZol reagent (Invitrogen). The RNA quality was confirmed by 1% agarose gel electrophoresis, stained with 25 mmol/L ethidium bromide, and had an OD260:OD280 ratio between 1.8 and 2.0. RNA was treated with DNase (Invitrogen) and quantitative real-time RT-PCR was performed in a Smart Cycler (Cepheid) using a Quantitect SYBR Green RT-PCR kit (Qiagen) according to the kit’s instructions.

**Calculations and statistical analyses.** The kinetic parameter estimates of alkaline phosphatase activity were obtained according to the Michaelis-Menten equation using the Fig. P curve fitting program (Fig. P, 1993, Biosoft, Cambridge, UK). The $V_{cap}$ of IAP with associated with the intestinal segments, the partitioned proximal jejunal fractions, and the whole small intestine was calculated according to the $V_{cap}$ definition by Weis et al. (52):

$$V_{cap} = \frac{[V_{max}\times\text{weight}_{\text{sample}}\times\text{protein}_{\text{sample}}] + [V_{max}\times\text{weight}_{\text{proximal jejunal}}\times\text{protein}_{\text{jejunal}}] + [V_{max}\times\text{weight}_{\text{distal jejunal}}\times\text{protein}_{\text{jejunal}}] + [V_{max}\times\text{weight}_{\text{duodenum}}\times\text{protein}_{\text{duodenum}}]}{(\text{BW} \times 14400 \text{min} / \text{d} \times 1000 \times 1000)}.$$

where $V_{cap}$ is the digestive capacity of the small intestinal mucosal IAP [mol/(kg body weight·d)], $V_{max}$ is the maximal enzyme-specific activity for all correspondingly labeled small intestinal segments and the further partitioned proximal jejunal fractions [μmol/mg protein·min], $\text{weight}$ is fresh tissue weight for all correspondingly labeled intestinal segments (g), protein is protein content in all correspondingly labeled intestinal tissues and the further partitioned proximal jejunal fractions (mg protein/g fresh tissue), and BW is body weight of corresponding individual piglet (kg).

The ratio of the expression of the target gene IAP relative to the housekeeping gene β-actin was calculated (53) as:

$$R = 2^{-\Delta\Delta Ct(\text{target-housekeeping})},$$

where $R$ is the relative expression ratio value of the target gene and Ct is the cycle number at the threshold at which both the target gene IAP and the housekeeping gene β-actin are amplified beyond 30 fluorescence units. Optimal real-time PCR efficiencies were acquired by amplification of dilution series of RNA according to the eq. $10^{-\Delta\Delta Ct}$ and were consistent between IAP and β-actin.

Homogeneity of variances was examined and confirmed by the Levene’s test for the endpoints, including, histology, IAP protein, and mRNA abundances and cdx1, using SAS (SAS Institute). Differences in these endpoints between the SU and WN groups were compared by ANOVA and Pearson correlation coefficients were calculated using General Linear Model of SAS. Comparison of the kinetic parameter
estimates for IAP activities was conducted by using the pooled t test (54). Where appropriate, data are presented as means ± SE or pooled SEM. P-values < 0.05 were considered significant.

Results

During the experimental period, the WN pigs were fed 162.8 ± 11.3 g of the weaning diet per day. WN pigs had lower (P < 0.05) daily body weight gains (55.8 ± 8.5 g, n = 12) than the SU pigs (172.2 ± 8.9 g, n = 12). Villus heights in the proximal and the distal jejunum were less (P < 0.05) by 55 and 51%, respectively, in the WN pigs than in the SU piglets (Table 1). Conversely, crypt depth in these intestinal segments increased (P < 0.05) by 128 and 98%, respectively, in the WN pigs compared with the SU piglets (Table 1). Furthermore, the total mucosal thickness in both the proximal and the distal jejunal segments was reduced (P < 0.05) by 20% in the WN pigs compared with the SU pigs (Table 1).

The kinetics and V_{cap} of the IAP in the small intestinal segments of the duodenum, proximal jejunum, distal jejunum, and ileum in the WN piglets are summarized compared with the SU piglets (Table 2; Supplemental Fig. 1A). In the proximal and distal jejunal segments, weaning decreased (P < 0.05) mucosal IAP K_{m}, V_{max}, and V_{cap} by 32, 19, and 51%, respectively, compared with the SU piglets (Table 2; Supplemental Fig. 1B,C). In the ileum, whereas mucosal K_{m} and V_{max} values did not differ between the WN and SU groups, V_{cap} was reduced (P < 0.05) by 26% in the WN group compared with the SU group (Table 2; Supplemental Fig. 1D) due to the greater (P < 0.05) ileal protein mass in the WN piglets (388.44 ± 5.84 mg protein/kg body weight) than in the SU piglets (485.08 ± 9.29 mg protein/kg body weight). Furthermore, weaning reduced the total gut mucosal IAP K_{m} by 26%, V_{max} by 22%, and V_{cap} by 19%, respectively, compared with the SU group.

The proximal and distal jejunal segments accounted for 84 and 86%, respectively, of the whole gut mucosal IAP V_{cap}. We next partitioned the distribution of IAP activity into the intracellular fraction and the apical membrane (Table 3). The apical membrane-associated V_{cap} accounted for 98% of the IAP.

| TABLE 1 | Mucosal morphology in the jejunum of SU and WN piglets fed a corn and soybean meal-based diet |
|---|---|---|
| Item | SU | WN | SEM |
| **Proximal jejunal segment** | | | |
| Villus height | 501.5 | 223.6* | 0.91 |
| Crypt depth | 138.4 | 315.1* | 0.59 |
| Villus height:crypt depth | 3.8 | 0.8* | 0.01 |
| Mucosal thickness | 639.9 | 512.0* | 0.95 |
| Smooth muscle thickness | 182.4 | 175.1 | 0.85 |
| **Distal jejunal segment** | | | |
| Villus height | 499.8 | 246.1* | 1.64 |
| Crypt depth | 128.9 | 255.2* | 0.60 |
| Villus height:crypt depth | 4.2 | 1.0* | 0.01 |
| Mucosal thickness | 628.7 | 501.4* | 1.83 |
| Smooth muscle thickness | 189.8 | 203.2 | 1.16 |

1 Values are means and the pooled SEM, n = 12. *Different from SU, P < 0.05.

2 Mucosal thickness = villus height + crypt depth.

| TABLE 2 | Small IAP enzyme K_{m}, V_{max}, and V_{cap} in SU and WN piglets |
|---|---|---|
| Item | Duodenum | Proximal jejunum | Distal jejunum |
| | mmol/L | μmol/(mg protein min) | μmol/(kg body weight d) |
| SU | 8.36 ± 0.72 | 0.421 ± 0.062 | 0.139 ± 0.010 |
| WN | 11.06 ± 1.69* | 0.342 ± 0.021* | 0.068 ± 0.011* |
| **Villus height:** | | | |
| SU | 6.99 ± 0.79 | 0.509 ± 0.048 | 0.958 ± 0.029 |
| WN | 7.87 ± 0.58* | 0.432 ± 0.032* | 0.831 ± 0.018* |

1 Values are parameter estimates ± SE, n = 60 measured in tissues from 10 piglets at 6 different substrate concentrations. *Different from SU, P < 0.05.

We conducted Western blot analyses to investigate the relationship between IAP V_{max} and IAP protein abundance in the proximal jejunal jejunum and ileal membrane fractions. A 60-kDa IAP protein was identified in the proximal jejunal tissue homogenate, the intracellular pool, and the apical membrane by 48 and 49%, respectively, compared with the suckling piglets (Table 3; Supplemental Fig. 2). Whereas weaning did not affect the proximal jejunal IAP V_{max} in the intracellular fraction, it decreased (P < 0.05) the proximal jejunal IAP V_{max} in the intracellular fraction by 17% due to the smaller (P < 0.05) intracellular protein mass in the WN piglets (280.65 ± 3.06 mg protein/kg body weight) than in the SU piglets (317.31 ± 4.09 mg protein/kg body weight). Furthermore, weaning reduced (P < 0.05) the proximal jejunal IAP V_{max} and V_{cap} in the apical membrane by 10 and 13%, respectively, compared with the SU piglets.

We conducted Western blot analyses to investigate the relationship between IAP V_{max} and IAP protein abundance in the proximal jejunal jejunum and ileal membrane fractions. A 60-kDa IAP protein was identified in the proximal jejunal tissue homogenate, the intracellular pool, and the apical membrane by 48, 53, and 64%, respectively, compared with the SU group (Fig. 1). There were positive correlations (P < 0.05) between the IAP V_{max} and the IAP protein abundances in the proximal jejunal jejunum and ileum in the WN piglets compared with the SU piglets (Table 3). Weaning reduced (P < 0.05) the proximal jejunal IAP K_{m} and V_{max} in the proximal jejunal jejunum in both the WN and the SU pigs (Table 3). Weaning reduced (P < 0.05) the proximal jejunal IAP K_{m} in both the intracellular fraction and the apical membrane by 48 and 49%, respectively, compared with the suckling piglets (Table 3). Weaning reduced (P < 0.05) the proximal jejunal IAP V_{cap} in the intracellular fraction by 17% due to the smaller (P < 0.05) intracellular protein mass in the WN piglets (280.65 ± 3.06 mg protein/kg body weight) than in the SU piglets (317.31 ± 4.09 mg protein/kg body weight). Furthermore, weaning reduced (P < 0.05) the proximal jejunal IAP V_{max} and V_{cap} in the apical membrane by 10 and 13%, respectively, compared with the SU piglets.

| TABLE 3 | Proximal jejunal intracellular and apical membrane-bound IAP enzyme K_{m}, V_{max}, and V_{cap} in SU and WN piglets |
|---|---|---|
| Item | Intracellular fraction | Apical membrane |
| | mmol/L | μmol/(mg protein min) | μmol/(kg body weight d) |
| | | | |
| SU | 1.44 ± 0.08 | 0.082 ± 0.001 | 0.018 ± 0.001* |
| WN | 2.13 ± 0.05* | 0.078 ± 0.001 | 0.015 ± 0.001* |
| **Villus height:** | | | |
| SU | 1.25 ± 0.05 | 0.728 ± 0.011 | 0.338 ± 0.032 |
| WN | 1.86 ± 0.07* | 0.662 ± 0.009* | 0.813 ± 0.021* |

1 Values are parameter estimates ± SE, n = 60 measured in tissues from 10 piglets at 6 different substrate concentrations. *Different from SU, P < 0.05.
homogenate \((r = 0.51; P = 0.023; n = 20)\), the intracellular pool \((r = 0.41; P = 0.032; n = 20)\), and the apical membrane \((r = 0.52; P = 0.020; n = 20)\) in the SU and WN piglets.

To understand the contributions of IAP gene transcription and its mRNA abundance to IAP \(V_{\text{max}}\) and protein levels, real-time RT-PCR analyses were conducted to measure changes in the relative abundance of IAP mRNA in the proximal jejunum of both WN and SU piglets. Weaning reduced \((P < 0.05)\) the relative abundance of the proximal jejunal IAP mRNA \((WN, 0.058 \pm 0.011 \text{ vs. SU, } 0.138 \pm 0.013, \text{ in arbitrary units})\) by 58\% compared with the SU piglets. Pearson correlation analyses indicated linear relationships \((r = 0.41; P = 0.029; n = 20)\) between the IAP \(V_{\text{max}}\) and the relative abundance of IAP mRNA in the proximal jejunum of both WN and SU piglets. There were also linear relationships \((P < 0.05)\) between IAP protein abundances in the proximal jejunal homogenate \((r = 0.48; P = 0.047; n = 20)\), the intracellular fraction \((r = 0.60; P = 0.005; n = 20)\), and the apical membrane \((r = 0.49; P = 0.027; n = 20)\) and the relative abundance of IAP mRNA in the proximal jejunum of both the WN and the SU piglets.

The abundances of the 23-kDa cdx1 did not differ between the proximal jejunal cytoplasmic protein extract and the nuclear protein extract in both the WN and the SU groups (Fig. 2). However, weaning decreased \((P < 0.05)\) the proximal jejunal cytoplasmic and nuclear cdx1 protein abundances by 27 and 29\%, respectively, compared with the SU piglets (Fig. 2). Furthermore, there were very similar levels of positive linear relationships \((P < 0.05)\) between the relative abundance of IAP mRNA and the cytoplasmic \((r = 0.60; P = 0.005; n = 20)\) and the nuclear \((r = 0.61; P = 0.004; n = 20)\) cdx1 protein abundances in the proximal jejunum in the WN and SU piglets.

The results of IAP mRNA abundance in the proximal jejunum and Pearson correlation analysis suggest that WN decreased the \(V_{\text{max}}\) and \(V_{\text{cap}}\) in part at the transcriptional level by reducing the steady-state IAP gene mRNA abundance. This observation is consistent with the notion that the expression of IAP is cell differentiation dependent and is partially regulated at the transcriptional level (18,30). Western blot analysis identified the porcine IAP protein at ~60 kDa in both the intracellular fraction and the apical membrane of the proximal jejunum, which is a similar size of the IAP protein as reported in humans (25). In postsmtial developing rats, the jejunal IAP protein was identified to be the 65-kDa IAP isomer (55). Cell-free translation of the rat IAP encoding mRNA has yielded 2 IAP protein

diagram1.png

**FIGURE 1** Proximal jejunal IAP protein abundance in the mucosal homogenate, intracellular pool, and the apical membrane in SU and WN piglets. Values are mean \pm SEM, \(n = 10\). *Different from SU, \(P < 0.05\).

diagram2.png

**FIGURE 2** The cdx1 protein abundance in the proximal jejunal mucosal (**A**) cytoplasmic protein extract and (**B**) the nuclear protein extract in SU and WN piglets. Values are mean \pm SEM, \(n = 10\). *Different from SU, \(P < 0.05\).

**Discussion**

Our major objectives in this study were to investigate the effect of weaning in parallel with a normal suckling process on the small intestinal IAP maximal enzyme activity, digestive capacity, catalytic affinity, and IAP gene expression in piglets. Results from this study indicated that weaning significantly reduced the small intestinal IAP digestive capacity and the maximal enzyme activity primarily in the jejunal region in pigs. There is a scarcity of literature reports regarding the effect of weaning on IAP activity and gene expression. Our observations of decreased IAP \(V_{\text{max}}\) and \(V_{\text{cap}}\) associated with weaning are in contrast to the findings by Miller et al. (40), who did not observe a weaning effect on IAP activity in piglets weaned at the ages of 3 and 5 wk. Two factors may help explain the discrepancy between this study and the study by Miller et al. (40). First, there were differences in weaning ages and weaning duration between this study with piglets weaned at the age of 10 d for 12 d and the study by Miller et al. (40) with piglets weaned at the age of 5 wk for 5 d. Second, IAP \(V_{\text{max}}\) values were measured in this study. However, only 1 substrate concentration at 1.5 mmol/L of anilide phosphate was used for measuring IAP activity by Miller et al. (40). Thus, IAP-specific activity far below the \(V_{\text{max}}\) level was likely reported by Miller et al. (40). As demonstrated in Supplemental Figures 1 and 2, IAP-specific activity values that are measured below the \(V_{\text{max}}\) level are affected by substrate concentrations used, which cannot reflect changes in the IAP digestive capacity and IAP protein abundances.

The results of IAP mRNA abundance in the proximal jejunum and Pearson correlation analysis suggest that WN decreased the \(V_{\text{max}}\) and \(V_{\text{cap}}\) in part at the transcriptional level by reducing the steady-state IAP gene mRNA abundance. This observation is consistent with the notion that the expression of IAP is cell differentiation dependent and is partially regulated at the transcriptional level (18,30). Western blot analysis identified the porcine IAP protein at ~60 kDa in both the intracellular fraction and the apical membrane of the proximal jejunum, which is a similar size of the IAP protein as reported in humans (25). In postsmtial developing rats, the jejunal IAP protein was identified to be the 65-kDa IAP isomer (55). Cell-free translation of the rat IAP encoding mRNA has yielded 2 IAP protein
isoforms with 62 and 65 kDa (56). In theory, the relative IAP protein abundances in the partitioned proximal jejunal intracellular fraction and the apical membrane, as analyzed by Western blotting, are the net balance between the biosynthesis of IAP and IAP trafficking via distinctive vesicular carriers and/or IAP degradation (33,35,38). For both the WN and SU groups, the apical membrane-associated IAP $V_{\text{max}}$ accounted for the majority (98%) of the IAP activity in the proximal jejunum, suggesting a very small intracellular IAP protein pool in the cell. Thus, the reduction in IAP protein abundance in the proximal jejunum associated with WN was largely due to decreased de novo synthesis of IAP protein, which needs to be further confirmed in future in vivo IAP labeling and biosynthesis studies. On the other hand, significant changes in IAP $K_m$ were also observed in the jejunal region in response to weaning. Postnatal decreases in jejunal IAP affinity from suckling to postweaning were reported in pigs in our previous studies (41). Changes in IAP $K_m$ and $V_{\text{max}}$ and its protein abundance in the partitioned proximal jejunal fractions collectively suggest that weaning modified jejunal IAP protein to be lower in its affinity. This change in IAP affinity is likely due to modifications that occurred in the posttranslational glycosylation of IAP protein, as discussed by Fan et al. (41). Thus, the reductions in the jejunal IAP $V_{\text{max}}$ and $V_{\text{cap}}$ resulted from the combined effects of decreases in IAP protein abundances and IAP affinity. On the other hand, it is known that the complete replacement of fetal enterocytes occurs at 19 d of age under normal suckling (16) and jejunal epithelial life span is 10 d in neonatal pigs (26). Therefore, it can be concluded that weaning decreased IAP digestive capacity by affecting IAP gene expression at the transcriptional and the post-transcriptional levels in newly replaced enterocytes. Given the important roles of IAP, including detoxification of toxins, maintaining lumen pH, digesting organic phosphate and fat absorption, decreases in the IAP digestive activity, digestive capacity, IAP catalytic affinity, and the jejunal IAP protein and mRNA abundances, as revealed from this study, provide information on the mechanisms regulating IAP enzymatic capacity in the small intestine. These findings could be useful in explaining the susceptibility of early-weaned piglets to increased occurrence of enteric diseases and growth-check.

The decreased jejunal IAP gene transcription and IAP protein expression in response to weaning might have been caused by hormonal and inflammatory factors. Epidermal growth factor is one of the milk-borne growth factors contributing to postnatal gut mucosal growth and development (57). Several lines of evidence suggest that oral administration of epidermal growth factor improved gut mucosal cell proliferation and differentiation (57,58) and IAP activity (59) in weaning piglets. Weaning removes milk-borne epidermal growth factor from the gut lumen of weaning piglets, thus partially contributing to weaning-associated villous atrophy and mucosal dysfunction, including a normal expression of IAP. It has been well documented that weaning causes intestinal inflammation marked by the upregulation of the proinflammatory cytokine genes such as tumor necrosis factor-$\alpha$ and interleukin-6 (60). Furthermore, the proinflammatory cytokine tumor necrosis factor-$\alpha$ and interleukin-6 have been shown to inhibit IAP gene expression (31). Thus, bowel inflammation associated with weaning might have contributed to reduced IAP mRNA abundance observed in this study. The lack of epidermal growth factor in the gut lumen and the increased bowel mucosal proinflammatory cytokine levels may be responsible for the reduced jejunal IAP gene expression in the renewed enterocyte of early-weaned piglets in this study.

The reductions in weaning-associated jejunal IAP gene transcription and IAP protein abundance were due to villous atrophy and increased crypt enterocyte population, which was likely further exacerbated by a decreased availability of enteral nutrition to weanling pigs via nutrient-dependent mechanisms. The daily feed intake of 163 g in the WN pigs in this study was lower than the projected feed intake level of ~240 g/d (~6% of the WN pigs’ live body weigh averaged at ~4 kg) according to the NRC (44). It has been well documented that weaning piglets on soybean meal-based diets have decreased feed intake (9). Furthermore, gut mucosal villous atrophy and crypt hyperplasia during the weaning transition were also observed in this study and these had been shown to result from weaning-associated anorexia (60). Intestinal epithelial enterocytes are polarized cells that are dependent upon the enteral supply of trophic nutrients for cellular proliferation and differentiation and this has been well demonstrated in the study by Burrin et al. (45), who established minimal levels of enteral nutrient requirements for the gut in neonatal pigs receiving total parenteral nutrition. Enteral nutrients can increase enterocyte differentiation, IAP gene transcription, and IAP protein expression via nutrient-dependent manner via the following mechanisms. First, enteral nutrients such as amino acids are essential energy substrates for providing metabolic fuels and for serving as precursors for intracellular protein synthesis during the epithelial proliferation and differentiation (34,61). Second, enteral nutrients such as trophic neutral amino acids are also signaling molecules and can stimulate the mammalian target of rapamycin-dependent signaling pathway (34), thereby increasing global synthesis of intracellular proteins, including IAP protein and the IAP gene cis transcriptional factor cdx1. cdx1 is known to positively enhance IAP gene transcription via interaction with IAP promoter in the formation of a viable transcription complex (27). Thus, cdx1 ought to be highly expressed in differentiated villus enterocytes. However, this is in contrast to the observation in the study by Silberg et al. (62) showing that cdx1 was highly expressed in the crypt cell. The reduced cdx1 protein abundance in the jejunum from this study further supports the findings that cdx1 promotes enterocyte differentiation and IAP gene expression, as demonstrated by Alkhoury et al. (27) and Soubeiran et al. (63). Two rodent studies have directly shown that adequate enteral nutrition is essential to maintaining IAP gene expression (25,64). Therefore, inadequate enteral nutrient availability due to weaning-associated anorexia may be partially responsible for the downregulation of jejunal IAP gene expression in the replaced enterocytes of early-weaned pigs. Research should be conducted to attempt to upregulate intestinal IAP gene expression and its digestive capacity via dietary supplementation of gut trophic nutrients for improving weaning pig nutrition.

In conclusion, weaning decreased the gut IAP digestive capacity, maximal activity, and IAP $K_m$ primarily in the jejunum at both the transcriptional and post-transcriptional levels in the renewed enterocyte of the pig. Early-weaned piglets are vulnerable to enteric infections and are susceptible to developing diarrhea and growth-check, which may in part be due to the compromised protective and functional roles of jejunal mucosal alkaline phosphatase.

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M.Z.F. had primary responsibility for final content. All authors read and approved the final manuscript.

**Literature Cited**


