Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs

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Moeser AJ, Ryan KA, Nighot PK, Blikslager AT. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. Am J Physiol Gastrointest Liver Physiol 293: G413–G421, 2007. First published May 24, 2007; doi:10.1152/ajpgi.00304.2006.—Our previous work has demonstrated that weaning at 19 days of age has deleterious effects on mucosal barrier function in piglet intestine that are mediated through peripheral CRF receptor signaling pathways. The objectives of the present study were to assess the impact of piglet age on weaning-associated intestinal dysfunction and to determine the role that mast cells play in weaning-induced breakdown of mucosal barrier function. Nursing Yorkshire-cross piglets were either weaned at 19 days of age (early-weaned, n = 8) or 28 days of age (late-weaned, n = 8) and housed in nursery pens. Twenty-four hours postweaning, segments of midjejunum and ascending colon from piglets within each weaning age group were harvested and mounted on Ussing chambers for measurements of transepithelial electrical resistance and serosal-to-mucosal [3H]mannitol fluxes. Early weaning resulted in reductions in transepithelial electrical resistance and increases in mucosal permeability to [3H]mannitol in the jejenum and colon (P < 0.01). In contrast, postweaning reductions in intestinal barrier function were not observed in piglets weaned at 28 days of age. Early-weaned piglet intestinal mucosa had increased expression of CRF receptor 1 protein, increased mucosal mast cell tryptase levels, and evidence of enhanced mast cell degranulation compared with late-weaned intestinal mucosa. Pretreatment of piglets with the mast cell stabilizer drug cromolyn, injected intraperitoneally 30 min prior to weaning, abolished the early-weaning-induced intestinal barrier disturbances. Our results indicate that early-weaning stress induces mucosal dysfunction mediated by intestinal mast cell activation and can be prevented by delaying weaning.

Previous research has shown that the weaning process has a deleterious impact on piglet intestinal mucosal health, highlighted by increased intestinal permeability and heightened baseline and agonist-stimulated secretory responses in the weaned intestine (8, 10, 30, 42). These disturbances in intestinal function likely play a significant role in postweaning enteric disorders. For example, increased intestinal permeability allows the transmigration of luminal antigens and toxins across the “leaky” epithelium and into subepithelial tissues, lamina propria, and underlying circulation, inciting inflammatory processes and systemic disease (24, 31). Furthermore, compromised barrier function can lead to activation and hypersensitization of enteric neural pathways by inflammatory mediators which can exacerbate postweaning diarrheal diseases such as enterotoxigenic E. coli, rotavirus, and Clostridium difficile (28). The underlying mechanisms of weaning-induced intestinal dysfunction or management interventions that may aid in the prevention of these intestinal responses are not well understood.

Our previous experiments have demonstrated that weaning occurring at 19 days of age (average weaning age employed in commercial swine production) triggers the breakdown of intestinal barrier function characterized by reductions in transepithelial electrical resistance (TER) and increased paracellular [3H]mannitol flux (30). These deleterious changes in mucosal function in the weaned piglet were shown to be mediated by activation of peripheral corticotropin releasing factor (CRF) receptors; however, the exact mechanisms are unclear.

One particular cell type that has been linked with CRF/CRF receptor signaling pathways and stress-induced alterations in barrier function is the mast cell. Mast cells and their products are becoming well recognized as central mediators of several intestinal disorders (3, 17, 21). Although mast cells are most well known for their role in allergy and parasitic intestinal diseases, there is mounting evidence for their prominent role in stress-related gastrointestinal disorders including irritable bowel syndrome (IBS) and the inflammatory bowel diseases (3, 4, 14, 33). Rodent stress models have demonstrated a link between stress (psychological and physical), CRF receptor signaling pathways, and mast cell function in intestinal pathophysiology (12, 20, 37). For example, CRF-induced colonic hyperpermeability and short circuit current elevations observed in stressed rats was prevented by pretreatment with the mucosal mast cell stabilizer drug, doxantrazole (37). It was also found that cholinergic and adrenergic nerves were important in these intestinal responses to stress, suggesting close

IN NATURE, WEANING IN THE PIG IS A GRADUAL PROCESS THAT OCCURS AT ~3 MO OF AGE AND REPRESENTS THE SHIFT FROM THE PIGLETS RELIANCE ON SOW’S MILK TO OTHER FOOD SOURCES (32). HOWEVER, IN MOST MODERN U.S. SWINE PRODUCTION SYSTEMS, WEANING IS AN ABRupt PROCESS OCCURRING EARLY IN LIFE AT AROUND 19 DAYS OF AGE. EARLY WEANING IS STRESSFUL BECAUSE THE PIGLET MUST RAPIDLY ADAPT TO DRAMATIC CHANGES IN ITS SOCIAL AND PHYSICAL ENVIRONMENT. SUCH CHANGES INCLUDE MATERNAL AND LITTERMATE SEPARATION, ABRupt CHANGES IN DIET, COMMINGLEING WITH UNFAMILIAR PIGS, AND PHYSICAL ESTABLISHMENT OF THE SOCIAL HIERARCHY. THESE COMBINED STRESSORS HAVE A SIGNIFICANT IMPACT ON POSTWEANING PIG HEALTH AND WELFARE THROUGH REDUCTIONS IN FEED INTAKE AND PERFORMANCE, DEVELOPMENT OF BEHAVIORAL VICES, AND INCREASED SUSCEPTIBILITY TO DISEASE (15, 19, 23).
interactions between CRF receptors, mast cells, and enteric nerves that mediate stress responses in the gut. The latter likely represents the close proximity of mast cells to enteric nerves within the intestinal mucosa (17, 33, 35). Moreover, conclusive evidence has been derived from experiments with mast cell-deficient rats, a genotype shown to be resistant to stress-induced defects in colonic epithelial barrier function (38).

The following experiments were conducted to assess the impact of management strategies aimed at reducing the stress of weaning such as delaying weaning on intestinal responses to weaning stress and to further define the mechanisms of weaning-induced breakdown in intestinal barrier function specifically investigating the role of mucosal mast cells.

METHODS

Animals and weaning protocol. The North Carolina State University Institutional Animal Care and Use Committee approved all studies. After farrowing, Yorkshire crossbred piglets of either sex were housed in standard farrowing crates with sows and subjected to routine management practices. A representative sample of piglets (adjusted for body weight) was randomly assigned to one of two weaning age groups: weaning at 19 days of age (early-weaned) or 28 days of age (late-weaned). On respective weaning days, two to three piglets from three different sows were weaned and housed in an on-site swine nursery facility until intestinal studies were conducted. The remaining unweaned pigs from each litter were handled briefly (sham stress) but were returned to the sow to continue to nurse and served as unweaned controls. Weaned pigs were immediately offered ad libitum access to water and a commercial nursery pig diet (Renaissance Nutrition, Roaring Spring, PA). Twenty-four hours after

Fig. 1. Effects of weaning age on jejunal transepithelial electrical resistance (TER) and [3H]mannitol permeability. Values represent means ± SE; n = 8. Pigs were weaned at 19 days of age (early-weaned) or 28 days of age (late-weaned). Age-matched control animals were left with the sow to continue to nurse (unweaned). Twenty-four hours postweaning, segments of midjejunum from weaned and unweaned pigs were mounted in Ussing chambers for measurement of TER and mucosal-to-serosal [3H]mannitol flux. Values from each tissue were averaged over a 1-h experimental period. A: early-weaned jejunal mucosa had reduced TER values compared with unweaned littermate controls. Late-weaned jejunal mucosa TER values did not differ from unweaned control tissues (* P < 0.01). B: early-weaned jejunum exhibited significant elevations in [3H]mannitol flux whereas late-weaned mucosa did not differ from unweaned controls (* P < 0.001).

Fig. 2. Effects of weaning age on colonic TER and [3H]mannitol permeability. Values represent means ± SE; n = 8. Pigs were weaned at 19 days of age (early-weaned) or 28 days of age (late-weaned). Age-matched control animals were left with the sow to continue to nurse (unweaned). Twenty-four hours postweaning, segments of ascending colon from weaned and unweaned pigs were mounted in Ussing chambers for measurement of TER and [3H]mannitol flux over a 2-h experimental period. A: early-weaned colonic mucosa had reduced TER values compared with unweaned littermate controls. Late-weaned colonic TER values were did not differ from unweaned control tissues (* P < 0.01). B: early-weaned colon exhibited significant elevations in [3H]mannitol flux whereas late-weaned colonic mucosa did not differ from unweaned controls (* P < 0.001).
weaning, n = 8 weaned pigs and n = 8 unweaned pigs were sedated with a combination of xylazine (1.5 mg im) and ketamine (11 mg/kg im) followed by euthanasia with an overdose of intravenous pentobarbital via a catheterized ear vein. Initial sedation was used to minimize stress before intestine was obtained for subsequent intestinal studies. Segments of midjejunum and ascending colon were immediately harvested following euthanasia and prepared for Ussing chamber studies. Ussing chamber experiments. Segments of midjejunum and ascending colon were harvested from the pig and the mucosa was stripped from the seromuscular layer in oxygenated (95% O2-5% CO2) Ringer solution (in mmol/l: 154 Na+, 6.3 K+, 137 Cl−, 0.3 H2PO4, 1.2 Ca, 0.7 Mg, 24 HCO3−; pH 7.4). Tissues were then mounted in 1.13-cm²-aperture Ussing chambers, as described in previous studies.(1) Tissues were bathed on the serosal and mucosal sides with 10 ml of Ringer solution. The serosal bathing solution contained 10 mM glucose, which was osmotically balanced on the mucosal side with 10 mM mannitol. Bathing solutions were oxygenated (95% O2-5% CO2) and circulated in water-jacketed reservoirs maintained at 37°C. The spontaneous potential difference (PD) was measured via Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes by a voltage clamp that corrected for fluid resistance. Tissues were maintained in the short-circuited state, except for brief intervals to record the open-circuit PD. Transepithelial electrical resistance (Ω·cm²) was calculated from the spontaneous PD and short-circuit current, as previously described (2). After a 30-min equilibration period on Ussing chambers, TER was recorded at 15-min intervals over a 1-h period and then averaged to derive the basal TER values for a given animal. Mucosal-to-serosal fluxes of 3H-labeled mannitol. Mucosal barrier properties were further assessed in terms of mucosal-to-serosal fluxes of 3H-labeled mannitol performed at the same time as TER measurements. After a 15-min period on Ussing chambers, 0.2 μCi/ml 3H-labeled mannitol was added to the mucosal side of Ussing chamber-mounted tissues. The isotope was allowed to equilibrate for 15 min, after which standards were taken from the mucosal side of each chamber (time t = 30 min), and a 60-min flux period was established by taking 0.5-ml samples from the serosal compartment at the beginning and end of the 60-min flux period. The presence of 3H was established by measuring β-emission in a liquid-scintillation counter (LKB Wallac, model 1219 Rack Beta, Perkin Elmer Life and Analytical Sciences, Boston, MA). Unidirectional 3H-labeled mannitol mucosal-to-serosal fluxes were evaluated by determining mannitol specific activity added to the mucosal bathing solution and calculating the net appearance of tritium over time in the serosal bathing solution on a chamber unit area basis. ELISA. Blood samples were obtained from pigs via venipuncture 24 h postweaning and prior to euthanasia. Pigs were sedated prior to blood collection to minimize the stress of sampling procedures. All samples were taken at the same time of day to minimize the effects of diurnal rhythms. Serum was separated by centrifugation (20 min, 10,000 g) and the serum was stored at −80°C until analysis. Serum levels of CRF, cortisol, and mast cell tryptase were determined by using commercial ELISA kits (CRF, Phoenix Pharmaceuticals, Belmont, CA; cortisol, R&D Systems, Minneapolis, MN; mast cell tryptase, Chemicon, Temecula, CA).

Histological analyses. Jejunal and colonic tissues were taken immediately after euthanasia and stored in 10% neutral buffered formalin until processing for routine histological evaluation. Jejunal and colonic tissues were sectioned (5 μm) and stained with hematoxylin and eosin and viewed under a light microscope. For quantification of mast cells, intestinal tissues (n = 4 animals/treatment group) were fixed in Carnoy’s fluid and sectioned for immunohistochemistry. Tissue sections were then processed for immunohistochemistry and stained with toluidine blue (Sigma, St. Louis, MO). Mast cells were counted at a ×40 magnification using a micrometer grid fitted within an eyepiece. At this magnification, the grid covered a 0.011-mm² area. For each tissue slide, six nonoverlapping areas above the muscularis

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\includegraphics[width=0.8\textwidth]{histological_images}
\caption{Histological appearance of early and late-weaned intestinal tissues. Figures are representative of n = 6 animals. Tissues were harvested 24 h postweaning. A: early-weaned jejunum. B: late-weaned jejunum. C: early-weaned colon. D: late-weaned colon. Bar = 100 μm. E-G: morphometric analysis jejunum of early- and late-weaned piglets.}
\end{figure}
mucosae were counted for the estimation of mucosal mast cell numbers and were expressed as cells per square millimeter. *G416 WEANING AGE INFLUENCES INTESTINAL DYSFUNCTION IN PIGS

**Gel electrophoresis and Western blotting.** Colonic mucosal scrapings from weaned and unweaned pigs were snap frozen and stored at −70°C before SDS-PAGE. Tissue aliquots were thawed at 4°C and added to 3 ml of chilled lysis buffer, including protease inhibitors at 4°C, as previously described. This mixture was homogenized on ice and then centrifuged at 4°C, and the supernatant was saved. Protein analysis of extract aliquots was performed (DC assay; Bio-Rad, Hercules, CA). Tissue extracts (amounts equalized by protein concentration) were mixed with an equal volume of 2× SDS-PAGE sample buffer and boiled for 4 min. Lysates were loaded on a 10% SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocols. Proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Science, Birmingham, UK) by using an electroblotting minitransfer apparatus. Membranes were blocked at room temperature for 60 min in Tris-buffered saline plus 0.05% Tween 20 and 5% dry powdered milk. Membranes were washed and incubated with primary antibody (goat CRF-r1 polyclonal antibody, Santa Cruz, Santa Cruz, CA, or mouse mast cell tryptase monoclonal antibody, Chemicon International, Temecula, CA). A β-actin antibody (Abcam, Cambridge, MA) was used as a control for protein loading. After additional washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody and developed for visualization of protein by the addition of enhanced chemiluminescence reagent (Amersham, Piscataway, NJ) as previously described. Following primary immunoblot analysis, membranes were stripped and reprobed a β-actin antibody (Abcam, Cambridge, MA) to ensure equal protein loading within lanes. Quantitative results were obtained by scanning the resulting images and analyzing them densitometrically using SigmaScan Pro 5 software (Point Richmond, CA).

**IF labeling.** Immunofluorescence (IF) labeling was performed on colonic tissues that were embedded and frozen in optimal cutting temperature medium. Frozen tissues were cut in 5-μm-thick sections and mounted on glass slides. Tissue sections were blocked with 2% BSA prior to incubation with either goat anti-CRF-r1 polyclonal antibody (1:250) or mouse anti-mast cell tryptase (1:150) in Blotto for 2 h at 4°C. For co-IF experiments, CRF-r1 and mast cell tryptase were incubated together. Sections were washed with Blotto and incubated for 45 min with Texas red-conjugated anti-goat secondary antibody (1:300; Santa Cruz) in the dark. Sections were mounted and well-oriented villi were examined with a Vanox AHS-3 photomicroscope linked to a Spot RT slider cooled-charge-coupled device digital camera.

**Mast cell stabilizer drug experiments.** Nineteen-day-old pigs nursing piglets of similar body weight were selected to receive intraperitoneal injection of saline vehicle or the mast cell stabilizer drug cromolyn (20 mg/kg) at 30 min prior to weaning. After weaning, piglets were redosed with saline or cromolyn treatments at 8 and 16 h postweaning. At 24 h postweaning, jejunal and colonic tissues from weaned and unweaned control piglets were mounted on Ussing chambers for measurement of TER and [3H]mannitol fluxes as described above.

**Statistical analyses.** Data were reported as means ± SE based on the experimental number (n). All data were analyzed by using a standard one-way ANOVA (Sigmastat, Jandel Scientific, San Rafael, CA). A post hoc Tukey’s test was used to determine differences between treatments following ANOVA. Statistical significance was set at a level of *P < 0.05. P values between *P > 0.05 and *P = 0.10 were considered trends.

**RESULTS**

**Impact of weaning age on intestinal barrier health and secretary activity.** Compared with unweaned littermates, early-weaned pigs exhibited marked disruption of intestinal barrier function demonstrated by significant reductions in TER and increases in mucosal permeability to [3H]mannitol (*P < 0.01) in the jejunum (Fig. 1) and colon (Fig. 2). In contrast, intestinal tissues from late-weaned pigs did not exhibit reductions in barrier function compared with unweaned control animals.

**Histological analyses of weaned intestine tissues.** There were no identifiable histopathological lesions observed in early or late-weaned jejunum and colon (Fig. 3, A–D). Villi from 28-day-old pigs were shorter (*P < 0.05) compared with 19-day-old pigs; however, weaning had no effect within respective weaning age groups (villus height = 348 ± 14 and 333 ± 10 μm in 19-day-old unweaned and weaned jejunum, respectively, and 249 ± 14 and 259 ± 3 μm in 28-day-old unweaned and weaned jejunum, respectively; Fig. 3E). No differences in villus width or crypt depth noted between different weaning age groups (Fig. 3, F and G).

**Serum CRF and cortisol levels in early- and late-weaned pigs.** To assess the impact of weaning age on activation of the hypothalamic-pituitary-adrenal (HPA) stress axis, we measured serum CRF and cortisol concentrations 24 h postwean-

![Figure 4](http://ajpgi.physiology.org/)
ing in pigs within each weaning age group. In general, both early-weaned and late-weaned pigs had significantly greater ($P < 0.05$) serum concentrations of CRF compared with their respective age-matched, unweaned littermate controls. However, a trend ($P = 0.10$) for lower serum CRF concentrations was observed in late-weaned pigs compared with early-weaned pigs (Fig. 4A). Serum cortisol concentrations were elevated ($P < 0.05$) in both early-weaned and late-weaned pigs but significantly greater cortisol levels were observed in late-weaned pigs compared with the early-weaned group ($P < 0.05$; Fig. 4B).

Expression of CRF receptors in weaned piglet intestine. We have previously shown that CRF-r1 is increased in early-weaned intestinal tissues and that blockade of peripheral CRF receptors prevented the deleterious impact of weaning on intestinal barrier function, suggesting that both CRF receptor expression and activation may play a critical role in this response to weaning (30). Therefore, we wanted to determine whether differences in mucosal barrier health observed in early and late-weaned piglets coincided with mucosal CRF-r1 protein expression. Western analyses from colonic mucosa revealed that CRF-r1 protein was detected as a predominant band of $\sim 100$ kDa with addition minor bands at observed between 50 and 59 kDa (Fig. 5A). Densitometric analysis of immunoblots revealed over a fourfold increase in CRF-r1 expression in early-weaned tissues compared with unweaned and late-weaned intestinal mucosa ($P < 0.01$; Fig. 5B). IF staining for CRF-r1 showed that CRF-r1 was expressed predominantly in the subepithelium and cells within the lamina propria of early-weaned and late-weaned porcine colonic mucosa with increased staining intensity in early-weaned tissues (Fig. 5C). Double-IF-labeling experiments revealed colocalization of CRF-r1 with mast cell tryptase in early-weaned porcine colonic mucosa (Fig. 5D).

*Mast cell tryptase expression in early and late-weaned pig intestinal mucosa.* Mast cell activity in early and late-weaned pig colonic mucosa was assessed by Western and IF analysis of mast cell tryptase. As shown in Fig. 6, A and B, early-weaned colon exhibited a 2.5-fold increase ($P < 0.05$) in mast cell tryptase protein compared with unweaned tissues. Although it appeared that late-weaned intestinal mucosa had increased mast cell tryptase levels compared with unweaned mucosa by Western analyses, there were no statistically significant differences in expression levels compared with unweaned mucosa ($P = 0.21$). IF staining for mast cell tryptase was enhanced in early-weaned pig colonic mucosa compared with unweaned and late-weaned tissues. It remains unclear why tryptase protein levels remained elevated in weaned piglet mucosa as levels might be expected to fall as mast cells as tryptase is released from degranulated mast cells and rapidly cleared from tissues as it enters the circulation. To investigate this further, we measured serum mast cell tryptase levels in unweaned and weaned piglet tissues and found no difference between unweaned and early-weaned piglets (serummast cell tryptase $= 17 \pm 1.9$ and $21.5 \pm 3.7$ g/ml for unweaned and early-weaned piglets). This may suggest that tryptase, once released from the mast cell, remains in the intestinal mucosa. In agreement with this finding, Barbara et al. (4) reported elevated mast cell tryptase protein levels in IBS patients in the presence of degranulated colonic mast cells.
Effect of mast cell stabilizer drug on early-weaning-induced intestinal dysfunction. To definitively assess the role of intestinal mast cell activation in weaning-induced changes in epithelial physiology, we performed experiments to assess the effects of in vivo mast cell blockade on early-weaning-induced intestinal dysfunction. We pretreated piglets with the mast cell stabilizer drug cromolyn (20 mg/kg ip) prior to weaning and measured its effect on intestinal barrier function. Cromolyn treatment prevented weaning-induced increases in $[^3H]$mannitol fluxes across the jejunum (Fig. 7A) and resulted in a trend ($P < 0.07$) toward reduced $[^3H]$mannitol fluxes across the weaned colon (7B). However, cromolyn treatment did not affect TER in either early-weaned jejunal or colonic tissues (TER = $37 \pm 7$ and $40 \pm 7 \Omega \cdot \text{cm}^2$ in saline and cromolyn-treated weaned jejunum; TER = $113 \pm 11$ and $111 \pm 7 \Omega \cdot \text{cm}^2$ in saline and cromolyn-treated weaned colon, data not shown). The lack of correlation between TER and flux measurements suggests some disparity in what these techniques assess in terms of barrier function. However, mannitol flux is likely of more clinical relevance because of its assessment of permeability rather than the calculated TER measurement of barrier function. To confirm that cromolyn’s effect was mediated through stabilization of mucosal mast cell populations in the piglet intestinal mucosa, intestinal sections were stained with toluidine blue and mucosal mast cell counts were performed. Weaned piglets receiving the saline vehicle had reduced numbers of jejunal (not shown) and colonic mucosal mast cell tryptase levels in early-weaned and late-weaned pig intestine. Figures are representative of experiments on 4 separate animals. A: mast cell tryptase protein expression in early- and late-weaned colonic mucosal lysates by Western blotting. B: densitometric analyses for levels of mast cell (MC) tryptase relative to β-actin. C: IF staining patterns of mast cell tryptase (indicated by white arrows) in early- and late-weaned colonic tissues. Statistical analysis was by ANOVA ($^*P < 0.05; n = 4$).
cells (Fig. 8) compared with unweaned piglet mucosa suggestive of mast cell degranulation. Mucosal mast cell numbers in piglets pretreated with cromolyn were similar to unweaned control piglets. Overall, these data indicate that early weaning resulted in degranulation of mucosal mast cells and breakdown in intestinal barrier function and that cromolyn was effective in stabilizing this population of mast cells, thus preventing disturbances in barrier function in response to weaning.

DISCUSSION

Weaning practices in commercial swine production impose considerable stress on the piglet in the form of maternal and littermate separation and abrupt changes in environment, nutrition, and social hierarchy. The postweaning period is often associated with reductions in feed intake and growth rate and increased susceptibility to enteric diseases (19, 23, 26). This paper reports that conventional, early weaning practices activate central and peripheral stress signaling pathways in the piglet that trigger breakdown of intestinal mucosal barrier function. Furthermore, results from this study demonstrate that weaning-induced intestinal barrier dysfunction is mediated by activation of mucosal mast cells and can be ameliorated by delaying weaning.

A well-characterized response to stress is activation of the HPA axis resulting in adrenal cortisol release (5). In the present study, serum concentrations of CRF and cortisol were elevated in both early- and late-weaned pigs, suggesting that weaning triggered activation of the HPA axis regardless of age at weaning. However, differences in postweaning CRF and cortisol hormone profiles between early- and late-weaned pigs were observed. For example, late-weaned piglets exhibited lower CRF levels but higher cortisol levels compared with early-weaned pigs. Elevated cortisol levels in late-weaned pigs has been reported by others (7, 46) and may relate to the maturity and development of the HPA axis in these piglets. Moreover, higher serum cortisol levels in late-weaned piglets may explain the lower serum CRF levels observed as cortisol inhibits CRF release from the hypothalamus (39). The direct role of the central HPA axis response in weaning-induced barrier dysfunction requires further investigation.

Rodent studies have demonstrated that both central and peripheral CRF/CRF-r1 signaling pathways are critical in stress-induced gastrointestinal dysfunction including disturbances in barrier function, hypersecretion, altered gastrointestinal motility, and visceral hypersensitivity (25, 29, 40). Previously published research from our laboratory (30) demonstrated that early weaning in the piglet (19 days of age) resulted in enhanced expression of CRF-r1 protein in intestinal mucosa and that administration of the nonselective, peripheral CRF receptor antagonist α-helical CRF-(9-41) attenuated intestinal barrier disturbances caused by early weaning, thus demonstrating a central role of peripheral CRF receptor pathways in early-weaning-induced intestinal dysfunction in pigs. In the present study, CRF-r1 protein expression was markedly increased in early-weaned colonic mucosa coinciding with increased mucosal permeability in these tissues. In late-weaned mucosa, which did not exhibit permeability disturbances, CRF-r1 upregulation was not observed, suggesting that CRF receptor expression as well as activation may be critical in this response. The mechanism by which CRF receptor activation induces breakdown in mucosal barrier function in the early-weaned piglets is unclear. In other species, CRF-r1 mRNA is expressed in several cell types within gastrointestinal mucosa.

![Fig. 8. A–C: toluidine blue staining for mast cells (indicated by arrows) in colonic mucosa from unweaned piglets (A) and weaned piglets pretreated with saline vehicle (B) or cromolyn (C) prior to weaning. Mucosal mast cell numbers were performed on tissue sections at ×40 magnification and expressed as the mean number of mast cells per millimeter squared (D). The number of toluidine blue-stained mast cells was significantly reduced in weaned mucosa from piglets receiving saline vehicle compared with unweaned control tissues. Intestinal sections from piglets receiving cromolyn were similar to unweaned control. *P < 0.05 vs. other treatments.](image-url)
including enteric neurons, epithelial cells, and mast cells, all of which influence intestinal barrier properties (9, 13, 47). Co-IF experiments in the present study revealed coexpression of CRF-r1 and mast cell tryptase in weaned porcine colonic tissues, suggesting a potential link between CRF receptors and intestinal mast cells. In support of this relationship, Santos et al. (37) showed that CRF-induced increases in intestinal permeability were prevented by mucosal mast cell stabilization in rodents. Likewise, pretreatment of piglets with the mast cell stabilizing agent cromolyn prevented weaning-induced disturbances in intestinal barrier function in the present study. This provides evidence for a critical role of mast cells in early-weaning-induced intestinal dysfunction in the piglet. Although cromolyn has been repeatedly shown to be selective for connective tissue-type mast cells with little effect on mucosal-type mast cells in rodents (34, 48), mast cell staining techniques in the present study revealed that cromolyn was effective in preventing degranulation of mucosal mast cells. Other studies have reported cromolyn’s influence on mucosal-type mast cells. Cromolyn inhibited TNF-α-dependent cytotoxicity from mucosal-type mast cells (6). In line with results from the present study, cromolyn treatment prevented increases in intestinal mucosal permeability induced by ischemia-reperfusion injury in the canine small intestine (43) and restored intestinal permeability to normal levels in allergic humans (18). Overall this suggests that significant species variation exists regarding the influence of cromolyn on specific mast cell populations.

There are several reports on the role of mucosal mast cells in stress-induced changes in mucosal permeability in rodents but the exact mechanisms are not well understood (3, 16, 36–38). Mast cells possess a variety of preformed and synthesized biologically active mediators that when released have profound effects on intestinal epithelium including secretion, increased mucosal permeability, and motility. Mast cell mediators such as histamine, serotonin, and prostanoids can alter epithelial function through direct activation of epithelial receptors and/or indirectly via stimulation of enteric secretomotor neurons (2, 44, 45). In addition, mast cells are capable of inducing robust inflammatory responses through the release of proinflammatory cytokines and arachidonic acid metabolites. These mediators are central to infectious and stress-induced intestinal disorders (11, 21, 48). Demaude et al. (16) showed that acute stress triggered mast cell degranulation and overproduction of IFN-γ coinciding with increased colonic permeability. There is also convincing evidence for a role of mast cell proteases in regulation barrier function. Scudamore et al. (41) showed that rat mast cell protease-II increased paracellular permeability when applied Madin Darby canine kidney (MDCK) cells in the absence of other inflammatory mediators. A similar role for rat mast cell protease-1 in intestinal barrier disruption in a mouse model of enteric nematode infection was observed (27). Jacob et al. (22) showed that release of mast cell tryptase and subsequent activation of protease activated receptor-2 on T84 monolayers was the critical event mediating redistribution of tight junction proteins occludin and zonula occludens-1 and increased monolayer permeability. In the present study, we observed increased mast cell tryptase levels in early-weaned piglet colonic mucosa as determined by Western blot and IF analysis. However, the exact role of mast cell tryptase in early-weaned mucosa is unknown. Furthermore, it is unclear whether the increased mast cell tryptase levels were due to a relative increase in mast cell numbers or an increase in mast cell tryptase synthesis by mast cells. Mucosal mast cell counts (as determined by toluidine blue staining) were similar between unweaned and weaned tissues from piglets treated with the mast cell stabilizer drug cromolyn, suggesting that the relative number of mucosal mast cells did not change as a result of weaning. Given these findings, it is plausible that the increased mast cell tryptase levels observed in early-weaned piglet intestinal mucosa are a result of increased tryptase synthesis. Barbara et al. (3) showed that mucosal mast cells from IBS patients released more tryptase than mast cells from normal control subjects (4). Further investigation of the effects of weaning stress on mucosal mast cell tryptase content and its direct role in mucosal barrier dysfunction in the weaned piglet is needed.

Overall results from this study reveal that piglet age at weaning has a profound impact on postweaning intestinal barrier function with delayed weaning resulting in amelioration of weaning-induced intestinal barrier dysfunction. Furthermore, mucosal mast cell activation appears to be a critical event mediating reductions in barrier function in early-weaned pigs.

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REFERENCES


