HISTAMINE AND ACTIVATION OF HISTAMINE RECEPTORS AS DRIVERS OF EARLY INFLAMMATION AND GUT FUNCTION IN PIGS

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ABSTRACT

Early life adversity has been established as a major risk factor for the development of inflammatory disease as well as increased mortality in humans. In swine production, weaning is a necessary practice, but is also the most stressful life event that a piglet will endure. Weaning stress-induced increases in inflammation and intestinal permeability occur during the window of developmental plasticity, which has been demonstrated to cause persistent detrimental effects on the gut, including chronic inflammation and epithelial barrier permeability. Despite efforts to improve growth performance and overall health, the lack of targeted interventions is a significant issue.

The critical problem regarding the limited understanding of the mechanisms driving early responses to weaning stress urgently needs to be addressed. The objective of this dissertation is to investigate the role of histamine receptors as a potential mechanism and target to mitigate the inflammatory response and gut dysfunction induced by weaning stress.

First, we sought to characterize the inflammatory response to weaning stress and the role of histamine receptors. As histamine has been shown to be upregulated in response to stress and histamine receptors are well known to be critical in allergic inflammation, we hypothesized that histamine receptors are responsible for early gastrointestinal (GI) inflammation. To test this, we utilized a porcine weaning model of early life stress and administration of pharmacological histamine receptor antagonists.

Next, we investigated the role of histamine receptors in early changes in gut function, including epithelial barrier permeability, nutrient transport, and neural-evoked secretions. Through the employment of Ussing Chambers analyses, we were able to make *ex vivo*

assessments of the aforementioned gut functions in response to weaning stress, as well as in response to selective inhibition of histamine receptors.

Together, our data indicate that histamine receptor subtypes play critical, yet distinct roles in mediating early weaning stress responses in pigs. This provides evidence that histamine and histamine receptors are novel biological targets in mitigating early inflammatory and functional responses to stress in the gut. Future studies should focus on long-term effects of histamine receptors in GI development post weaning stress, as well as determining their role in secondary immune challenges.

To Jax

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
ADHD	Attention-deficit/Hyperactivity Disorder
cAMP	Cyclic Adenosine Monophosphate
cfDNA	Cell-free Deoxyribonucleic Acid
Ca^{2+}	Calcium
ChAT	Choline acetyltransferase
cm	Centimeter
COL1A1	Collagen Type 1 Alpha 1
COX2	Cyclooxygenase 2
CRF	Corticotropin Releasing Factor
CRFR2	Corticotropin Releasing Factor Receptor 2
DMV	Dorsal motor nucleus of the vagus
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
ELN	Elastin
FD4	FITC-dextran (4 kDa)
FITC	Fluorescein isothiocyanate
GCPR	G-Protein Coupled Receptor
GI	Gastrointestinal
H1R	Histamine 1 Receptor
H_2O_2	Hydrogen Peroxide
H2R	Histamine 2 Receptor

H3R	Histamine 3 Receptor
H4R	Histamine 4 Receptor
HNMT	Histamine N-methyltransferase
IBD	Irritable Bowel Disease
IBS	Irritable Bowel Syndrome
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular Adhesion Molecule 1
IFNγ	Interferon-gamma
IFNγR1	Interferon-gamma Receptor 1
IgE	Immunoglobulin E
IgG1	Immunoglobulin G1
IL17A	Interleukin-17A
IL17AR	Interleukin-17A Receptor
IL4	Interleukin-4
IL4R	Interleukin-4 Receptor
IL6R	Interleukin-6 Receptor
I_{sc}	Transepithelial short-circuit current
kg	Kilogram
LPS	Lipopolysaccharide
MCPT4	Mast Cell Protease 4
mg	Milligram
min	Minute
MLN	Mesenteric Lymph Nodes

mM	Milli-molar
MMP9	Matrix Metallopeptidase-9
MPO	Myeloperoxidase
MUC2	Mucin-2
ng	Nanogram
OVA	Ovalbumin
PKA	Protein Kinase A
PTGS1	Prostaglandin H2 Synthase 1
mRNA	Messenger Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RNA	Ribonucleic Acid
RNA-seq	RNA-sequencing
ROS	Reactive Oxygen Species
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
STAT3	Signal transducer and activator of transcription 3
TER	Transepithelial resistance
T_h1	T helper Type 1 cells
T_h1	T helper Type 2 cells
TNC	Tenascin C
TNFα	Tumor Necrosis Factor alpha
T_{reg}	Regulatory T cells
UW	Unweaned
VEGF	Vascular Endothelial Growth Factor

μM	Micro-molar

CHAPTER ONE: HISTAMINE AND HISTAMINE RECEPTORS AS POTENITAL MEDIATORS OF STRESS-INDUCED INFLAMMATORY RESPONSES AND GUT FUNCTIONS

Abstract

The roles of histamine and histamine receptors in allergic inflammation, gastric acid secretion, neuronal activity, and cellular trafficking have been well established. To mitigate these responses, histamine receptor antagonists have been developed and utilized to manage food and environmental allergies and prevent gastric ulcers. Early life adversity has been shown to cause persistent changes in gastrointestinal immune responses and functions, but the mechanism driving these changes remain poorly understood. Histamine is a major mediator synthesized, stored, and released by mast cells, which are innate immune cells classically linked to inflammatory responses. More recently, mast cells have been shown to be associated with altering the developmental trajectory of gut function. In this review, we discuss the classical roles of histamine receptors in inflammation, the current use of antagonists, and how the receptors may mediate the gastrointestinal responses to early life stress.

Introduction

Mast cells are innate immune cells that quickly respond to stress and immune challenges by synthesizing and storing potent mediators in granules, including histamine. Histamine acts through four major receptors, which have been widely studied for their roles in allergy, gastrointestinal disease, and cognitive disorders. However, their role in stress-induced inflammatory responses remains less defined, especially during critical developmental periods early in life. This chapter aims to review the current knowledge of mast cells, histamine, and its receptors and their potential as targets for mitigating inflammation and progression of subsequent diseases.

Mast Cells

For decades, mast cells have been studied for the critical role they play in allergic inflammatory responses and diseases.[1-4] These unique cells are able to respond within seconds to stimuli due to their ability to synthesize and store mediators in granules, including histamine, proteases, tryptases, cytokines, and chemokines. Mast cells act as major immune effector cells during stress and have been shown to mediate responses in vasculature and intestinal permeability, activation of neuroimmune signaling and activation of other immune cells.[5-8] Although mast cells can be activated by a wide number of ligands, including lipopolysaccharide (LPS), a protein found in Gram-negative bacteria, -IL-33, and Substance P, they are most commonly recognized for their activation through binding of IgE to the surface protein FcεR1; binding of this receptor initiates a cascade that ultimately results in mobilization of calcium and translocation of NF-κB to the nucleus.[9,10] Corticotropin-releasing factor (CRF) has also been shown to induce mast cell degranulation, releasing mast cell proteases and TNFα, which results

in injury to the intestinal epithelial barrier.[11] However, binding of the CRF receptor 2 (CRFR2) has been shown to limit mast cell degranulation.[5] The immune mediators released by mast cells have profound effects on local and peripheral tissues, as well as on recruitment of other immune cells. In a study of various mammals, treatment with compound 48/80, which also activates mast cells, increased circulating histamine levels and blood pressure, as well as induced hyperventilation and hyperemia of the skin.[12] Numerous studies have utilized *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice, which lack mast cells, to assess immune responses coordinated by mast cells. These mast cell knockout mice, as well as mast cell protease (MCPT4) knockout mice, have increased transepithelial resistance (TER) and decreased transepithelial flux with reduced cadherin-3 protein levels.[13] Other studies utilizing mast cell-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice show decreased intestinal permeability during infection compared to wild type controls,[14] as well as show increased circulating neutrophils and platelets compared to wild type controls.[15] These data indicate that mast cells, and more specifically the MCPT4 that is released by mast cell granules, regulate intestinal epithelial cell migration and barrier function.

In the adult pig, tissue histamine levels are highest in the stomach, duodenum, small intestine, and lung, and lowest in the salivary glands and pancreas.[12] Along the gastrointestinal (GI) tract, histamine levels increase from the esophagus to the jejunum and then decrease from there to the rectum.[12] In the young pig, histamine levels are much lower throughout the entire body, but each tissue showed varying degrees of lower histamine content relative to measures in the adult.[12] This has also been shown to be correlated to increased enterochromaffin cells in the ileum mucosa.[16] Studies have shown that IBS patients with increased serotonin release from enterochromaffin cells

have higher abdominal pain severity.[17,18] Other studies have demonstrated that mast cells may play a role in GI cancer tumor progression.[19] This illustrates the crucial role that mast cell activation, their degranulation, and release of their mediators play in immune responses, regulation of blood vessel contraction, intestinal permeability, and cellular trafficking.

Histamine Synthesis and Degradation

Histamine is a major mediator synthesized and stored in mast cell granules. Histamine is synthesized by the decarboxylation of L-histidine by histidine decarboxylase (HDC).[20,21] Several studies have proven a strong correlation between upregulated HDC, activity as well as increased levels of histamine itself, and a poor prognosis in human colorectal cancer and small cell lung carcinoma.[22-24] However, administration of the HDC producing probiotic *Lactobacillus reuteri* has been shown to decrease colorectal tumor progression in mice.[19]

Histamine is degraded intracellularly by the histamine N-methyltransferase (HNMT) and extracellularly by the diamine oxidase (DAO).[20,21,25] By-products of this process are N-methyl-histamine and imidazole-4-acetate respectively.[20,21] Recently, it has been demonstrated that histamine degrading enzyme expression in the gut is upregulated in response to weaning stress and is correlated to increased levels of circulating histamine,[26] indicating that these enzymes are sensitive to histamine content. Histamine intolerance occurs when there is a dysregulation in the degradation of histamine by HNMT and DAO, and thus a buildup of endogenous histamine. New treatments have been designed to treat intolerance by administering histamine degrading enzymes with a catalase, to eliminate H_2O_2 and prevent toxic accumulation in the gut.[27] Together, this

suggests that the downstream effects of histamine may be mitigated by histamine degradation, but further studies are necessary.

Classical Functions of Histamine Receptors

The various actions of histamine are conducted through four G-protein coupled receptors (GPCR),[28] the functions of which have been widely reviewed and studied for their implications in allergic inflammatory responses.[19,28-30] Studies of BALB/c mice demonstrate that agonism of any of the four histamine receptor subtypes induce a scratching response, similar to that seen in allergic responses, that is reversed when mice are treated with respective antagonists.[31] Although expression of these receptors is ubiquitous, they do have varying degrees of expression levels throughout the body. Of the four histamine receptors, Histamine 1 Receptor (H1R) and Histamine 2 Receptor (H2R) have the highest expression in the gut.[32] Further, gene expression of histamine receptor subtypes H1R and H2R are upregulated in patients with IBS and food allergies compared to controls, but there was no indication of variable expression across differing regions of the small and large intestine.[32] Moreover, while H1 and H2 receptors are found throughout the gut, Histamine 4 Receptor (H4R) have been localized to circulating leukocytes, intraepithelial cells (similar to neuroendocrine cells), and enterocytes at the apical end of crypts.[32] However, immune cells, including mast cells and PBMCs, express histamine receptors 1, 2 and 4.[32] These data implicate the crucial role of histamine and histamine receptors in the function of numerous cell types.

Histamine 1 Receptor (H1R)

As previously stated, histamine receptors are GPCRs. The H1R is coupled to the $G\alpha_{q/11}$ protein whose ligand binding results in activation of phospholipase C beta (PLC-β).

PLC-β then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which activates protein kinase C (PKC) and increases intracellular Ca^{2+} respectively (**Figure 1.1**).[33,34] H1R is ubiquitously expressed,[35] and is highly expressed on immune cells, including mast cells, dendritic cells, and macrophages.[36,37] Classically, activation of H1R drives cell trafficking, vasodilatation, and bronchoconstriction in allergic inflammatory responses,[34] but has also been shown to play a role in nociception and neurotransmission of enteric ganglia.[34,38-40] Further, elevated expression of H1R is correlated with worse survival outcomes in patients with colorectal cancer and worse prognosis in patients with pancreatic cancer,[41] which may suggest a role in the development of tumor microenvironments.

Activation of H1R on dendritic cells activates antigen presentation to promote IL-12 cytokine secretion and promote the differentiation of T_h1 cells.[42,43] The activation of H1R on T_h1 cells then promotes production of IFNγ.[44] It has also been demonstrated that H1R knockout mice have decreased IFNγ-producing T cells and more ovalbumin (OVA)-specific IgG1 and IgE,[45] which are hallmarks of auto-immune disorders. However, expression of H1R is reduced on human monocyte-derived dendritic cells when stimulated with LPS,[46] which may indicate a stimulus and cell-type specific response. Together, this indicates that H1R is a key player in inflammatory response mechanisms.

Histamine 2 Receptor (H2R)

The H2R is coupled to the $G\alpha_s$ protein that when activated, in turn activates adenylate cyclase to promote cyclic adenosine monophosphate (cAMP) formation and activation

of protein kinase A (PKA), which is then responsible for the phosphorylation of other proteins (**Figure 1.1**).[47] H2R is responsible for gastric acid secretion, mucus production, and relaxation of smooth muscle in airway, uterus, and vasculature.[48,49] Similar to H1R, H2R is also ubiquitously expressed.[35] In the well-studied HDC knockout mice model, increased histamine levels result in upregulated expression of H1R, but reduces expression of H2R.[47] However, recent studies have shown contrasting data in the pig intestine where increased circulating histamine levels correlate with increased H2R gene expression.[26] These opposing results could be due to length of exposure to histamine. HDC knockout mice received consistent doses of histamine in their diet for 15 days, while exposure in the pigs was more acute, lasting less than 24 hours. In another opposition to H1R, H2R signaling suppresses MAPK signaling pathways that are induced by H1R activation.[41] H2R signaling has been shown to suppress cell proliferation and IL-6 expression in human colon cell cultures.[41] Further, simultaneous inhibition of H1R and activation of H2R suppresses proinflammatory signaling in macrophages.[41] Although H2R is well-known for its modification of gastric acid secretion, airway mucus production, vascular permeability,[50] elevated expression of H2R has also been correlated with improved patient outcomes in colorectal cancer.[41] Gao et al. 2015 demonstrated that suppression of colitis inflammation by probiotics is mediated by activation of the H2R and not H1R.[51] This may indicate that these two histamine receptor subtypes have differing roles in the inflammatory response.

Similar to H1R, H2R is also expressed on immune cells, including mast cells, neutrophils, macrophages, dendritic cells, B cells, and T cells.[36,37] Expression of H2R has been shown to increase on human monocyte-derived dendritic cells when activated

with LPS,[46] which is the opposite response seen in expression of H1R. Activation of H2R expressed on dendritic cells stimulates production of IL-10 and T_h2 responses;[42,43] however, activation of H2R on T_h2 cells has a negative feedback on IL-4 production and proliferation.[28,30] In fact, activation of H2R on T cells in mice has been shown to negatively regulate both T_h1 and T_h2 responses.[45,52,53] Even more evidence of this is that H2R knockout mice show upregulation of both T_h1 and T_h2 phenotypic cytokines, but reduced OVA-specific IgE production.[45] This suggests that in addition to playing a critical role in regulating gastric secretions, H2R also plays a role in inflammatory responses, specifically in modulating T helper cell activity.

Histamine 3 Receptor (H3R)

The H3R is coupled to the $G\alpha_{i/0}$ protein that once activated inhibits adenylate cyclase and reduces cAMP levels (**Figure 1.1**).[54] The H3R was first described in 1983 as being expressed in axon terminals,[54] and then later as being expressed in histaminergic neurons.[55] Although it has been demonstrated that the control of mast cell trafficking is mediated by neuropeptide fibers,[56] expression of H3R is not found within the human intestinal mucosa at the gene or protein level, but instead has been shown to be highly expressed in the central and enteric nervous system.[32,57] The numerous actions of histamine receptors in the nervous system have been thoroughly reviewed by Haas et al.,[58] and in summary, H3R primarily serves three functions: 1) as presynaptic inhibitory receptors that suppress release of neurotransmitters from axon terminals,[54] 2) as inhibitory somadendritic receptors that suppress the firing rate along the neuron,[55] and 3) inhibition of the synthesis of histamine in the cerebral cortex and synaptosomes.[59] In a study of preoptic/anterior hypothalamic area cultures of mice, it was shown that

activation of H3R reduces neuronal firing and movement of Ca^{2+} through gated channels.[60] In rats, antagonism of H3R increased synthesis of histamine in the lung and spleen, but did not have an effect on histamine levels in the jejunum.[56] H3R knockout mice show decreased locomotion and basal body temperature,[61] a shift in metabolic homeostasis including hyperphagia, late-onset obesity,[62] increased insulin and leptin levels,[63] and an increased severity of neuroinflammatory diseases compared to wild type controls.[64] Together, this indicates that H3R plays a critical role in numerous homeostatic processes coordinated by both the central and peripheral nervous systems.

Histamine 4 Receptor (H4R)

The H4R is similar to the H3R in that it is coupled to the Gαi/0 protein and thus when activated also inhibits adenylate cyclase and reduces cAMP levels (**Figure 1.1**). First reported by Oda et al.,[65] H4R is expressed in the thymus, small intestine, and peripheral blood leukocytes.[32] Although H1R and H2R expression is elevated in patients with food allergies and irritable bowel syndrome, H4R does not exhibit increased intestinal expression in inflammatory disease states.[32] This most recently characterized histamine receptor subtype is responsible for calcium mobilization within mast cells and for mast cell trafficking via GTPases, RAC1 and RAC2.[66,67] H4R has also been shown to be responsible for chemotaxis of dendritic cells[28] and for inhibition of adhesion-dependent degranulation of neutrophils.[68] In a BALB/c mouse peanut allergy model, treatment with both loratadine, a H1R antagonist, and JNJ7777120, a H4R antagonist, prevents the development of diarrhea and intestinal inflammation as well as reduces the number of presenting dendritic cells in the mesenteric lymph nodes and lamina propria.[69] Another study utilizing a BALB/c mouse model of peritonitis demonstrated reduced neutrophil

infiltration with administration of H4R antagonist. Together, these studies reinforce the role of the H4R in inflammation and chemotaxis of immune cells.

Antagonism of Histamine Receptors and Mediation of the Inflammatory Response

Histamine receptor antagonists have been primarily used to reduce or prevent allergic inflammation, including rhinitis and urticaria, or to reduce secretion of gastric acid. Treatment with mast cell stabilizing agents has also been utilized to prevent the release of histamine. In the last 30 years, second generation antihistamines have been more widely used as they are more receptor specific and do not cross the blood brain barrier. Although effective in suppressing primary functions of histamine receptors, more recent studies have demonstrated that antagonists have other downstream effects that are shedding light on larger roles of histamine receptors.

H1R

As the H1R is mostly responsible for allergic responses, there has been numerous antagonists developed to prevent or suppress allergy symptoms. Upon allergic stimulation, H1R knockout mice do not exhibit increased immune cell infiltration in the lungs compared to their wild type controls.[70] In another murine model, H1R antagonism was shown to reduce paw edema after stimulus with histamine.[71] Multiple studies have also shown that the H1R antagonist desloratadine prevents histamine release from mast cells, but also reduces release of the cytokine IL-4 to an even greater degree.[72,73] Desloratadine has also been demonstrated to decrease expression of IL-6 and IL-8 mRNA.[74] In further support of being able to attenuate inflammatory responses, H1R antagonists have been shown to reduce the expression of adhesion molecules.[75] In one study, nasal epithelial cells incubated with desloratadine for 24 hours demonstrated

significantly reduced expression of ICAM-1 after histamine exposure.[76]

H1R antagonism has also been shown to disrupt homeostasis of T cell populations. Antagonism of H1R reduces T cell migration in response to histamine,[70] and reduces proliferation of Jurkat T cells in a dose-dependent manner.[77] Under basal conditions, histamine blocks CD4+/CD25+ T_{reg} suppressive functions towards T_{resp} via H1R activation; however, administration of the H1 receptor antagonist, loratadine, rescues this suppression[78]. H1R antagonists can also shift T cell polarization between T_h1 and T_h2 cells.[79] H1R knockout mice show decreased lung inflammation post-allergen challenge and increased T_h2 polarization.[70] Furthermore, the H1R knockout mice have decreased IFNγ and increased IL-5 and IL-13, indicating recruitment of T_h2 cells.[70] These data suggest that H1R is important in regulating the inflammatory response to allergens, but that antagonists are capable of mitigating these responses.

Antagonism of H1R has also been shown to have effects beyond blocking histamine. The sedative effects of first generation H1R antagonists in humans is associated with their high binding affinity to H1R in the central nervous system.[80] Morphine is known to release histamine from mast cells,[81] which may impact nociceptor ability to sense stimuli and relay that signal to the spinal cord and brain. Multiple studies by Mobarakeh and coauthors show that morphine increases antinociception and that this is exacerbated by the addition of an H1R antagonist. They were also able to show this increase of antinociception in a H1R knockout mouse.[82,83] H1R antagonism has also been shown to alter feeding responses. Human patients that have been prescribed antipsychotic drugs that have high affinity to the H1R, thus preventing histamine binding, have shown weight gain.[84] Moreover, H1R antagonism in the ventromedial hypothalamus has been

associated with increased feeding in rodents,[85,86] but does not have the same effect if blocked in the lateral hypothalamus or the paraventricular nucleus.[85] Together, these data indicate that the histamine H1R may be responsible for nociception in the body, but also that H1R antagonists may serve a secondary purpose in altering hypothalamic responses including pain management.

H2R

Antagonists for H2R are most commonly utilized to reduce gastric acid secretion. These ligands have also been found to have effects on cytokine production and neuronal responses. H2R antagonists block increases in cyclic AMP levels, but do not block histamine release itself.[87] Antagonism of H2R suppresses anti-inflammatory mechanisms of action by increasing IL-12 and decreasing IL-10 production.[88] Recently, it was observed that early elevated levels of IL-10 in SARS-CoV-2 infections predicted poor outcomes in patients.[89,90] More pertinent, COVID-19 positive patients treated with the H2R antagonist, famotidine, showed reduced inflammation and improved rate of symptom reduction.[91] Further, it was demonstrated that famotidine functions through anti-inflammatory mechanisms, not anti-viral.[92] Given these data, H2R may play larger role in inflammatory responses, which was originally thought to be more H1R specific. Although there may be evidence that H2R does play a role in inflammatory responses, it may be stimulus specific. For example, H1R antagonists have been shown to decrease inflammation severity in response to LPS, H2R antagonists do not have the same effects. In response to administration of LPS, dendritic cells traffic to the mesentery and are found in increased numbers compared to controls; however, this response is mitigated with administration of a H1R antagonist but not with a H2R antagonist.[93] LPS

also increases AP-1/NF-κB activation, which is then suppressed by H2R activation; antagonism of H2R by famotidine is able to reverse this and bring back the level AP-1/NF-κB activation matching that induced by LPS.[46] This indicates that acute inflammatory responses induced by LPS may be mediated by H1R and activation of H2R may have an opposing anti-inflammatory response to the same pathogenic protein. Activation of the vagus nerve, and its subsequent release of acetylcholine, has been shown to have profound effects on the inflammatory response and in the breakdown of the epithelial barrier of the gut.[94] Vagal nerve stimulation has been shown to reduce intestinal injury and inflammation, as well as be preventative of intestinal permeability by maintaining tight junction integrity.[95] However, famotidine activates the vagus nerve inflammatory reflex to attenuate cytokine release.[96] Although there has been association with H1R antagonism and feed intake, H2R antagonism in the ventromedial hypothalamus is not associated with increased feeding in rodents.[85,86] Famotidine has also been shown to increase the threshold of electro-convulsions and decrease the ED_{50} of the antiepileptic drug valproate, but also impairs motor activity.[97] This suggests that H2R may also be involved in neuronal sensitivity and neural-evoked responses.

H3R

As H3R are largely expressed in the nervous system, H3R antagonists have largely been utilized to target nerves to reduce nasal congestion,[98] reduce body weight and fat deposits,[99] and manage cognitive disorders, as well as being widely studied in rodent models of behavior.[100] Utilization of thioperamide and clobenpropit in memory processing models have shown improvement in avoidance behaviors[101,102] and object recognition.[103] H3R antagonists have also been shown to reduce food intake when

administered orally or by intraperitoneal injection to rodents[104-106] and when given via intragastric or subcutaneous injection to pigs or rhesus monkeys.[107] The H3R antagonists thioperamide and ABT-239 have been shown to increase acetylcholine levels in the rat hippocampus.[100,103] ABT-239 has also been shown to improve inhibitory behaviors in a model of attention-deficit/hyperactivity disorder (ADHD) and memory in a model of Alzheimer's disease.[108] Together these data show a clear role of H3R in the central nervous system, but little is known of the role of H3R in the enteric nervous system other than its regulation of peristalsis.[109] This could be due to lower expression levels of H3R in the gut compared to other histamine receptor subtypes or lesser H3R+ innervation within the gut.

H4R

Antagonists for H4R have been more recently developed and studied and have brought out the role of H4R in mediating immune cell trafficking. Although mast cells under normal conditions migrate in response to stimulus by histamine, mast cells from H4R knockout mice do not migrate in response to histamine.[67] Further, in response to activation by histamine, H4R mediates chemotaxis of other immune cells, including eosinophil and basophils, and mast cells.[110-112] Similar to H1R antagonism, H4R antagonism also decreases T cell migration in response to histamine.[70] A study by Thurmond et al., demonstrated that histamine stimulus plus H4R antagonism by JNJ7777120 in mice inhibits mast cell chemotaxis and accumulation of mast cells in the trachea by reducing Ca^{2+} mobilization.[71] H4R antagonists also have been studied in the context of cellular adhesion. Activation of H4R by histamine results in Akt activation, which can mediate junctional permeability between endothelial cells.[113] It has been

shown that H4R antagonism reduces adhesion of human eosinophils to the endothelium, but the same is not true with antagonism of the other histamine receptor subtypes.[114] Less is known about the role that H4R plays in the gut function, but studies have implicated a role in tumor development. In a study of osteosarcoma cells, JNJ7777120 recruits β-arrestin to H4R scaffolding without activating G-protein pathways,[115] indicating that this receptor may have agonist specific activations and may also be involved in production of reactive oxygen species. However, studies utilizing multiple H4R antagonists have shown decreased tumor cell proliferation and increased cell cycle arrest in gastric and intestinal cancers,[116,117] making these new antagonists and this histamine receptor a promising target for treatment of cancers. Altogether, more studies are necessary in determining the full scope of responsibilities held by the H4R and developing antagonists to reduce these responses.

Stress Responses in the Gut

The brain-gut axis has more recently been demonstrated to play a key role to overall health as cross talk between the gut microbiome and the brain has been shown to play an important role in inflammatory responses to stress and development of gut disorders.[118] During a stress response, CRF is released by the hypothalamus, which then stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) which stimulates the adrenal glands to release cortisol.[119] Further, stress induced inflammation has been linked to increased disease risk across the lifespan.[120,121] Across species, increased inflammation during pre and postnatal developmental periods have been demonstrated to increase risk of developing chronic inflammatory and metabolic diseases later in life.[120-123] More specifically, stressors associated with swine weaning

practices have demonstrated lasting, detrimental effects to intestinal epithelial barrier function, the enteric nervous system, and the mucosal innate immune system.[124-130] Pigs that are weaned at an earlier age show increased gut permeability compared to their later weaned counter parts.[7,127] Early life stress has also been indicated to increase neuro-secretory functions later in life,[126] despite the fact that afferent neuro-sensitivity is reduced with age.[131] However, the mechanisms responsible for triggering early inflammatory responses and changes in gut function remain poorly defined.

As previously discussed, histamine and histamine receptors have profound effects on gut function, especially during immune responses to pathogens or stressors. Previous studies in humans with either IBS or food allergy were shown to express elevated levels of H1R and H2R within the ileum, cecum, and rectum.[32] Moreover, histamine content in mesenteric tissues is increased with LPS and activation of H1R but not H2R.[93] Mesenteric peri-lymphatic mast cells also have a greater percentage of degranulation in response to LPS when H1R is activated compared to when H2R is activated.[93] When stimulated with peptidoglycan, a protein found in Gram-positive bacteria, mast cell degranulation causes increases in dendritic cell trafficking to the mesenteric lymph nodes. Dendritic cell migration is unaltered by pre-treatment with a H1R or H2R antagonist, indicating that responses to stimulus by Gram-positive bacteria is not mediated by either H1R or H2R.[132] In a murine model of stress in conjunction with induced inflammatory response, mice that experienced water avoidance stress and that were given OVA to induce an inflammatory response had reduced ileal transepithelial resistances (TERs) and increased ileal transepithelial flux of FITC-dextran, demonstrating the sensitivity of the gut to stimuli.[133] Additionally, treatment with H1R

antagonist, pyrilamine, reduces intestinal permeability during infection.[14] These data indicate that histamine receptors mediate early inflammatory and functional changes in the gut and would be a novel target to mitigating stress-induced inflammatory responses.

Summary and Objectives

Histamine and histamine receptors are well known for their ability to drive allergic inflammatory responses, but the individual receptor subtypes have been more recently shown to play diverse roles throughout the body. Despite this, the role that histamine receptors play in stress-induced inflammatory responses and dysfunction, specifically in the gut, has yet to be elucidated. As weaning is an unavoidable swine production practice, targeted interventions for mitigating responses to this major life stress must be identified. Early life inflammation and increased gut permeability have been associated with disease risk later in life.[120-123] Further, early weaning used in swine production has been shown to cause persistent changes in immune responses as well as GI barrier functions, including epithelial barrier permeability, nutrient transport, and neural-evoked secretion.[6,124,127,128,134,135] These changes have also been associated with upregulation of mast cell numbers and activity[7,127] and given that histamine is a major mediator released by mast cells makes it a novel target for mitigating weaning stress-induced gut dysfunction. The objective of this dissertation is to fill the knowledge gaps discussed above.

In Chapter 2, I further characterized the weaning stress-induced inflammatory response in pigs, which has been used as a large animal model of early life adversity. As previously stated, inflammation during early life is associated with increased disease

risk later in life.[120-123] In swine production specifically, early weaning has been shown to cause a large inflammatory response,[136-139] yet the mechanism that drives this response is unknown. Given that mast cells conduct inflammatory responses to allergens by releasing mediators, including histamine, we hypothesized that histamine signaling through H1R and H2R is critical in the initiation and progression of GI inflammation induced by early weaning.

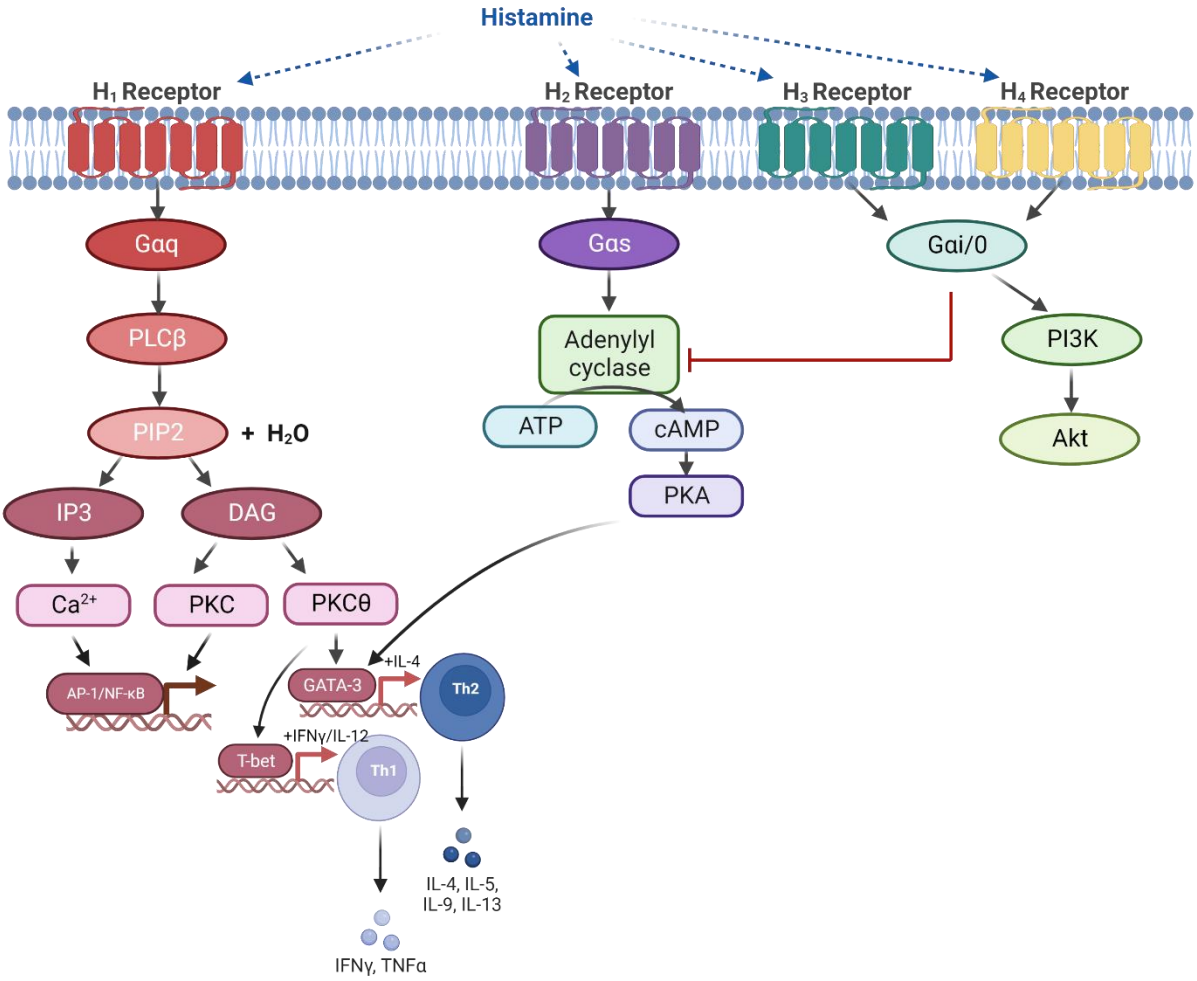
In Chapter 3, I will target the immediate impacts of weaning stress on GI epithelial barrier functions mediated by histamine receptors. Previously, our lab has demonstrated that early weaning stress shifts the developmental trajectory of GI function and causes increases in GI permeability, alterations in nutrient transport, and increase in neural-evoked secretions well into adulthood.[124,126,127] Here, we seek to evaluate the role of histamine and histamine receptors in mediating these functions. Given that mast cell activity is increased quickly in response to stressors and that the H1R and H2R are the major histamine receptor subtypes that are activated within the gut, we hypothesized that H1R and H2R are potent modulators of early weaning-induced alterations in GI epithelial barrier, nutrient transport, and secretory functions.

In summary, the work presented here demonstrates that weaning stress-induced changes in GI inflammatory responses, permeability, nutrient transports, and neural-evoked secretory functions are mediated by histamine receptors. Considering the role that mast cells have already been shown to play in inflammatory responses and mediation of enteric diseases, the new knowledge generated here can be used to identify targets for therapeutics in production practices. Further, these results indicate that histamine and its receptors are a novel therapeutic target to mitigating stress-

induced changes in gut function and reducing enteric disease risk later in life.

Figures

Figure 1.1: Activation Pathways of Histamine Receptors
Histamine Receptors are transmembrane G-Protein Coupled Receptors (GCPR) that activate intracellular cascades, which ultimately result in inflammation, gastric acid secretion, neuronal activation, and cellular trafficking. Created with BioRender.com.



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CHAPTER TWO: HISTAMINE RECEPTOR MEDIATION OF THE INFLAMMATORY RESPONSE TO WEANING STRESS IN PIGS

Abstract

Early weaning (EW), a necessary but stressful early life production practice, induces gastrointestinal (GI) inflammation, which is detrimental for short and long-term health. The mechanism by which GI inflammation is triggered is unknown and thus early targeted anti-inflammatory interventions are lacking. Previous studies have demonstrated that histamine and histamine receptors are upregulated early in response to weaning stress. Here we tested the hypothesis that histamine receptors mediate the inflammatory response to weaning in pigs. Histamine receptor antagonists were administered 30 minutes prior to weaning and jejunal mucosa and mesenteric lymph nodes were isolated for inflammatory marker analyses at 0, 3, and 24 hours post weaning. In response to weaning, Histamine 1 Receptor (H1R) exhibited upregulated expression in the jejunal lamina propria while Histamine 2 Receptor (H2R) exhibited upregulated expression in the jejunal epithelium. Weaning stress and histamine receptor antagonism largely downregulated inflammatory gene expression. Famotidine inhibited weaning-induced elevation of jejunal myeloperoxidase and weaning-induced reduction of hydrogen peroxide in the jejunal mucosa (P=0.0038 & P=0.0166 respectively). Famotidine also reduced weaning-induced increases of jejunal IL1β (P=0.0415). Weaning and treatment with H1R or H2R antagonists did not have an effect on IFNγ, TLR4, COX2, or ChAT expression compared to saline controls (P>0.05). However, H2R antagonism did significantly reduce TGFβ (P=0.0010) and β-Integrin (P=0.0002) expression in the mesenteric lymph nodes (MLN). These data demonstrate that weaning stress induces early inflammatory responses in the gut and that specific aspects of this inflammation are mediated by H1R and H2R, including cytokine

responses, oxidative stress, and cell trafficking. This provides a novel target to mitigating early inflammation in swine production that leads to enteric disease, as well as a model for human health and disease risk.

Introduction

Early weaning (EW), a necessary but stressful early life management practice, induces gastrointestinal (GI) inflammation, which is detrimental for short and long-term health. Early life stress and inflammation had been associated with increased GI and inflammatory disease risk and mortality in people.[1-4] The mechanism by which GI inflammation is triggered is unknown and thus early targeted anti-inflammatory interventions are lacking. Previous studies have indicated that early life adversity induces upregulation of mast cell activation, increased functional gastrointestinal disorders, and increased immune activation.[5-7] Histamine, a major immune mediator that is synthesized, stored, and released by mast cells, is very quickly upregulated following response to stress in rodents and pigs.[8-10] In humans, histamine has been shown to play a major role in intestinal allergy and inflammatory bowel diseases.[11-14] The various actions of histamine are conducted through four receptors.[12] Given the ubiquitous expression of the histamine 1 receptor (H1R) and histamine 2 receptor (H2R) throughout the body and of the four histamine receptor subtypes, are the most highly expressed in the gut,[15] here we investigated the hypothesis that administration of histamine 1 and 2 antagonists prior to weaning will decrease inflammatory responses in the gut of pigs.

Methods and Materials

Animals

All animal studies were first approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University under the animal use protocol PROTO202200130. Male-castrate Yorkshire cross-bred piglets were selected based on

mean body weight from multi-parous sow litters without cross-fostering. At 18 days of age, selected pigs were randomly assigned a treatment group based on average body weight and given an intramuscular injection 30 minutes prior to weaning with one of 4 treatments: Saline (2 mL), Desloratadine (2 mg/kg; PHR1680, Sigma-Aldrich), Famotidine (4 mg/kg; NDC# 0641-6021-01; 07-890-7011, Patterson Veterinary Supply), or a combination of Desloratadine and Famotidine (Figure 2.1). At weaning, piglets were removed from their sow and moved to an adjacent nursery facility and cohoused with ad libitum access to water. Pigs were fasted to recapitulate industry procedures. At 3, 8, and 24 hours post weaning, piglets were first sedated using a combination of Telazol (100 mg/mL), Ketamine (100 mg/mL), and Xylazine (100 mg/mL) at a dose of 0.03 mL/Kg body weight administered via intramuscular injection (gluteus medius). Once sedated, piglets were euthanized via intracardiac overdose of pentobarbital sodium (Euthasol; 85.9 mg/kg). Tissue samples were immediately collected for subsequent histopathologic and gene and protein expression analyses. A control group of piglets was immediately harvested at weaning (unweaned; UW).

Histopathological Analyses

Mid-jejunum was excised and immediately fixed in Carnoy's 2000 (FXCAR2GAL, StatLab, McKinney, TX). Samples were transferred into 70% ethanol 24 hours post collection. Samples were then taken to the Michigan State University Investigative Histopathology Lab for paraffin embedding, sectioning (5 µm longitudinal cross-sections), and staining.

H1R and H2R Stains

Sections were labeled for Histamine Receptor 1 (orb331289, Biorbyt, Cambridge,

United Kingdom) and Histamine Receptor 2 (NLS1175, Novus Bio, Centennial, CO) at 1:100 dilutions and for Histamine Receptor 4 (LS-C146254, LifeSpan Biosciences, Seattle, WA) at a 1:200 dilution. Detection of Histamine Receptor 1 was performed using anti-mouse-on-HRP Polymer secondary for 10 minutes and treatment with Romulin AEC. Detection of Histamine Receptor 2 and Histamine Receptor 4 was performed using anti-rabbit-on-Farma HRP Polymer secondary for 30 minutes and treatment with Betazoid DAB. All sections were counter stained with Endure Hematoxylin (Cancer Diagnostics, Inc., Durham, NC).

qPCR Gene Expression Assays

Mucosal scrapes were isolated from longitudinal jejunal sections excised from piglets. Collected scrapes were immediately flash frozen in liquid nitrogen. Total RNA was isolated from 30 mg of mucosal scrapes using the RNeasy Mini kit (74106, Qiagen, Hilden, Germany) and RNase-Free Dnase Set (79254, Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 480 ng RNA using Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR with dsDNase (K1671, Thermo-Fisher, Waltham, MA). Real-time qPCR was used to determine the relative quantities of transcripts of the genes of interest. Primer sequences for all gene primers are listed in **Table 1**. All PCR reactions were done using *Power* SYBR Green (4367659, Applied Biosystems, Waltham, MA) and the QuantStudio™ 3 system (Applied Biosystems, Waltham, MA) and subjected to a melt curve analysis to validate the absence of nonspecific products. The data are presented as $2^{-\Delta\Delta CT}$ in gene expression relative to unweaned control group, first normalized to the reference gene RPL4, before statistical analysis.

Protein Isolation

For all protein expression assays, mucosal scrapes were obtained from longitudinal jejunal sections and mesenteric lymph nodes (MLN) from mid-ileum excised from piglets. Collected scrapes and MLN were immediately flash frozen in liquid nitrogen. Total protein was isolated using Mammalian Protein Extraction Reagent (MPER, PI78501, Thermo-Fisher, Waltham, MA) with protease (11697498001, Roche, Basel, Switzerland) and phosphatase (PI78426, Thermo-Fisher, Waltham, MA) inhibitors, and protein was quantified using the Pierce BCA Protein Assay Kit (PI23227, Thermo-Fisher, Waltham, MA).

Protein Expression: ELISAs and Multiplex

Protein expression assessed via ELISA was done using commercially available kits (Myeloperoxidase [LS-F4326-1, LifeSpan Biosciences, Seattle, WA], Hydrogen Peroxide [A22188, Thermo-Fisher, Waltham, MA], Interleukin-1-β [PLB00B, R&D Systems, Minneapolis, MN], Interleukin-6 [ELP-IL6, Ray Biotech, Peachtree Corners, GA], IFNγ [KSC4021 Thermo-Fisher, Waltham, MA], and TGFβ [MB200, R&D Systems, Minneapolis, MN]) and performed according to respective manufacturer instructions.

Protein Expression: Western Blots

Protein expression assessment via Western blots using the Protein Simple Wes™ capillary electrophoresis system (Protein Simple, Minneapolis, MN) for β-Integrin (ab183666, Abcam, Cambridge, United Kingdom) and TLR4 (ab22048, Abcam, Cambridge, United Kingdom) was done as described previously.[16] Briefly, samples were diluted to 0.324 µg/µL in provided sample buffer and fluorescent molecular weight marker/reducing agent (Protein Simple, Minneapolis, MN). Samples were then vortexed,

Table 1: Gene Primer Sequences

Gene	Primer	*5' to 3'* Sequence
COL1A1	Forward	*CCTGGACGCCATCAAAGTCT*
	Reverse	*GGTTACCCAGGATCAACCCC*
COL1A2	Forward	*GAAGAACCCAGCTCGCACAT*
	Reverse	*CCAAATCTTTTGGGGAGGGGAA*
ELN	Forward	*ATGGCGGGTCTGACGGCGG*
	Reverse	*CCCCTGTCCCTGTTGGGTAACCAGC*
MMP9	Forward	*CGGGAGACCTACGAACCAAT*
	Reverse	*TCCAGGGACTGCTTTCTGTC*
TNC	Forward	*CACACACGAAATCACTGCTAAC*
	Reverse	*TTTTCCTTCTCTTGGGTGGCT*
ITGB1	Forward	*ACTTGTGAGATGTGTCAGACC*
	Reverse	*CAATCATCAACGTCCTTCTCC*
ICAM1	Forward	*GAGGAGCTGTTCAGGCAGTC*
	Reverse	*CATCCGGAACGTGACATTG*
MADCAM1	Forward	*ACAGAGCAGGGGACAAAGC*
	Reverse	*CCGTGGCCAGGCTGGAG*
VCAM1	Forward	*CATTCCATGGTGTCCCAGA*
	Reverse	*TCCAAACTCTTCGTTTCCTTG*
IFNγ	Forward	*GGCCATTCAAAGGAGCATGG*
	Reverse	*GCTCTCTGGCCTTGGAACAT*
IL-1β	Forward	*GAAAGCCCAATTCAGGGACC*
	Reverse	*GGCGGGTTCAGGTACTATGG*
IL-4	Forward	*GGACACAAGTGCGACATCA*
	Reverse	*GCACGTGTGGTGTCTGTA*
IL-6	Forward	*AAAGAATCCAGACAAAGCCACC*
	Reverse	*TCCACTCGTTCTGTGACTGCA*
IL-17a	Forward	*CCCTGTCACTGCTGCTTCTG*
	Reverse	*TCATGATTCCCGCCTTCAC*
MCP1	Forward	*GAAGCTCTGCGTGACTGTCC*
	Reverse	*CAGTCATCACTGGGGTTGG*
MIP1b	Forward	*CCTGCTGCTTCACATACACC*
	Reverse	*CAGTCATCACTGGGGTTGG*
TNFα	Forward	*GGGGTCCTTGGGTTTGGATT*
	Reverse	*TTGGAACCCAAGCTTCCCTG*
IFNγR1	Forward	*CCAGACTTCCTGAACGGCTT*
	Reverse	*CTTGGCGGTTTCACTGTTGG*
IL4R	Forward	*GCAGCGGCATTGTCTATTCA*
	Reverse	*TCGTCTTGGCCGTGACACT*
IL6R	Forward	*CACCCGATCTTCATTCACTG*
	Reverse	*AAGGAGGCAATGTCTTCCAC*
IL17AR	Forward	*CACAGGAATCGACACACCCA*
	Reverse	*TCTCATCCTGACCCCCTCTG*

Table 1 (cont'd)

TLR4	Forward	*TCAGTTCTCACCTTCCTCCTG*
	Reverse	*GTTCATTCCTCACCCAGTCTTC*
CHAT	Forward	*AGCTAGCCTTCTACAGGCTCCAT*
	Reverse	*CGCTCTCATAGGTAGGCACGA*
CRFR1	Forward	*CCTTCCTGTACTGAATGGTCTC*
	Reverse	*CTGCGCAAGTGGATGTTTATC*
CRFR2	Forward	*CCTGCTGCTTCACATACACC*
	Reverse	*CAGTCATCACTGGGGTTGG*
MUC2	Forward	*GGCTGCTCATTGAGAGGAGT*
	Reverse	*ATGTTCCCGAACTCCAAGG*
DAO	Unique Assay Primer - BIO RAD	
	qSscCID0005156	
HDC	Unique Assay Primer - BIO RAD	
	qSscCID0004146	
HNMT	Unique Assay Primer - BIO RAD	
	qSscCID0003833	
PTGS1	Forward	*CAACACTTCACCCACCAGTTCTTC*
	Reverse	*TCCATAAATGTGGCCGAGGTCTAC*
PTGS2	Forward	*TCGACCAGAGCAGAGAGATGAGAT*
	Reverse	*ACCATAGAGCGCTTCTAACTCTGC*
VEGF	Forward	*GTCTGGAGTGTGTGCCCA*
	Reverse	*GTGCTGTAGGAAGCTCATC*
RPL4	Forward	*GGCGTAAAGCTGCTACCCTC*
	Reverse	*GGATCTCTGGGCTTTTCAAGATT*

heat-denatured for 5 minutes at 95°C and loaded into the Wes™ assay plate (Protein Simple, Minneapolis, MN). Primary antibodies were loaded at dilutions indicated in **Table 2**.

Blocking solution, horseradish-peroxidase conjugated secondary antibodies, and chemiluminescent substrate were loaded per kit instructions (Separation Module SM-W004 and Detection Modules DM-001 [Rabbit] and DM-002 [Mouse], Protein Simple, Minneapolis, MN). The assay plate was then loaded into the Wes™ machine for automated electrophoresis (375 Volts for 30 minutes). Protein expression was quantified via densitometry of protein bands performed using ImageJ software (National

Institute of Health) and normalized to β-Actin (ab8226, Abcam, Cambridge, United Kingdom).

Table 2: Western Blot Antibodies

WES System				
Primary Antibodies				
Target	**Host**	**Vendor**	**Catalog Number**	**Dilution**
β-Integrin	Rabbit	Abcam	ab183666	1:10
TLR4	Mouse	Abcam	ab22048	1:10
β-Actin	Mouse	Abcam	ab8226	1:100
Conventional System				
Primary Antibodies				
COX2	Goat	Millipore Sigma	SAB2500267	1:1,000
ChAT	Mouse	Millipore Sigma	MAB5270	1:1,000
MHCII	Mouse	Kingfisher Biotech	WS0589S-100	1:2,000
Secondary Antibodies				
Anti-Mouse IgG	Rabbit	Abcam	ab97046	1:10,000
Anti-Goat IgG	Donkey	Santa Cruz	sc-2020	1:10,000

Protein expression assessment via conventional Western blot for COX2 (SAB2500267, Millipore Sigma, Burlington, MA), Choline acetyltransferase (MAB5270, Millipore Sigma, Burlington, MA), and MHCII (WS0589S-100, Kingfisher Biotech, St. Paul, MN) were done as described previously.[17] Briefly, jejunal mucosa and mesenteric lymph node protein samples were diluted to 1 µg/µL in 20% Pierce Lane Marker Reducing Sample Buffer (39000, Thermo-Fisher, Waltham, MA) and heat denatured at 100 °C for 5 minutes. Protein samples (15 µg) was run on a TGX-Stain Free gel (5678094, Bio-Rad, Hercules, CA). Electrophoresis, wet to wet transfer, and total protein quantification was performed as published in Criterion™ Precast Gels: Instruction Manual and Application Guide and Western Blot Normalization Using Image Lab™ Software (Bio-Rad, Hercules, CA). The PVDF membrane was blocked in 5% BSA at room temperature for 90 minutes prior to incubation with antibodies listed in Table 2 in 1X TBS+5%

BSA+0.1% Tween-20 (1X TBST) overnight at 4°C. The following morning, the blot was washed with 1X TBST and incubated for 1 hour at room temperature with an HRP-linked IgG secondary antibody (host-species specific listed in Table 2) in 1X TBS+5% Milk. Chemiluminescence was performed using Clarity ECL (1705060, Bio-Rad, Hercules, CA). Total lane protein was assessed via Pierce Reversible Protein Stain (P24585, Thermo-Fisher, Waltham, MA). Densitometry was performed utilizing ImageJ Software v1.54b (National Institute of Health, Bethesda, MD) and band density was normalized to total lane protein.

Statistical Analyses

Data are reported as means ± SEM. Experimental *n* are indicated in each figure legend. All data were analyzed using a One-Way ANOVA between treatment groups ANOVA or a Two-Way ANOVA between treatment groups and timepoints using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A post-hoc Fisher's LSD test was used to determine the effects of weaning, treatment, time, or interaction between groups. Differences were considered significant at $P \leq 0.05$.

Results

H1R and H2R Show Different Localization Patterns in the Jejunum Mucosa

Given that H1R and H2R have the highest expression of the 4 histamine receptors within the gut, we investigated the expression localization of H1R and H2R under basal conditions and in response to weaning stress within the jejunal mucosa. We observed an increased expression of H1R localized to the lamina propria of the jejunal mucosa at 8 hours post weaning compared to 0 hours (P=0.0082; **Figure 2.2A**) as well as significantly higher expression compared to H2R at the same timepoint (P=0.0060). We

also saw an increase in H2R expression localized to the epithelium in response to weaning stress (*P*=0.0105; **Figure 2.2B**). We also observed that H2R was increased compared to H1R in the epithelium at 8 hours post weaning. These data demonstrate different localization patterns within histamine receptor subtypes.

Antagonism of H1R and H2R Inhibits Weaning Stress-Induced Upregulation in Gene Expression of Jejunal Mucosa

To identify the effects of histamine receptors on the immune response in the gut to weaning, pigs were treated with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. Jejunal mucosa was collected at 0, 3, and 24 hours post weaning. RNA was then extracted to be utilized in real time semi-quantitative PCR assessment of relative mRNA expression of genes related to extracellular matrix (ECM), immune trafficking, inflammatory cytokines, neural mediators, and enzymes within the jejunal mucosa. Surprisingly, we saw that many genes were down regulated in response to weaning at both the 3 and 24 hour post-weaning timepoints (**Figure 2.3**). Weaning itself has been demonstrated to increase acute cortisol levels and inflammation,[18,19] making the gene expression patterns from our study especially interesting. In assessing the jejunum at 3 hours post weaning, we observed that genes related to ECM trended towards significance and were significantly upregulated in response to weaning stress (**Figure 2.4B and C**; *P*=0.0659 and *P*=0.0275 respectively). Treatment with desloratadine or famotidine alone did not significantly alter expression of genes related to ECM, but treatment with both desloratadine and famotidine upregulated expression of *MMP9* (**Figure 2.4D**; *P*=0.0031). At 24 hours post weaning, genes related to the ECM were downregulated in response to weaning and showed no

response to histamine receptor antagonism (**Figure 2.4A-E**). At 3 and 24 hours post weaning, genes related to immune trafficking showed no change in expression in response to weaning stress or antagonism of histamine receptors (**Figure 2.4F-I**). However, genes related to ECM and immune trafficking did have a significant effect of time between 3 and 24 hour post weaning timepoints (**Table 3**).

At 3 and 24 hours post weaning, genes responsible for the expression of classical inflammatory cytokines or chemoattractants were largely unchanged in response to weaning stress or antagonism of histamine receptors (**Figure 2.4J-P**). At 24 hours post weaning, expression of *IL17A* was downregulated in response to weaning stress (P=0.0462) but was not affected by antagonism of histamine receptors (**Figure 2.4N**). Further, at both 3 and 24 hours post weaning expression of *TNFα* was also significantly downregulated in response to weaning (P=0.0001 and P<0.0001 respectively) but showed no change in expression in response to histamine receptor antagonism at either timepoint (**Figure 2.4Q**). However, at 24 hours post weaning, gene expression of *IFNγ* and *IL4* were upregulated in response to weaning stress and significantly downregulated in response to histamine receptor antagonism (**Figure 2.4J and L**). Genes related to cytokine and pattern recognition receptors had variable responses to weaning stress and histamine receptor antagonism. At 3 hours post-weaning, expression of *IFNγR1* and *IL17AR* was upregulated in response to weaning (**Figure 2.4R and U**; P<0.0001 and P=0.0008 respectively). Further, histamine receptor antagonism prevented weaning-induced upregulation in both genes. At 24 hours post weaning, there was no significant differences in expression seen with weaning or with histamine receptor antagonism, but there was a significant effect of time with both

IFNγR1 and *IL17AR.* In contrast, gene expression of *IL4R* and *IL6R* was downregulated in response to weaning stress at both 3 and 24 hours post weaning (**Figure 2.4S and T**). Treatment with desloratadine prevented weaning-induced downregulation while treatment with famotidine or desloratadine + famotidine had no effect on relative expression levels. At 3 and 24 hours post-weaning, *TLR4* expression was unchanged in response to weaning stress or histamine receptor antagonism (**Figure 2.4V**).

Expression of genes relating to neural mediators also had variable responses. At 3 hours post weaning, expression of *CHAT* was downregulated in response to weaning (Figure 2.3W; P=0.0008); there was no effect of histamine receptor expression. At 24 hours post weaning, expression of *CHAT* was also downregulated in response to weaning (P<0.0001); treatment with either desloratadine or famotidine prevented weaning-induced downregulation in expression (P=0.0486 and P=0.0113 respectively), although there was no effect of treatment with desloratadine + famotidine. At 3 hours post weaning, there was no effect of weaning or histamine receptor antagonism on expression of *CRFR1* or *CRFR2* (**Figure 2.4X and Y**). Although there was no significant difference in expression of *CRFR1* in response to weaning or histamine receptor antagonism at 24 hours post weaning, expression of *CRFR2* was downregulated in response to weaning, but was not affected by histamine receptor antagonism (**Figure 2.4Y**).

At 3 hours post weaning, there was no response to weaning or histamine receptor antagonism in gene expression of *MUC2* (**Figure 2.4Z**). At 24 hours post weaning, expression of *MUC2* was downregulated in response to weaning (P=0.0004); treatment with either desloratadine or famotidine prevented weaning-induced downregulation in

expression (*P*=0.0199 and *P*=0.0158 respectively), although there was no effect of treatment with desloratadine + famotidine.

Expression of genes regulating the enzymes involved in histamine synthesis and degradation were not affected by weaning or antagonism of histamine receptors (**Figure 2.4AA-AC**). Gene expression of HNMT did exhibit a significant effect of time between 3 hours and 24 hours post weaning (**Figure 2.4AC**). At both 3 and 24 hours post weaning, expression of PTGS1 was downregulated in response to weaning (Figure 2.3AD; *P*=0.0274 and *P*=0.0022). Expression of PTGS1 was not changed with histamine receptor antagonism at either timepoint. At 3 and 24 hours post-weaning, *PTGS2* expression was unchanged in response to weaning stress or histamine receptor antagonism (**Figure 2.4AE**). At 3 hours post weaning, expression of VEGF was not changed in response to weaning or antagonism of histamine receptors (**Figure 2.4AF**). However, at 24 hours post weaning, expression was downregulated in response to weaning (*P*<0.0001) but was not affected by histamine receptor antagonism.

H2R Antagonism Reverses Weaning-Induced Upregulation of Myeloperoxidase and Downregulation of Hydrogen Peroxide

As we saw expression of classical inflammatory genes were downregulated in response to weaning or histamine receptor antagonism treatment, we assessed associated inflammation and protein expression in the jejunal mucosa. Since increased neutrophil infiltration and activation are hallmarks of a marked inflammatory response, we assessed protein expression of myeloperoxidase (MPO) within the jejunal mucosa. We saw no differences in expression in response to weaning or with treatment of antagonists at 3 hours post weaning. However, at 24 hours post weaning, there was a

Table 3: Two-Way ANOVA P Values of RT-qPCR Gene Expression Analysis

Gene	Treatment Effect	Time Effect	Interaction
COL1A1	0.0003***	<0.0001****	0.2520
COL1A2	0.6102	0.0141*	0.6220
ELN	0.3357	0.0015**	0.2194
MMP9	0.1630	0.0247*	0.0324*
TNC	0.1161	0.0046**	0.6710
ITGB1	0.6726	0.5625	0.3768
ICAM1	0.4078	0.0006***	0.1202
MADCAM1	0.4710	0.8352	0.9962
VCAM1	0.7807	0.1124	0.0601#
IFNγ	0.0159*	0.1361	0.0662#
IL1β	0.2149	0.3775	0.4999
IL4	0.0863#	0.2586	0.2215
IL6	0.1782	0.4617	0.9667
IL17A	0.1044	0.0187*	0.5643
MCP1	0.2196	0.7645	0.9062
MIP1b	0.6627	0.6311	0.9841
TNFα	<0.0001	0.0932#	0.8024
IFNγR1	<0.0001****	<0.0001	<0.0001****
IL4R	<0.0001****	0.7569	0.2498
IL6R	0.0008***	0.0710#	0.5390
IL17AR	0.0233*	0.0021**	0.0141*
TLR4	0.3000	0.9997	0.3091
CHAT	<0.0001****	0.4436	0.8569
CRFR1	0.8083	0.4824	0.5029
CRFR2	0.0260*	0.0037**	0.6057
MUC2	0.0003***	0.4546	0.2862
DAO	0.2866	0.6344	0.1549
HDC	0.5486	0.1628	0.6166
HNMT	0.8244	0.0048**	0.4715
PTGS1	0.0049**	0.0119*	0.5322
PTGS2	0.5884	0.2346	0.5244
VEGF	0.0003***	<0.0001****	0.2270
#*P*≤0.1, **P* ≤0.05, ***P* ≤0.01, ****P* ≤0.001, *****P* ≤0.0001,			

significantly higher expression of MPO in response to weaning stress (*P*=0.0068). There was no effect seen in the desloratadine treatment group (*P*=0.8177), but treatment with

famotidine and treatment with desloratadine + famotidine was able to prevent this weaning-induced increase (**Figure 2.5A**; *P*=0.0006 and *P*=0.0484 respectively). Since MPO is also a mediator of oxidative stress, we measured the reactive oxygen species, hydrogen peroxide (H_2O_2) in the jejunal mucosa. At 3 hours post weaning, we saw that weaning reduced levels of H_2O_2 compared to unweaned controls (**Figure 2.5B**; *P*=0.0093). While we saw no significant differences between saline treated animals and those treated with desloratadine alone or with both desloratadine and famotidine, we saw treatment with famotidine prevented weaning-induced decreases in H_2O_2 (*P*=0.0166). At 24 hours post weaning, we saw that weaning reduced expression of H_2O_2 (*P*=0.0228), but there was no effect of histamine receptor antagonism. This indicates that H2R mediates neutrophil activity in the jejunum.

H2R Antagonism Reduces Weaning-Induced Upregulation of IL-1β and IL-6

In measuring protein expression of IL-1β in jejunal mucosa at 24 hours post weaning, weaning increased expression compared to unweaned animals (**Figure 2.6A**; *P*=0.0369) and treatment with famotidine prevented the weaning-induced increases in expression (*P*=0.0415). However, treatment with desloratadine did not change protein expression (*P*=0.7920). In measuring protein expression of IL-6 at 3 hours post weaning, we saw that weaning increased expression compared to unweaned controls (**Figure 2.6B**; *P*=0.0061) and that this weaning-induced increase in expression was prevented with administration of famotidine (*P*=0.0402). We did not see any expression differences in the treatment with desloratadine alone or desloratadine and famotidine combined. We also didn't see any responses to weaning or antagonism at 24 hours post weaning. In assessing protein expression of IFNγ, we saw that it did not match the

gene expression analysis in that we saw no effect of weaning or antagonism (**Figure 2.6C**). However, we did see a significant time effect where expression of IFNγ was downregulated within each treatment group from 3 hours to 24 hours post weaning. This indicates that H2R mediates expression of specific proinflammatory cytokines. This could also be an indicator that there is a decreased macrophage presence in the intestinal tissue, but possibly increased infiltration into the mesentery and lymph system.

Antagonism of H1R and H2R Does Not Change Expression of β-Integrin, TLR4, COX2, and ChAT

Similar to our gene expression analysis, assessment of β-Integrin showed no effect in protein expression in response to weaning (**Figure 2.7A**; *P*=0.9607). In contrast to our gene expression analysis, we saw no effect in treatment with desloratadine (*P*=0.4596) or famotidine (*P*=0.1348). Further, in assessment of TLR4, COX2, and ChAT, we saw that weaning and histamine receptor antagonism had no effect on protein expression (**Figure 2.7B, C, and D**).

H2R Antagonism Reduces Weaning-Induced Expression of TGFβ but not IL-1β in the MLN

A trend towards increased expression of TGFβ was observed in response to weaning in the mesenteric lymph node (MLN) compared to unweaned controls (*P*=0.0712). Both desloratadine and famotidine reduced the weaning-induced increases in expression of TGFβ compared to saline treated animals (*P*=0.0016 and *P*=0.0010 respectively; **Figure 2.8A**). No differences in protein expression of IL-1β were observed in response to weaning or treatment with desloratadine or famotidine (**Figure 2.8B**). As this expression pattern resembles that of tolerogenic dendritic cell secretions, this may

indicate their presence, but further assessment of phenotype and resulting T cell polarization is necessary.

H2R Antagonism Reduces Expression of β-Integrin but Increases Expression of ChAT in the MLN

Similar to what we saw in the jejunum, weaning had no effect on β-Integrin expression in the MLN (*P*=0.6277; **Figure 2.9A**). However, antagonism of H1R trended to reduce β-Integrin expression (*P*=0.0553) and antagonism of H2R showed significantly reduced expression compared to saline treated animals (*P*=0.0002; **Figure 2.9A**). Although we did not see any significant differences in response to weaning stress or histamine receptor antagonism in the expression of MHCII in the MLN, we did see a trend toward increased expression in animals treated with desloratadine + famotidine at 3 hours post weaning (**Figure 2.9B**; *P*=0.0791). However, this was lost at 24 hours post weaning. Expression of ChAT was not significantly changed in the MLN in response to weaning at either 3 or 24 hours post weaning timepoints (**Figure 2.9C**). At 3 hours post weaning, treatment with desloratadine or famotidine alone did not have an effect on ChAT expression, but treatment with desloratadine + famotidine trended towards increased expression (*P*=0.0755). At 24 hours post weaning, treatment with famotidine significantly increased expression of ChAT compared to saline treated animals (**Figure 2.9C**; *P*=0.0071). These data indicate that histamine receptors mediate some facets of antigen presentation and immune cell activation in the draining lymph node.

Discussion

Early life adversity has been shown to cause inflammation during developmental periods and is linked with increased disease risk later in life, including gastrointestinal

disorders.[2,3,20] More specifically, weaning stress in pigs increases immune responses and intestinal inflammation[21-24] that persist into adulthood.[6,7,17] However, the mechanisms that trigger early inflammatory responses remain poorly understood. Mast cell activation and increases in histamine levels have been shown to increase quickly in response to stressors,[8] and histamine receptors are well known for their role in allergic inflammation.[25] Here we demonstrate that H1R and H2R differentially mediate distinct aspects of weaning-induced inflammatory responses, including gene and protein expression relating to cytokines, ECM, oxidative stress, and cellular trafficking. Previous work has focused on later timepoints and have not investigated the role of histamine receptors in early inflammatory responses to weaning stress.

H1R and H2R Show Differing Localized Protein Expression Patterns in Jejunum Mucosa

Early life adversity is a significant risk factor for GI and inflammatory disease later in life.[1,2,26] However, the mechanisms by which early life adversity initiates early inflammatory responses remain poorly understood. Previous work has indicated that mast cells, histamine, and histamine receptors play a critical role in regulating intestinal inflammatory responses.[6,11,12,27] In our study, expression of H1R was localized to the lamina propria and muscle, while expression of H2R was localized to epithelium and lamina propria. Further, weaning increased expression of H1R within the lamina propria and expression of H2R within the epithelium. This confirms previous work that showed that lamina propria mononuclear cells have a higher expression of H1R than H2R.[15] The lamina propria contains stromal cells and numerous immune cells, including T cells, macrophages, mast cells, and dendritic cells.[28] Considering the expression of H1R is

localized to the lamina propria, this may indicate that H1R mediates activation or trafficking of immune cells. Cells within the epithelium play crucial roles in maintaining the intestinal barrier and antigen presentation during the inflammatory response.[29] Given the expression of H2R is localized to the epithelium, this may indicate that H2R meditates these functions. However, colocalization of H1R and H2R to specific cell types within the mucosa would further elucidate the role that histamine receptors play in weaning stress-induced inflammatory responses.

Antagonism of H1R and H2R Selectively Attenuates Weaning Stress-Induced Gene Expression of Jejunal Mucosa

In this study, we investigated the gene expression profiles of ECM and immune-related genes during the inflammatory response to weaning stress. We were surprised to see our data demonstrated a broad downregulation in gene expression of ECM and immune-related genes, despite observed increases in inflammatory protein expression. Interestingly, a select group of genes, including *IFNγ*, *IL4*, *IL17AR*, and *ELN* exhibited upregulation during the same inflammatory response. IFNγ and IL-17A are proinflammatory cytokines that play critical roles in the activation and regulation of immune cell, including neutrophils, and progression of inflammation.[30] The upregulation of these cytokines is consistent with the increases in inflammatory proteins we observed, which suggests they play a crucial role in the inflammatory response. IL-4 is an anti-inflammatory cytokine that is secreted by mast cells, T_h2 cells, eosinophils and basophils and is known to play an important role in antibody production, inflammation, and effector T cell responses.[31] Although largely recognized for its role in T_h2 cell responses, there is also evidence that IL-4 plays a proinflammatory role in the

intestines.[32] The upregulation of *IL-4* may indicate a unique inflammatory response induced by weaning, a complex stressor. However, further studies would be necessary to determine the expression of IL-4 on the protein level. Elastin is an ECM protein responsible for providing elasticity and resilience to tissues.[33] In chronic inflammatory diseases, such as ulcerative colitis, patients express decreased levels of elastin.[34] However, gene expression of *ELN* was found to be upregulated in our data set but was not affected by histamine receptor antagonism. This upregulation could be indicative of tissue remodeling or repair processes occurring in response to weaning stress. Increased expression of elastin may also be an adaptive response aimed at maintaining the structural integrity of the affected tissue during acute inflammation. Further studies investigating elastin protein expression and ECM remodeling in response to weaning stress are necessary.

This marked downregulation of ECM and immune-related genes during an inflammatory response is an unexpected finding. A possible explanation for this unique profile is negative feedback mechanisms that act to prevent an excessive inflammatory response, which might otherwise lead to tissue damage or autoimmune reactions. Here, downregulation of these genes might represent a regulatory mechanism aimed at maintaining homeostasis and preventing immunopathology.

Another potential explanation for the observed downregulation is the involvement of previously unidentified regulatory elements or pathways that modulate the expression of immune-related and ECM-related genes. Further studies are needed to elucidate these potential mechanisms and to determine whether the downregulation observed is a specific response to the inflammatory stimulus used in this study or a more general

response to inflammation.

Another possible explanation of downregulated gene expression is transcriptional regulation by unidentified regulatory elements or pathways that modulate the expression of immune-related and ECM-related genes, such as glucocorticoids. Glucocorticoids are regulated by the hypothalamic–pituitary–adrenal (HPA) axis, which is activated by weaning stress,[19] and have been implicated in the downregulation of inflammatory responses.[35-37] However, further studies, including an analysis of the mucosal transcriptome, are required to determine potential mechanisms of the observed downregulation, if the expression pattern here is specific to the weaning stress stimulus or if it is a previously uncharacterized response to inflammation.

Another potential explanation for these results is transient gene expression in response to a complex stressor to reduce excessive inflammation and tissue damage. Previous studies have suggested that gene expression of inflammatory cytokines within the gut is transient in response to weaning stress.[38,39] As many of the genes we assessed were not significantly changed in response to weaning at the 3 or 24 hour post weaning timepoints, it possible that they have transient expression in response to stress and they are upregulated at other timepoints, but future studies including a more inclusive time-course would be necessary.

Antagonism of H1R and H2R Selectively Attenuates Weaning Stress-Induced Protein Expression of Jejunal Mucosa and Mesenteric Lymph Nodes

Here we also demonstrate a unique inflammatory protein response within the jejunal mucosa and MLN. We observed increased expression of MPO, IL-1β, IL-6, and TGFβ in response to weaning stress and a reduced expression with H2R antagonism. It has

been indicated that heat stress and heat shock protein 70 induce intestinal inflammation and increase neutrophil activity and therefore MPO expression.[40,41] However, it has also been demonstrated that H2R antagonists have anti-oxidant properties in MPO-catalyzed reactions.[42] It is undetermined here whether or not the weaning-induced increase in MPO expression is due to increased activation of resident neutrophils or due to increased trafficking of neutrophils to the tissue. Further, MPO is a major catalytic enzyme in the production of reactive oxygen species (ROS).[43] It has been demonstrated that circulating H_2O_2 levels are increased in response to weaning stress in piglets.[44] Moreover, *in vitro* cultures of IPEC-J2 cells show reduced viability when supplemented with H_2O_2, a ROS.[45] In our study, expression of H_2O_2 in the jejunal mucosa was decreased in response to weaning stress at both 3 and 24 hours post weaning, but this weaning-induced decrease was prevented by famotidine at 3 hours post weaning. Although toxic in high concentrations, production of H_2O_2 in intestinal epithelial cells has been shown to be an important signal for wound repair and recruiting immune cells in response to injury.[46] It has been previously shown that leukocyte binding of VCAM1 results in the production of H_2O_2 and activation of MMP-mediated degradation of extracellular matrix to allow for immune cell migration.[47,48] A possible explanation is that there is a high antioxidant capacity within the jejunum mucosa allowing for reduced levels of ROS upon weaning and this capacity is not mediated by histamine receptors. However, assessment of antioxidant capacity would be needed. Given our data demonstrating downregulation of genes related to the extracellular matrix and adhesion molecules, it is also possible that early weaning stress causes a reduction in leukocyte binding by specific adhesion molecules and reduced production in ROS at the

timepoints assessed here. However, further assessment of transcriptional regulation and localization of immune cells is necessary. Together, these data indicate that weaning stress increase in neutrophil activity in the gut is mediated by H2R. Further, combined with our gene expression data, this also indicates that early weaning stress may uniquely regulate ROS production.

We also demonstrate weaning induced expression of IL-1β and IL-6 is attenuated with antagonism of H2R. IL-1β and IL-6 are proinflammatory cytokines shown to increase epithelial barrier permeability.[49,50] Similar to our observations, previous studies have shown that levels of IL-6 are quickly elevated in response to immune challenge, but then are reduced by 22 hours post challenge.[51] It has been demonstrated that glucocorticoids are anti-inflammatory, but it has also been shown that they do not regulate expression of IL-6 in the inflammatory response.[52] This would align with the hypothesis of glucocorticoid regulation as a potential explanation for observed downregulated inflammatory gene expression, but increases in inflammation at the protein level. Further, we observed no significant changes in response to weaning or histamine receptor antagonism in other inflammatory proteins, which may be tied to the downregulation in inflammatory genes. This may also be due to the expression localization patterns of histamine receptors and that the mucosa was analyzed as a homogeneous sample. H1R may be more responsible for inflammatory responses in the lamina propria and H2R may be more responsible for inflammatory responses in the epithelium. Future studies evaluating cell-specific gene and protein expression would be beneficial in elucidating the role of histamine receptor-mediated inflammatory responses.

The mesenteric lymph nodes play a critical role in draining the small intestine and in the activation of T cells. Antigen presenting dendritic cells migrate from the intestines to release cytokines and prime naïve immune cells. Dendritic cell precursors mature into differing phenotypes based on the combination of stimulatory factors they are exposed to. The mature dendritic cell subpopulations have differing surface receptor expression and cytokine secretion patterns.[53] It has been shown that stress reduces expression of MHCII and IFNγ protein expression in antigen presenting cells.[54,55] We observed that H2R antagonism reduces weaning induced expression of TGFβ and β-Integrin expression but had no effect on IL-1β or MHCII expression. As this weaning-induced protein expression pattern resembles that of tolerogenic dendritic cells,[53,56] this may indicate that weaning stress alters dendritic cell populations and that this may be mediated by H2R. Future studies characterizing the populations of dendritic cells and T cell populations within the MLN in response to weaning stress and histamine receptor antagonism would be necessary to determine the extent by which histamine receptors mediate dendritic cell maturation and subsequent adaptive immune responses including T cell polarization.

Limitations and Future Directions

A limitation in this study is the limited knowledge of the short-term effects of weaning stress. Previous studies have focused on investigating the long-term effects of weaning stress on immune and gut health[2,6,57] and the role of histamine receptors in mediating allergic inflammation and gastric acid secretions.[11,12] However, there remains a large gap in knowledge in the early transcriptional regulation of genes in response to weaning stress as well as the functional consequences of histamine receptor expression or

activation. To our knowledge, our study is the first to assess the intestinal inflammatory response to weaning stress in a short-term time course. Moreover, we here demonstrate that inflammatory gene and protein responses are selectively altered by antagonism of H1R or H2R, such as extracellular matrix, adhesion molecules, and cytokine expression. Although future studies including more timepoints may be necessary to fully understand the implications of histamine receptor activation in the inflammatory response to weaning stress, we have provided here a foundation for future studies aimed at targeting the histamine system as a regulatory mechanism of stress-induced intestinal inflammation.

Another limitation in our study is the use of male castrates and not including females. Previous human studies have indicated that females have increased inflammatory response compared to males after puberty.[58,59] Female pigs also have been shown to have longer time with diarrhea and increased mast cell tryptase activity when early weaned compared to male castrates.[6] However, this does not negate the significant data generated here as male castrates have an increased mortality rate in the nursery phase of swine production compared to females.[60] Sexual dimorphic programming of mast cells and inflammatory responses following early life adversity have been recently characterized and attributed to perinatal androgens,[10,16] which indicates a key contribution of biological sex in inflammatory responses of neonates as well as adults. Inclusion of females in future studies is necessary to determine the sex specific roles of histamine receptors in inflammatory responses to weaning stress.

An additional limitation is that we cannot conclude if the effects of the famotidine are due to anti-inflammatory properties or its ability to reduce gastric acid secretions.

Famotidine is also commonly utilized to reduce gastric acid in both humans and animals. Here, we are unable to determine if the changes in the intestinal inflammatory response are attributed to a reduction in gastric acid secretion or another mechanism of action by famotidine. Future studies may include the use of a proton pump inhibitor to determine if inflammatory responses can be attributed to changes in gastric acid secretion itself or if famotidine has previously uncharacterized properties in mediating inflammatory responses to weaning stress.

Future experiments should also include a transcriptome analysis of the jejunal mucosa to assess the effects of weaning and histamine receptor antagonism on gene expression. Utilization of RNA sequencing (RNA-seq) would give a more comprehensive understanding of the gene expression pathways regulated by weaning stress and histamine receptors within the intestine. Given that the intestinal mucosa is complex and contains numerous cell types, utilizing single-cell RNA-seq would provide critical knowledge of the cell-specific gene expression changes that histamine receptors mediate in response to stress. Together, these would provide a more comprehensive understanding of gene expression regulation and potentially reveal additional targets that influence the progression of inflammation.

Future studies would also benefit from characterizing expression and effects of other histamine receptor subtypes. The histamine 3 receptor (H3R) and histamine 4 receptor (H4R) are commonly recognized for their mediation of neuronal function and immune cell trafficking, respectively. Although these receptor subtypes have a lesser expression in the intestine compared to H1R and H2R under basal conditions, little is known of their expression or ramifications of their activation in response to early weaning stress. The

enteric nervous system is plays a large role in the pathogenesis of enteric diarrheal and inflammatory bowel disorders.[61,62] Given the expression if H3R in the nervous system, it is possible that H3R mediates neuro-secretory functions that underlie these disease progressions. The importance of immune cell migration in the induction of an immune response also positions H4R as a probable target in mediating early inflammation. Further experiments including the use of antagonists specific to these receptor subtypes would help to identify the role that they, and histamine, play in inflammatory responses to early weaning stress. Along with antagonists, additional studies utilizing histamine receptor agonists could also provide insight to the role histamine receptors play in inflammatory responses. Receptor-specific agonists, such as dimethylhistaprodifen and impromidine, could be utilized to induce receptor-specific response and allow for a more thorough characterization of the mechanism by which histamine receptors regulate inflammatory responses.

Conclusion

In summary, the present studies demonstrate that weaning stress largely downregulates extracellular matrix and inflammatory gene expression yet increases inflammatory protein expression in the jejunum. We also demonstrate that H1R and H2R individually mediate differing aspects of these responses, such that antagonism of each receptor regulates specific individual gene or protein expressions related to cellular adhesion, trafficking, or cytokines, but not all. Given that early life inflammation is a major risk factor for later life development of chronic inflammatory diseases and GI disorders, these findings serve as a first step to characterizing early inflammatory responses to early weaning stress and the role of histamine receptors. This also provides new

knowledge of the early short term time course at which inflammatory responses to stress occur, although future studies are necessary to assess an increased number of time points. Future directions should include evaluation of immune cell populations colocalized to histamine receptors as well as a transcriptomic analysis to identify pathways regulated by histamine receptors and identify other potential anti-inflammatory targets.

Acknowledgments

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Histological Staining

Special thanks to Amy and Jessie at the Michigan State University Investigative Histopathology Laboratory for their assistance in the histological tissue mounts and staining.

Figures

Figure 2.1: Experimental design
Piglets were selected based on body weight and randomly assigned a treatment group. At 18 days of age, piglets were given an injection 30 minutes prior to weaning of either saline (control), desloratadine (2 mg/kg), famotidine (4 mg/kg), or desloratadine + famotidine. At 0, 3, and 24 hours post weaning, blood and tissue (jejunum and mesenteric lymph node) samples were harvested. RNA and protein were isolated from jejunal mucosa and mesenteric lymph nodes for subsequent analyses. Created with BioRender.com.

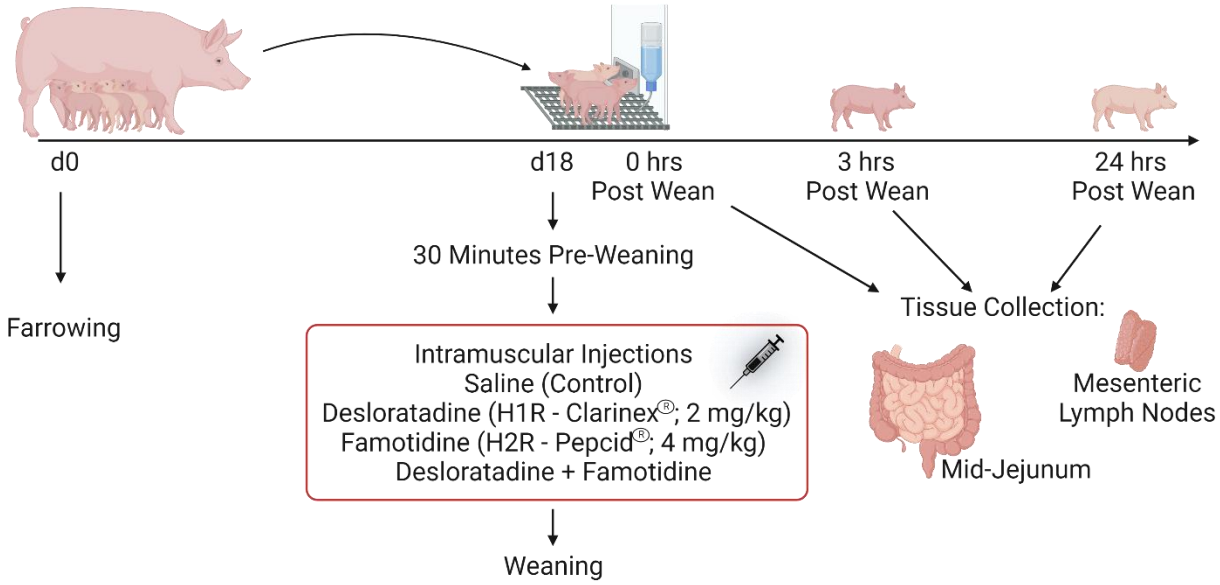


Figure 2.2: H1R and H2R is Localized to Lamina Propria and Epithelium Respectively in Jejunal Mucosa

Jejunum isolated from pigs directly at weaning and at 8 hours post weaning was stained for H1R (A, C) and H2R (B, D) to assess localized expression within the jejunal lamina propria (E) and epithelium (F). Scale bars = 100μm. Data are presented as mean±SEM per animal. *n*=3-6 per treatment group. Two-way ANOVA comparison as done between timepoints and receptors. (A) Receptor Effect - *P*=0.0138, Time Effect - *P*=0.1731, Interaction - *P*=0.1907; (B) Treatment Effect - *P*=0.1399, Time Effect - *P*=0.0367, Interaction - *P*=0.0530. **P*≤0.05 ***P*≤0.01.

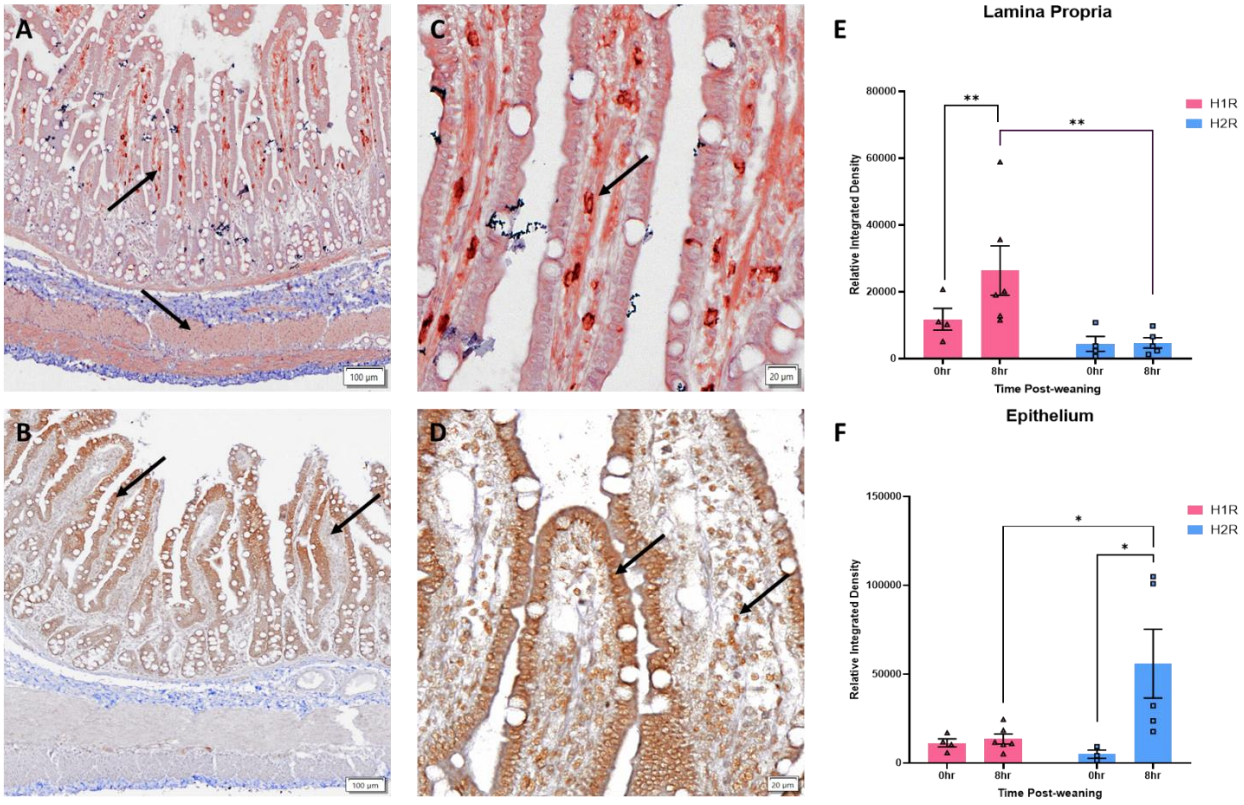


Figure 2.3: Effects of Weaning and Histamine Receptor Antagonism on Inflammatory Gene Expression

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, jejunum was collected and analyzed for gene expression via SYBR Green RT-qPCR. Heat map data are presented as mean ΔΔCT per treatment group compared to unweaned controls at (A) 3 hours post weaning and (B) 24 hours post weaning.

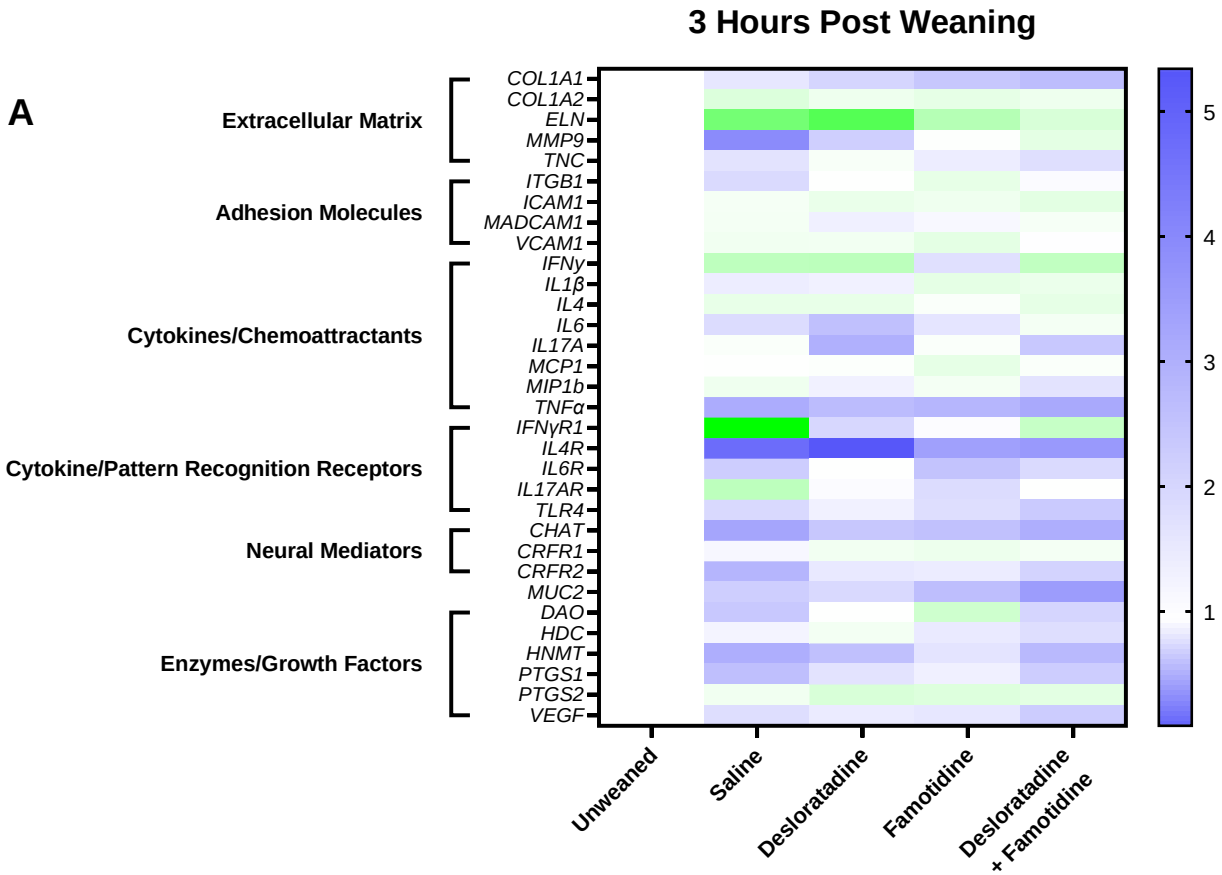


Figure 2.3 (cont'd)

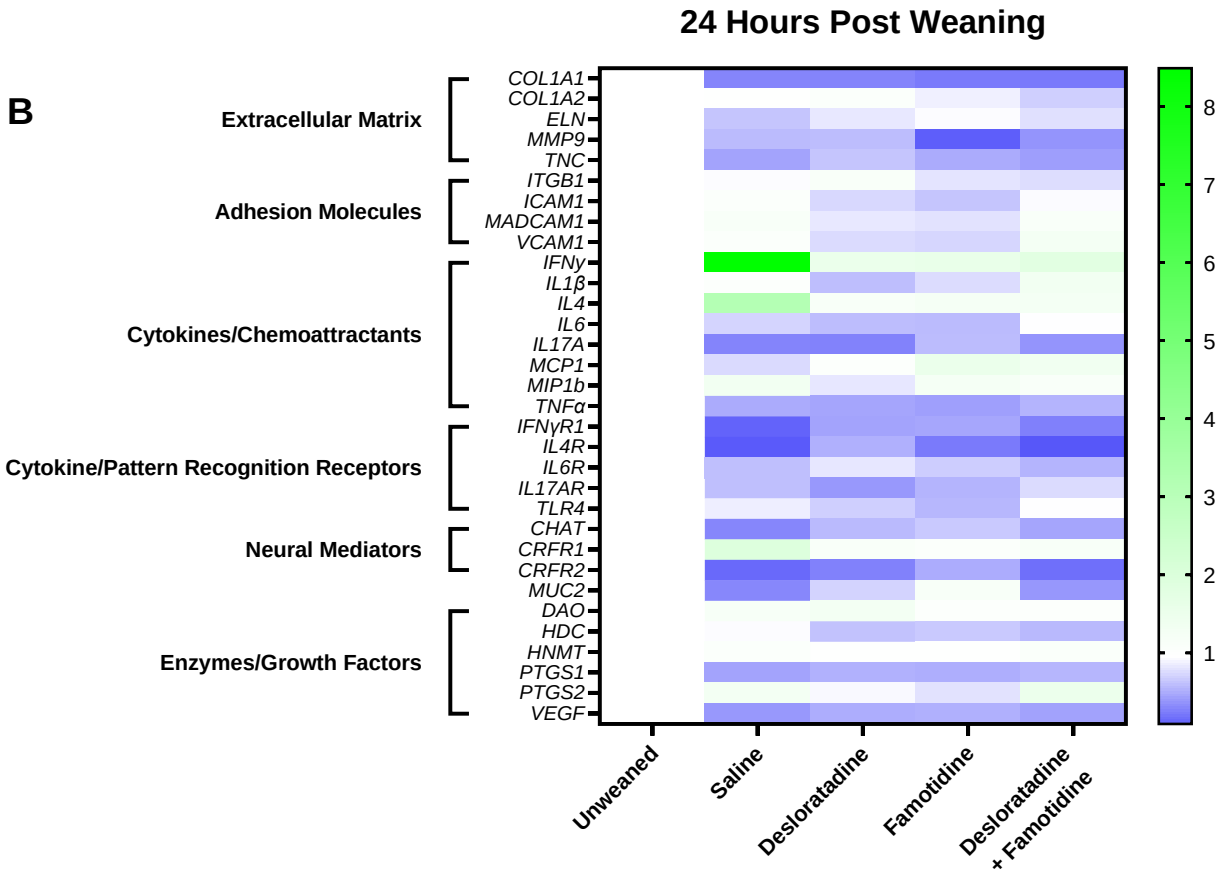


Figure 2.4: Antagonism of Histamine Receptors Decreases Weaning-Induced Upregulated Gene Expression of Inflammatory Cytokine and Cellular Trafficking Genes

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, jejunum was collected and analyzed for gene expression via SYBR Green RT-qPCR. Data are presented as mean±SEM per animal. *n*=4-6 per treatment group. Two-way ANOVA comparison between treatment groups and timepoints. *P* values indicated in Table 3. **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

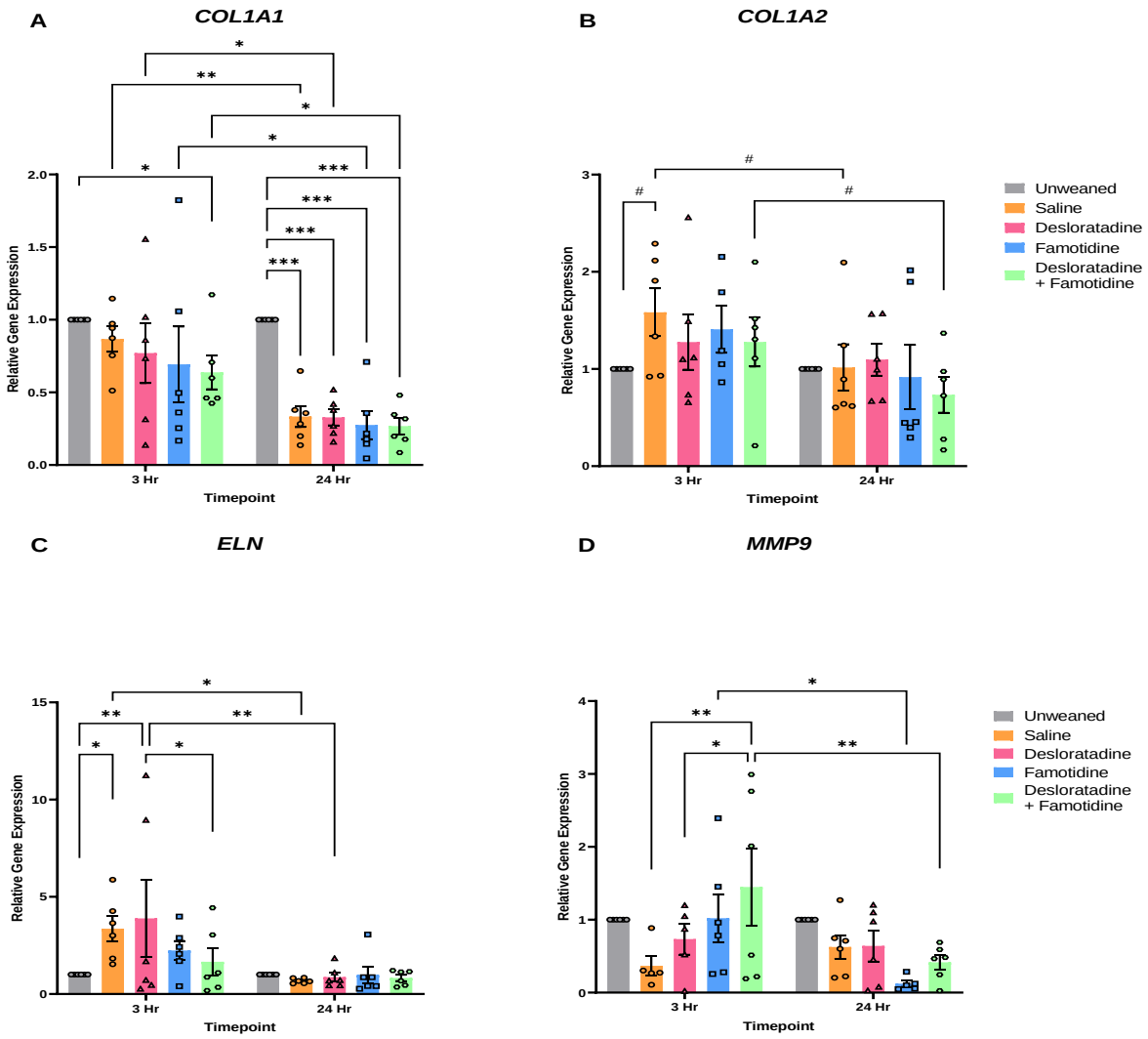
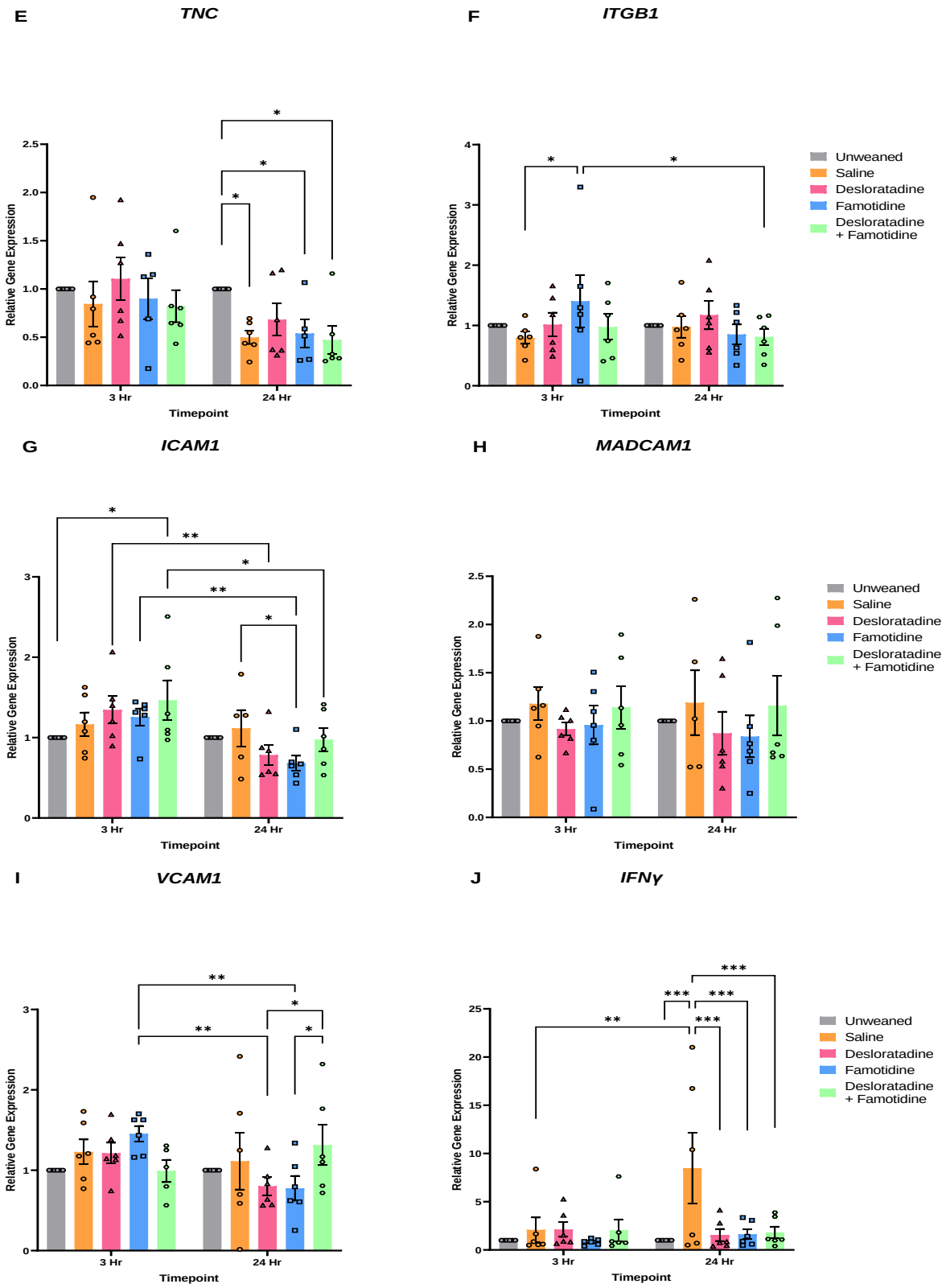


Figure 2.4 (cont'd)



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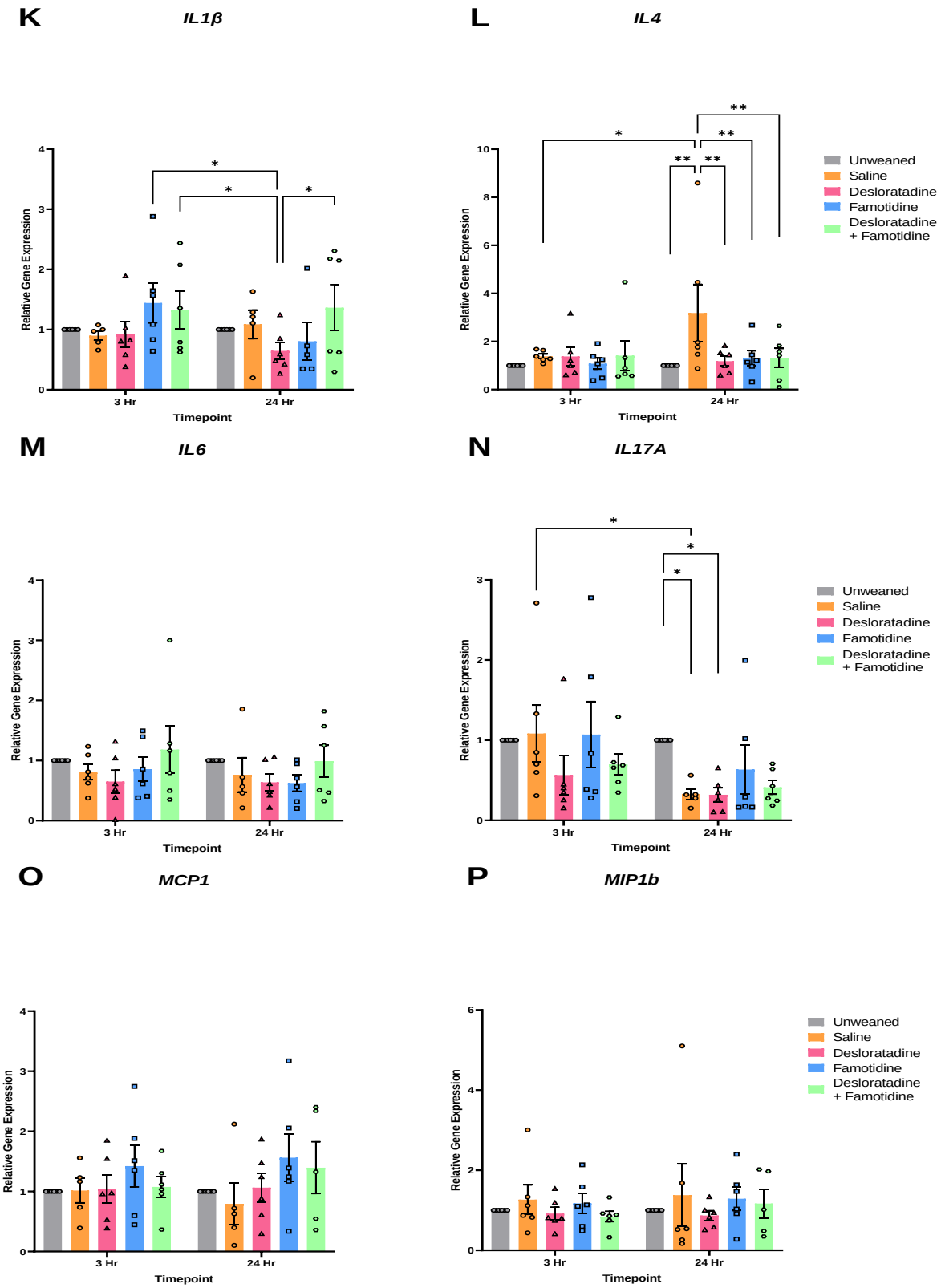

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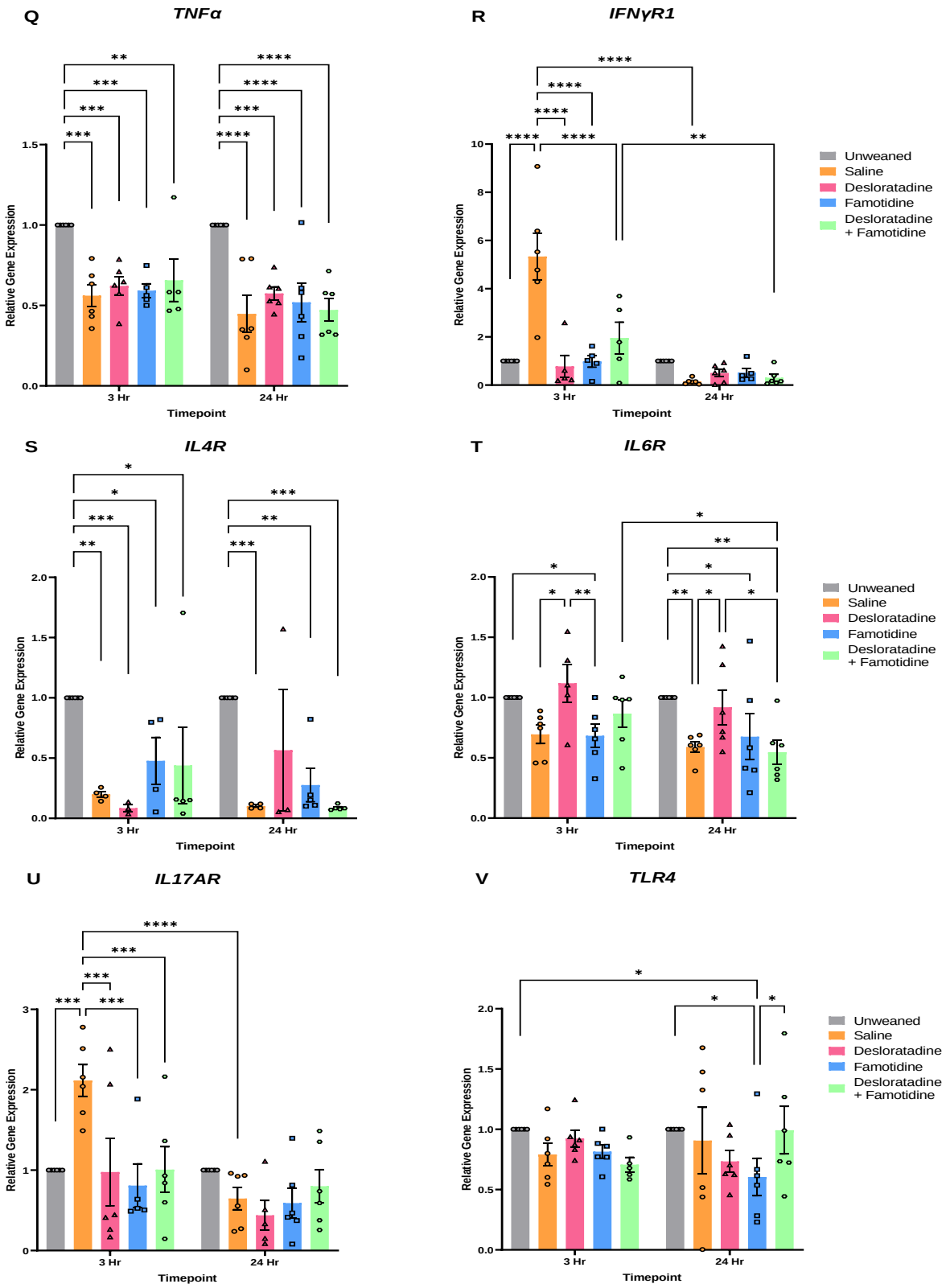

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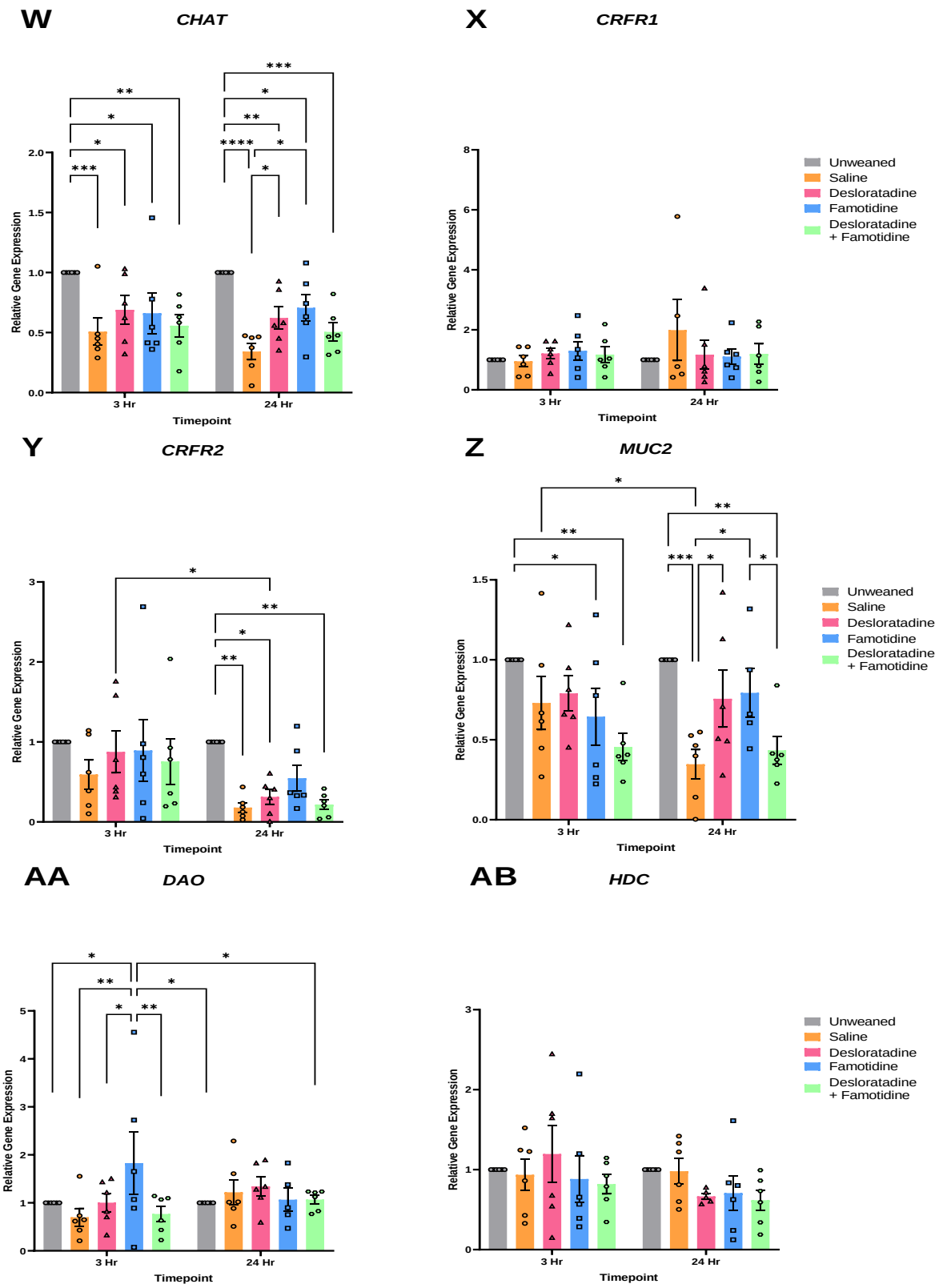

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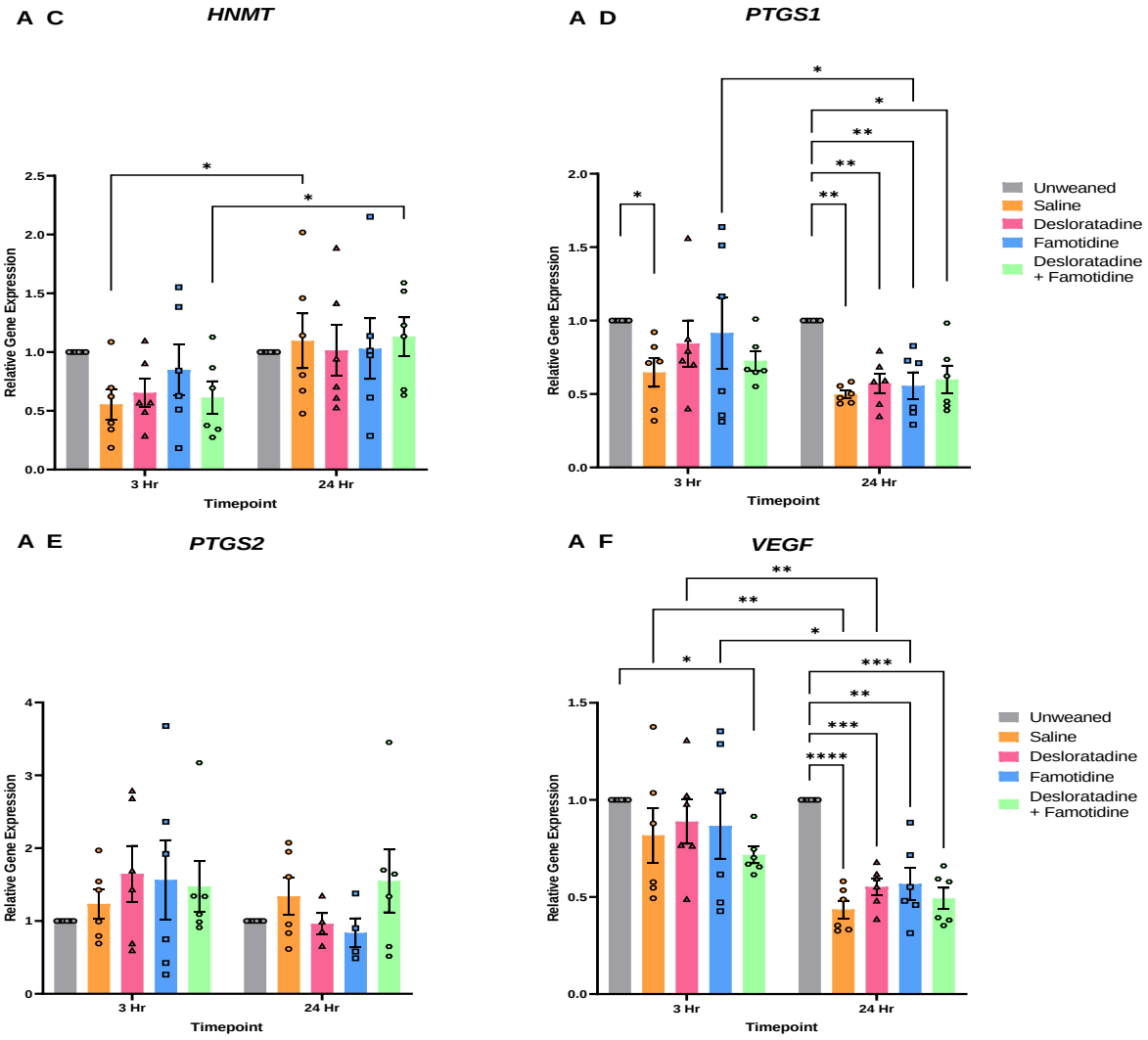


Figure 2.5: Antagonism of H2R but not H1R Prevents Weaning-Induced Expression Changes in Myeloperoxidase and H_2O_2 in Jejunal Mucosa
Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, jejunum mucosa was collected and analyzed for protein expression of (A) myeloperoxidase and (B) hydrogen peroxide. Data are presented as mean±SEM per animal. *n*=5-23 per treatment group. Two-way ANOVA comparison between treatment groups and time points. (A) Treatment Effect - *P*=0.0070, Time Effect – *P*=0.3801, Interaction - *P*=0.6073; (B) Treatment Effect - *P*=0.0137, Time Effect – *P*=0.4081, Interaction - *P*=0.3640. **P*≤0.05, ***P*≤0.01, ****P*≤0.001, *****P*≤0.0001.

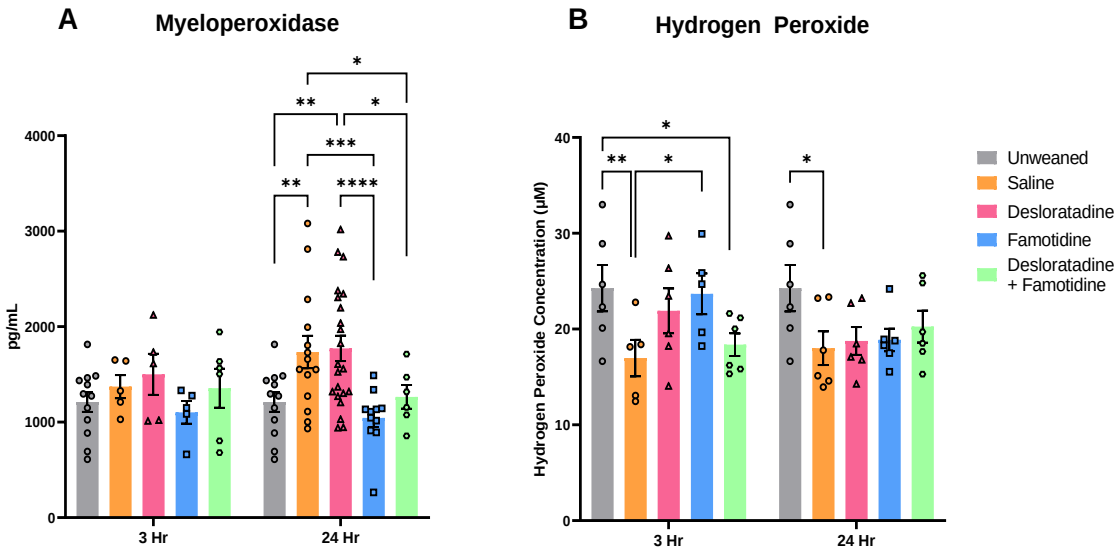


Figure 2.6: H2R Antagonism Reduces Weaning-Induced Protein Expression of IL-1β and IL-6, but not IFNγ in Jejunal Mucosa

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post-weaning, jejunum mucosa was collected and analyzed for protein expression of (A) IL-1β via ELISA. At 0, 3, and 24 hours post weaning, jejunum mucosa was collected and analyzed for protein expression of (B) IL-6 and (C) IFNγ via ELISA. Data are presented as mean±SEM per animal. *n*=4-10 per treatment group. One-way ANOVA comparison between treatment groups (A) or Two-way ANOVA comparisons between treatment groups and timepoints (B & C). (A) *P*=0.5395; (B) Treatment Effect - *P*=0.0133, Time Effect – *P*=0.2213, Interaction - *P*=0.6151; (C) Treatment Effect - *P*=0.7012, Time Effect – *P*<0.0001, Interaction - *P*=0.2106. **P*≤0.05, ***P*≤0.01.

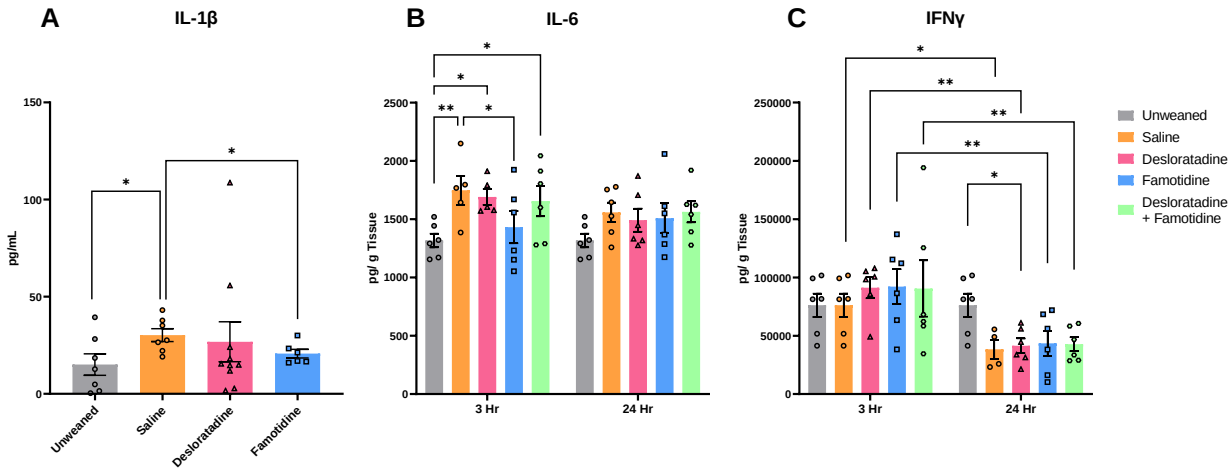


Figure 2.7: H2R Antagonism Does Not Change Protein Expression of β-Integrin, TLR4, COX2, or ChAT in Jejunal Mucosa

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, jejunum mucosa was collected and analyzed for protein expression of (A) β-Integrin, (B) TLR4, (C) COX2, and (D) ChAT via Western Blot. (E) Representative blots from WES analysis of β-Integrin and TLR4 normalized to β-Actin. (F) Representative Blots from conventional western blot analysis of COX2 and ChAT normalized to total protein. Data are presented as mean±SEM per animal. *n*=5-8 per treatment group. One-way ANOVA comparison between treatment groups (A & B) or Two-way ANOVA comparisons between treatment groups and timepoints (C & D). (A) *P*=0.5395; (B) *P*=0.9407; (C) Treatment Effect - *P*=0.4255, Time Effect – *P*=0.0022, Interaction - *P*=0.5050; (D) Treatment Effect - *P*=0.9512, Time Effect – *P*=0.8249, Interaction - *P*=0.3425. *$P \leq 0.05$.

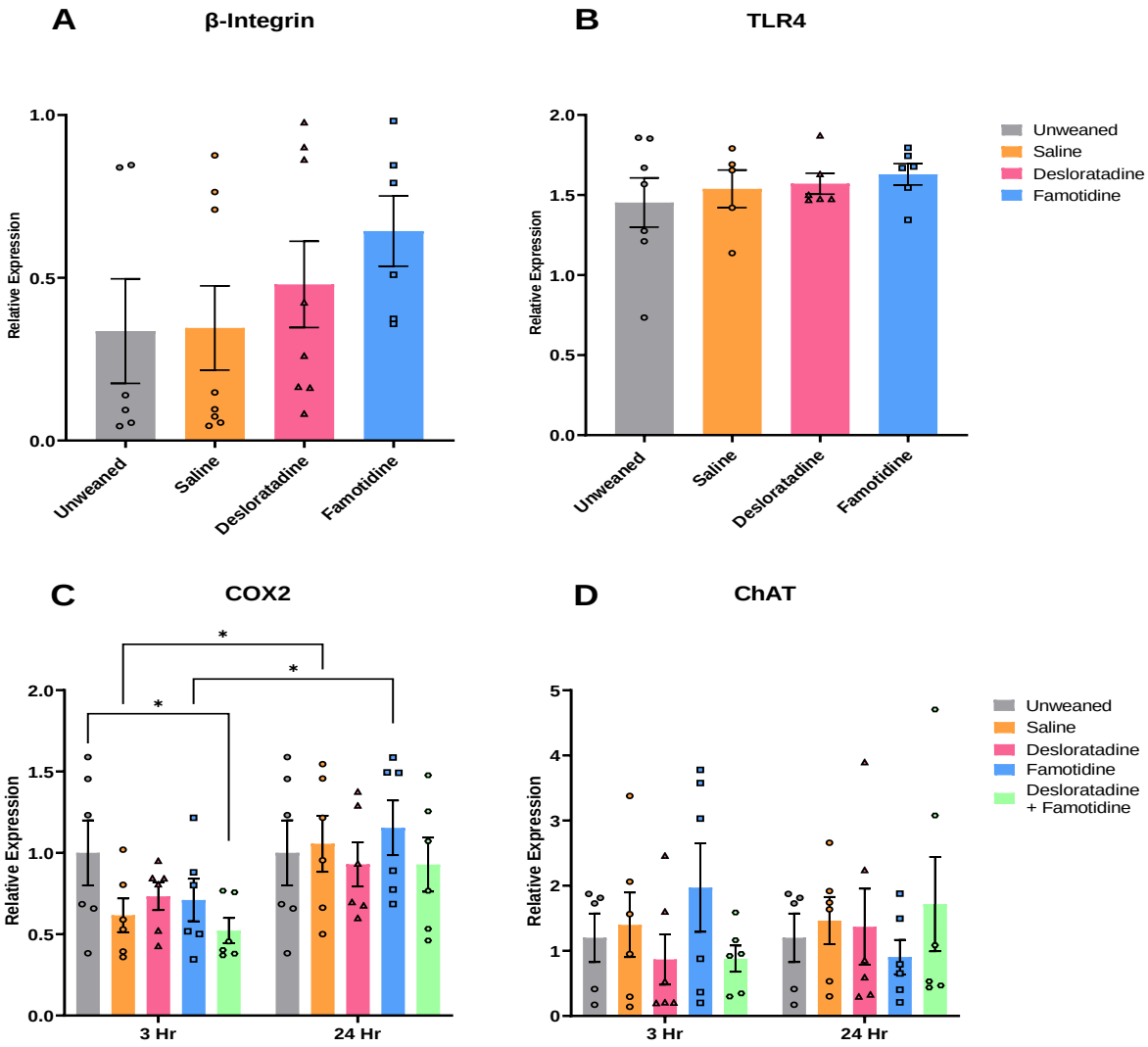


Figure 2.7 (cont'd)

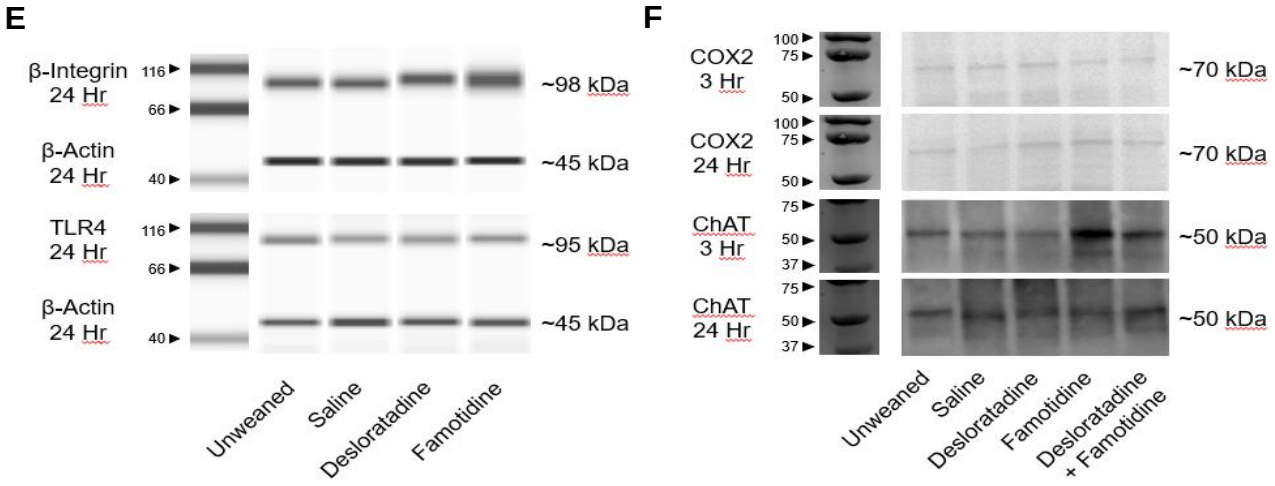


Figure 2.8: H2R Antagonism Reduces Protein Expression of TGFβ and but not IL-1β in the Mesenteric Lymph Node

Pigs were injected with saline, desloratadine, or famotidine 30 minutes prior to weaning. At 0 and 24 hours post weaning, mesenteric lymph node was collected and analyzed for protein expression of (A) TGFβ and (B) IL-1β ELISA. Data are presented as mean±SEM per animal. *n*=3-8 per treatment group. One-way ANOVA comparison between treatment groups. (A) *P*=0.0031, (B) *P*=0.2584. #*P*≤0.10, ***P*≤0.01.

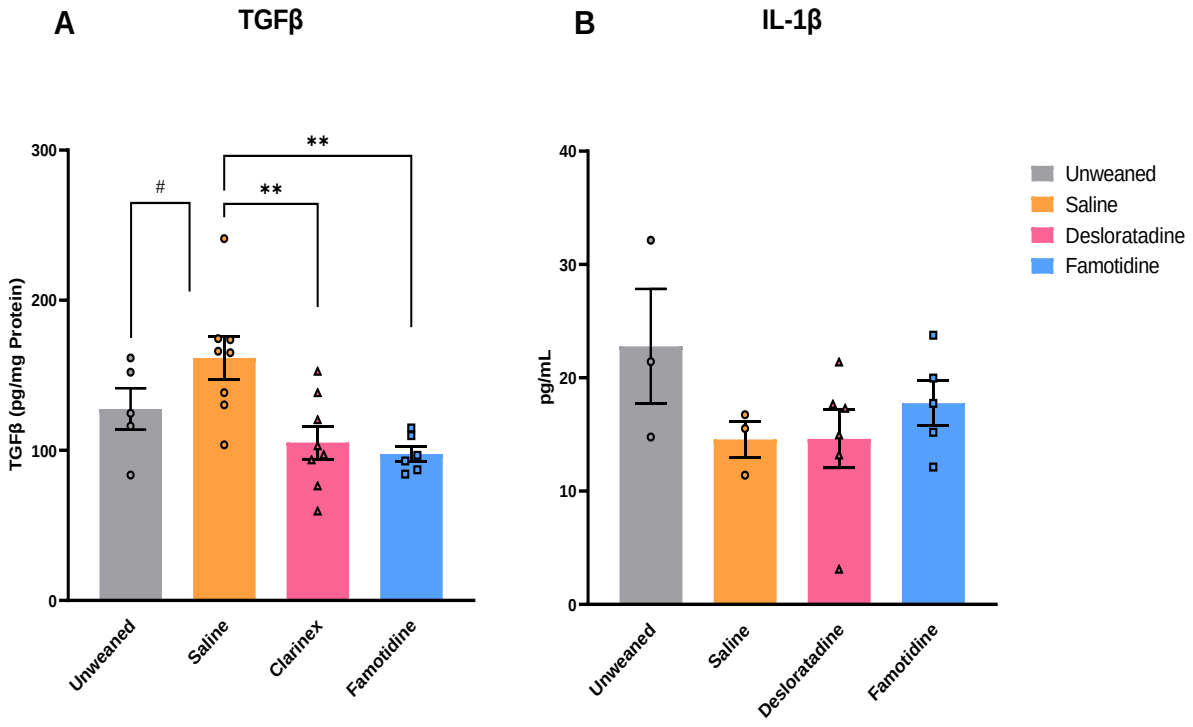
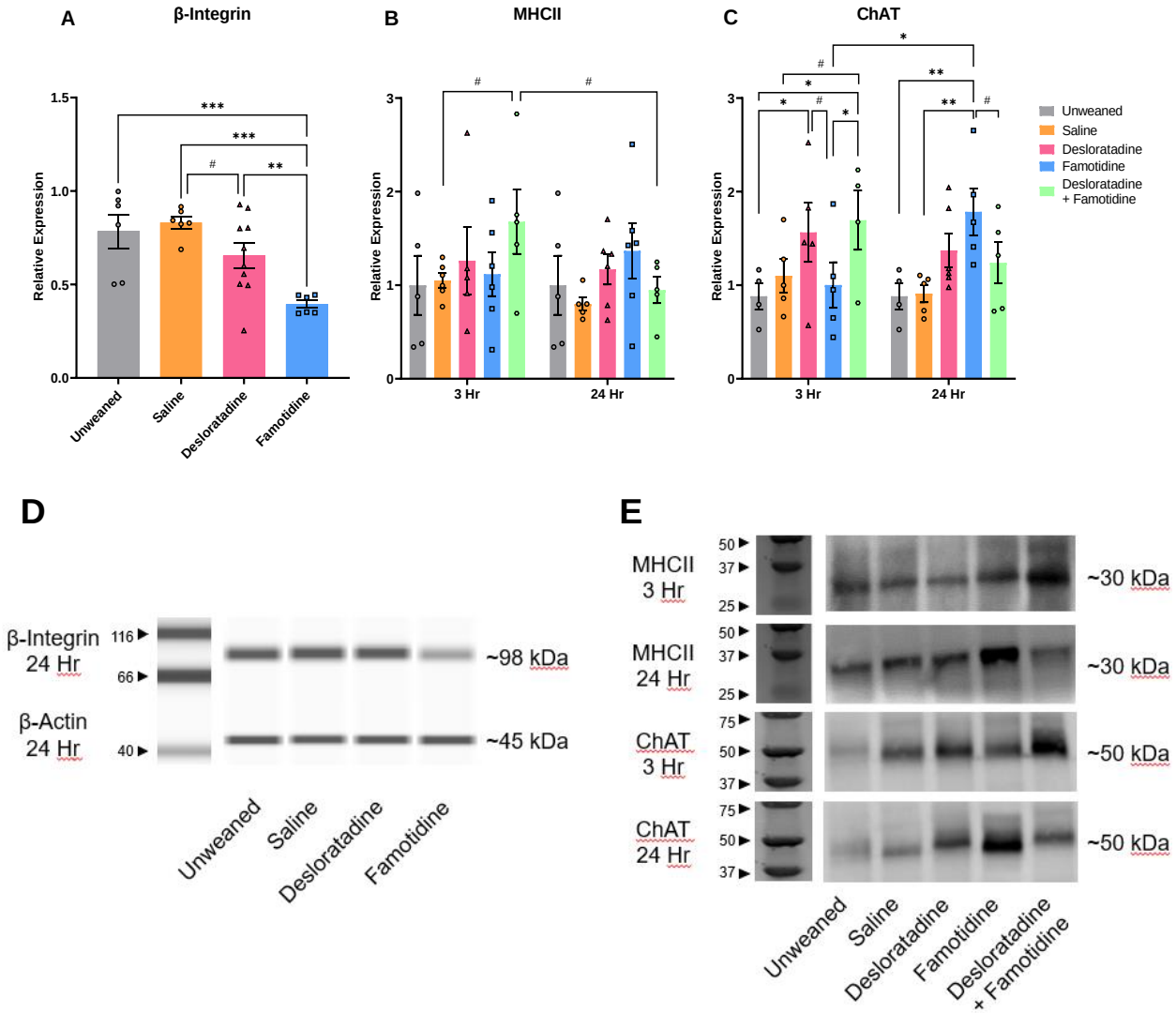


Figure 2.9: H2R Antagonism Reduces Protein Expression of β-Integrin but Increases Expression of ChAT in Mesenteric Lymph Node

Pigs were injected with saline, desloratadine, or famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mesenteric lymph node was collected and analyzed for protein expression of (A) β-Integrin, (B) MHCII, and (C) ChAT via Western Blot. (D) Representative blots from WES analysis of β-Integrin normalized to β-Actin. (E) Representative Blots from conventional western blot analysis of MHCII and ChAT normalized to total protein. Data are presented as mean±SEM per animal. *n*=4-10 per treatment group. One-way ANOVA comparison between treatment groups (A) or Two-way ANOVA between treatment groups and timepoints (B&C). (A) *P*=0.0008*;* (B) Treatment Effect - *P*=0.4767, Time Effect – *P*=0.3005, Interaction - *P*=0.3865; (C) Treatment Effect - *P*=0.0302, Time Effect – *P*=0.9394, Interaction - *P*=0.0745. #*P*≤0.05, **P*≤0.05, ***P*≤0.01, ****P*≤0.001.



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CHAPTER THREE: HISTAMINE AND HISTAMINE RECEPTORS MEDIATE INTESTINAL PERMEABILITY, NUTRIENT TRANSPORT, AND NEURAL-SECRETORY FUNCTIONS IN RESPONSE TO WEANING STRESS

Abstract

Early weaning (EW), a necessary but stressful early life production practice, induces changes in gastrointestinal (GI) epithelial barrier permeability as well as nutrient transport and secretory functions, which is detrimental for short and long-term health. The mechanism by which GI dysfunction is triggered is unknown and thus early targeted interventions are lacking. Previous studies have demonstrated that histamine and histamine receptors are upregulated early in response to weaning stress. Here we tested the hypothesis that histamine receptors mediate changes in GI epithelial barrier permeability, nutrient transport, and secretory functional responses to weaning in pigs. Histamine receptor antagonists were administered 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, whole blood was isolated to measure 16S rRNA expression. Whole jejunum was mounted on Ussing chambers where glucose and veratridine-evoked short circuit current (I_{sc}) responses were recorded as indices of nutrient transport and neuro-secretory function, respectively. Flux of FITC-dextran (FD4) was also recorded as a measure of transepithelial permeability. At 3 hours post weaning, histamine receptor antagonism prevented weaning-induced increases in plasma 16S rRNA expression. At both 3 and 24 hours post weaning, we observed that dual antagonism of H1R and H2R reduced transepithelial flux of FD4 (P=0.0497 and P=0.0244 respectively). At 24 hours post weaning, antagonism of H2R significantly reduced SGLT1-mediated glucose transport (P=0.0255) as well as neural-evoked secretion (P=0.0007) compared to saline controls. These data demonstrate that weaning stress induces early functional responses in the gut that are mediated by H2R, including epithelial permeability, nutrient transport, and neuro-secretory functions. This

provides a novel target to mitigating early GI dysfunction in swine production, a hallmark of enteric disease, as well as a model for human health and disease risk.

Introduction

Gastrointestinal disorders are the second most common health challenge in swine production, second only to respiratory disorders.[1] These include decreased gastrointestinal function, nutrient transport, suboptimal feed efficiencies, and pathogenic disease. Early weaning (EW), a necessary but stressful early life management practice, induces GI dysfunction, decreasing performance and increasing costs. Current practices to improve gut health include the incorporation of probiotics, prebiotics, and antioxidants into diets; however, targeting interventions are lacking as the specific mechanism of gut dysfunction has yet to be elucidated. The intestinal epithelial barrier plays a major role in gut, and overall, health. As the largest barrier to the outside world, the intestinal epithelial barrier prevents pathogens from entering the body, mediates nutrient transport, and secretes ions and fluids. Regulated by tight junction proteins, increased space between epithelial cells results in leaky gut and passage of bacteria across the mucosal layer into the blood stream. This increase in intestinal wall permeability has been seen in response to early life stress and increases disease risk into adulthood in both swine and humans.[2,3] Another major function of the intestinal epithelial barrier is the absorption of nutrients. The primary sodium-linked glucose transporter on the apical membrane of the epithelium is SGLT1. Expression and function of SGLT1, as well as the facilitated glucose transporter GLUT2, have been shown to increase with infectious challenge,[4] but less is known about the short- or long-term effects of early life adversity on glucose transport outside of the context of inflammatory responses. Previous work has indicated that stress increases SLGT1-mediated glucose transport and expression.[5,6] However, the mechanism driving

changes in glucose transport is unknown. The secretory functions of the epithelial barrier allow for regulation of ion gradient and fluid transport within the lumen of the intestine. It was been previously shown that these secretory functions are largely regulated by enteric neurons[7] and that neural-secretory functions are increased in response to early life adversity and stress.[8,9] Further, activation of the corticotropin-releasing hormone system has been linked to increases in both SLGT1-mediated glucose transport and neural-secretion in the gut.[7,9] Mast cells synthesize, store, and release histamine. They are also known to be highly sensitive to stressors, have increased activation and numbers in the tissue in response to stressors, and have been demonstrated to orchestrate functional changes in the gut in response to early life stress.[2,8] The various actions of histamine are conducted through four receptors.[10] Histamine 1 receptor (H1R) and histamine 2 receptor (H2R) have the highest expression in the gut of the four histamine receptor subtypes[11] and display unique localization patterns to the lamina propria and epithelium, respectively. Given H2R localization to the epithelium and the effects of histamine receptor antagonism on the inflammatory response to early weaning stress, we investigated the hypothesis that H1R and H2R mediate weaning stress-induced changes in epithelial barrier permeability, glucose transport, and neural-evoked secretory functions in pigs.

Methods and Materials

Animals

All animal studies were first approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University under the animal use protocol PROTO202200130. Male-castrate Yorkshire cross-bred piglets were selected based on

mean body weight from multi-parous sow litters without cross-fostering. At 18 days of age, selected pigs were randomly assigned a treatment group based on average body weight (n=5-6) and given an intramuscular injection 30 minutes prior to weaning of one of 4 treatments: Saline (2 mL), Desloratadine (2 mg/Kg; PHR1680, Sigma-Aldrich), Famotidine (4 mg/Kg; NDC# 0641-6021-01; 07-890-7011, Patterson Veterinary Supply), or a combination of both desloratadine and famotidine (2 mg/Kg and 4 mg/Kg respectively; **Figure 3.1**). At weaning, piglets were removed from their sow and moved to an adjacent nursery facility and cohoused with ad libitum access to water. Pigs were fasted to recapitulate industry procedures. At 3 and 24 hours post weaning, piglets were first sedated using a combination of Telazol (100 mg/mL), Ketamine (100 mg/mL), and Xylazine (100 mg/mL) at a dose of 0.03 mL/Kg body weight administered via intramuscular injection (gluteus medius). Once sedated, piglets were euthanized via intracardiac overdose of pentobarbital sodium (Euthasol; 85.9 mg/kg). Blood plasma and intestinal tissue samples were immediately collected for subsequent anatomical and functional analyses. A control group of piglets was immediately harvested at weaning (unweaned; UW).

Gastric pH

Immediately following euthanasia of animals collected at 3 hours post weaning, a 2-centimeter incision was made in the fundus of the stomach. A pH probe (Mettler Toledo, Columbus, OH) was then inserted through the incision to reach the stomach pylorus and record pH of the gastric acid.

Tissue Sample Collection for Histopathologic Analysis

Immediately following euthanasia, mid-jejunum was excised and immediately fixed in

Carnoy's 2000 (StatLab, McKinney, TX). Samples were transferred into 70% ethanol 24 hours post collection. Samples were then taken to the Michigan State University Investigative HistoPathology Lab for paraffin embedding, sectioning (10 μm longitudinal cross-sections), and staining.

Hematoxylin and Eosin Staining and Tissue Image Analysis

To assess anatomical changes, jejunal sections were stained with Endure Hematoxylin (Cancer Diagnostics, Inc., Durham, NC) and 1% Alcoholic Eosin-Phloxine B (Cancer Diagnostics, Inc., Durham, NC) and assessed under a light microscope. Images were taken at 10X magnification with final resolution at 1600x1200 pixels using imaging software (LAS-EZ, Leica Microsystems, Inc., Deerfield, IL) and a high-resolution digital camera (Leica ICC50 W, Leica Microsystems, Inc., Deerfield, IL) affixed to a light microscope (DM 750RH Leica Microsystems, Inc., Deerfield, IL). Villus height and crypt depth were measured as described in previous experiments.[9] Briefly, for each histologic slide prepared for each pig, five different areas were located on the slide within the 10X field of view that contained at least three, well-oriented villi and crypt visible in the cross section in their entirety and with the central lacteal present. Thus, a minimum of 15 individual villi measurements per pig were taken and then averaged to derive the mean villi height and crypt depth for each pig. Data are represented as villus to crypt ratio.

Tissue Sample Collection for Functional Studies

Immediately following euthanasia, 20 cm of mid-jejunum was isolated and placed in warm oxygenated (95% O_2–5% CO_2) porcine ringer solution (154 mM Na^+, 6.3 mM K^+, 137 mM Cl^-, 0.3 mM H_2PO_3, 1.2 mM Ca^{2+}, 0.7 mM Mg^{2+}, 24 mM HCO_3 at pH 7.4) for Ussing chamber analyses, as described in previous experiments.[9] Briefly, after the

jejunum section was rinsed in oxygenated ringer, the section was opened longitudinally and the seromuscular layer was removed from the mucosa while still bathed in Ringer solution. The mucosa was then mounted on 0.71 cm^2 aperture Ussing chambers (Physiologic Instruments, Inc., Sand Diego, CA). Once mounted, the tissue was incubated with 5 mL oxygenated ringer solution containing 10 mM mannitol on the mucosal side and 10 mM glucose on the serosal side and maintained at 37°C. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using 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 (TER; $\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current (I_{sc}). After a 30 minute equilibration period on Ussing chambers, TER and I_{sc} were recorded over a 10-min period and then averaged to derive the basal TER and basal I_{sc} values for a given pig.

Epithelial Barrier Permeability

In vivo permeability studies were performed as measures of bacterial 16S rRNA content in plasma collected from pigs at the time of euthanasia. Blood collected in EDTA tubes was centrifuged to separate plasma. Cell-free DNA (cfDNA) was then isolated from plasma using the Mag-Bind® cfDNA Kit (M3298-01, Omega Bio-Tek, Norcross, GA) and following manufacturer instructions. Relative abundance of 16S rRNA was determined by amplifying cfDNA via RT-PCR using *Power* SYBR™ Green PCR Master Mix (4367659, Applied Biosystems, Waltham, MA) and universal 16S rRNA primers (Forward 5'-*AGAGTTTGATCCTGGCTCAG*-3'; Reverse 5'-

ACGGCTACCTTGTTACGACTT-3'). Data are represented as relative gene expression to unweaned controls.

Ex vivo permeability studies were performed as mucosal-to-serosal fluxes of FITC-dextran 4 kDa (FD4) (Sigma-Aldrich, St. Louis, MO) as described in previous experiments.[2] Briefly, after tissue was equilibrated for 30 minutes on Ussing chambers, 0.25 mM FD4 was added to the mucosal side of Ussing chamber-mounted tissues. The probe was allowed to equilibrate for 3 minutes after which standards were taken from the mucosal and serosal side of each chamber. A 60-minute flux period was established by taking 60 µL samples in triplicate from the serosal compartment at the beginning and end of the 60-minute flux period. Prescence of FD4 was measured using Excitation/Emission readings at 488nm/525nm wavelengths respectively on a fluorescent plate reader and concentrations were determined from standard curves generated by serial dilution of FD4. Unidirectional FD4 mucosal-to-serosal flux were evaluated by determining the net appearance of FD4 in the serosal bathing solution on a chamber over time and presented as ng/cm^2/minute. Data are presented as the fold change in treated pigs relative to unweaned pigs.

Glucose Transport

Functional studies of Na^+-dependent glucose transport were conducted as described in previous experiments.[9] Briefly, after a 20-minute equilibration period, a dose of 10mM glucose (Sigma-Aldrich, St. Louis, MO) was added to mucosal side of Ussing chamber and osmotically balanced on the serosal side with equimolar amounts of mannitol and I_{sc} was recorded at 1 second intervals. Electrogenic glucose transport was determined as the change in I_{sc} (ΔI_{sc}) in response of glucose addition over a 20-minute period and

represented as area under the response curve.

Histamine Receptor Mediation of Functional Neural-Evoked Secretion

Functional studies of veratridine-evoked short circuit current were conducted as described in previous experiments.[8] Briefly, after a 20-minute equilibration period, 30 μM veratridine (Abcam, Cambridge, MA) in DMSO (1% of total chamber volume, previously demonstrated to have no measurable effect on I_{sc}) was added to the serosal side of jejunal mucosa mounted on Ussing chamber and I_{sc} was recorded at 1 second intervals. Neural evoked secretion was determined as the change in ΔI_{sc} in response to veratridine addition over a 20 minute period and represented as area under the response curve.

Statistical Analyses

Data are reported as means ± SEM. Experimental *n* are indicated in each figure legend. Data were analyzed using a One-Way or Two-Way ANOVA using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). A post-hoc Fisher's LSD test was used to determine the effects of weaning, treatment, or interaction between groups. Differences were considered significant at $P \leq 0.05$.

Results

H2R Antagonism Increases Gastric pH

To confirm that the H2R antagonist was able to reach systemic active concentrations, gastric pH was measured in animals collected at 3 hours post weaning. We observed that weaning itself had no effect on gastric pH levels (**Figure 3.2**). Desloratadine also did not change pH, but famotidine significantly increased gastric pH (**Figure 3.2;** P=0.0169). Treatment with desloratadine + famotidine trended towards a significant

increase in pH (*P*=0.0880). This indicates that famotidine was in fact active at the time of tissue collection.

Histamine Receptor Antagonism Does Not Inhibit Weaning Induced Reduction in Villus to Crypt Ratio

To determine the effects of histamine receptors on morphological changes in the jejunum mucosa at 24 hours post weaning, whole tissue sections were stained with hematoxylin and eosin to quantify the ratio between villus length and crypt depth. Histopathological analysis revealed that weaning stress induced blunting of the villi (**Figure 3.3A**) but no change in crypt depth was observed (**Figure 3.3B**). This resulted in reduced villus to crypt ratios between unweaned and saline treated animals (**Figure 3.3C**; *P*=0.0200). Antagonism of H1R or H2R had no effect on villus to crypt ratios compared to saline treated animals. These data suggest that histamine receptors do not mediate villus atrophy or crypt hyperplasia in response to weaning stress.

Antagonism of H1R and H2R Decreases Jejunal Epithelial Barrier Permeability In Vivo

Plasma samples were collected at 3 and 24 hours post weaning and bacterial 16S rRNA was quantified as a measure of intestinal permeability. At 3 hours post weaning, we observed upregulation of 16S rRNA between saline treated animals and unweaned controls (**Figure 3.4).** Antagonism of histamine receptors significantly reduced expression of 16S rRNA in circulation compared to saline controls (**Figure 3.4**). No significant differences were seen with weaning or treatment with antagonist at 24 hours post-weaning. This indicates that histamine receptor antagonism is reducing early changes transepithelial permeability at 3 hours post weaning.

Histamine Receptor Antagonism Does Not Change Basal TER

Jejunum isolated from weaned pigs was mounted on Ussing chambers to assess the effects of histamine receptors on gut function in response to weaning stress. After tissues were mounted and allowed to equilibrate, basal TER was assessed. Basal TER levels at 3 and 24 hours post-weaning were reduced in response to weaning. Further, all weaned animals regardless of antagonist treatment showed significantly reduced basal TER levels compared to unweaned control animals (**Figure 3.5A**; $P \leq 0.05$), but there were no differences between saline and histamine receptor antagonism groups. This indicates that at although weaning and histamine receptors do not affect electrogenic properties of epithelial barrier integrity at 3 hours post-weaning, which weaning itself does reduce electrogenic epithelial barrier integrity and 24 hours post-weaning.

Dual Antagonism of H1R and H2R Decreases Jejunal Epithelial Barrier Permeability Ex Vivo

To assess epithelial barrier permeability, FD4 was added to the mucosal side of Ussing chamber mounted jejunum and determined as mucosal-to-serosal flux. Weaning itself did not significantly increase FD4 flux at either 3 or 24 hours post weaning (**Figure 3.5B**; $P=0.1210$ and $P=0.1622$ respectively). Although treatment with either desloratadine or famotidine alone did not affect FD4 flux at 3 hours ($P=0.0823$ and $P=0.1721$ respectively) or 24 hours post-weaning ($P=0.6525$ and $P=0.1943$ respectively), treatment with both antagonists significantly decreased flux of FD4 at both 3 and 24 hours ($P=0.0497$ and $P=0.0244$ respectively). This indicates that H1R and H2R antagonists have an additive effect and that H1R and H2R may work together to

regulate epithelial barrier permeability in the jejunum in response to weaning stress.

H1R Receptor Antagonism Increases Basal I_{sc}

Jejunum isolated from weaned pigs was mounted on Ussing chambers to assess the effects of histamine receptors on gut function in response to weaning stress. After tissues were mounted and allowed to equilibrate, basal I_{sc} was assessed. Basal I_{sc} levels were similar between unweaned controls and weaned animals regardless of treatment with saline or antagonists at the 3 hours post-weaning. At 24 hours post-weaning, basal I_{sc} was higher in saline treated pigs compared to unweaned control animals (**Figure 3.6**; P=0.0145). Animals treated with desloratadine had higher basal I_{sc} compared to saline treated animals (P=0.0451) but was not different in animals treated with famotidine or both desloratadine and famotidine (**Figure 3.6**).

Antagonism of H2R Decreases SGLT1-Mediated Glucose Transport

To assess Na^+-dependent glucose transport via SGLT1, glucose was added to the mucosal side of Ussing chamber mounted jejunum and transport was determined as change in ΔI_{sc}. At 3 hours post-weaning, there was no differences seen with weaning or treatment with antagonists (**Figure 3.7C**). Weaning significantly increased ΔI_{sc} from unweaned controls at the 24 hour timepoint (P=0.0154). No difference was seen in animals treated with desloratadine or both desloratadine and famotidine combined compared to animals treated with saline (P=0.0792 and P=0.0707 respectively). However, animals treated with famotidine alone showed significantly reduced levels of glucose transport compared to saline treated controls (P=0.0255). This indicates that H2R mediates Na^+-dependent glucose transport in the jejunum.

Antagonism of H2R Decreases Neural-Evoked Secretion

To assess neural-evoked secretory function, veratridine was added to the serosal side of Ussing chamber mounted jejunum and secretion was determined as change in ΔI_{sc}. At 3 hours post weaning, no differences were seen between unweaned and saline treated animals or between animals treated with saline and antagonists (**Figure 3.8C**). At 24 hours post-weaning, weaned animals had significantly higher secretory function compared to unweaned controls (P=0.0001). No difference was seen between saline treated animals and desloratadine treated animals (P=0.1834). However, animals treated with famotidine or both desloratadine and famotidine showed reduced secretory function compared to saline treated controls (P=0.0007 and P=0.0015 respectively).

Discussion

Early life adversity has been demonstrated as being a significant risk factor in disease development later in life, especially GI diseases in both humans and pigs.[12-15] Further, it has been well established that nutrition, pathogen exposure, and stressors play crucial roles in intestinal and overall health of weaned pigs, including gut permeability.[2,8,16,17] However, the specific mechanisms that drive changes in GI function at early timepoints are yet to be elucidated. Histamine receptors are well known for their role in allergic inflammation and gastric acid secretion.[18,19] Here we demonstrate that H1R and H2R mediate different aspects of jejunal epithelial barrier and that H2R also mediates glucose transport and neural-secretory functions.

Histamine Receptor Antagonism Does Not Inhibit Weaning Induced Reduction in Villus to Crypt Ratio

Numerous studies have reported that weaning and intestinal inflammation results in

villus atrophy and crypt hyperplasia,[20-22] and thus a reduced villus to crypt ratio. Our study reaffirmed these results in that weaning stress reduced the villus to crypt ratios. However, treatment with histamine receptor antagonism did not prevent weaning induced villus atrophy. This indicates that histamine receptors do not mediate morphological changes to villi in response to weaning stress. We also saw that reduced villus:crypt ratios were attributed to villus blunting and not to changes in crypt depth. Similar results were also seen in a study by Boudry et al., where changes in intestinal morphology was attributed to changes in villus height but changes in crypt depth are more prominent at later post-weaning timepoionts.[23] Villus atrophy is attributed to contraction of villi and reduced cell regeneration. Villus contraction is an energy-dependent and neurally mediated event where smooth muscle microfilaments in the lamina propria, as well as tight junctions, condense to reduce injured tissue area.[24,25] Given the localization patterns of H1R and H2R, it is surprising that histamine receptor antagonism has no effect on villus atrophy. Compensatory cell regeneration in the crypts to make up for rapid epithelial cell loss doesn't occur until 5 days post weaning,[21] which is well outside our timepoint of investigation. It is possible that histamine receptors do not regulate villus contraction, but given the localization of H2R to the epithelium, it is still a regulator of other epithelial barrier functions.

Antagonism of H1R and H2R Decreases Jejunal Epithelial Barrier Permeability In Vivo and Ex Vivo

Intestinal epithelial barrier dysfunction, or increased intestinal permeability, is a primary pathophysiologic mechanism in progression of GI disease.[3,26] Numerous studies have demonstrated that early life stressors reduce epithelial barrier integrity.[2,6,9,17,23] Previous

studies have assessed barrier integrity at much later timepoints, but here we investigate histamine receptors as an early mechanism regulating epithelial barrier permeability prior to upregulation of an inflammatory response. Quantification of bacterial 16S rRNA in blood has recently been used as a marker for loss of GI barrier function and bacterial translocation in humans with inflammatory bowel disease, as well as in animal models of GI disease.[27-29] Our data demonstrate that antagonism of histamine receptors reduces circulating levels of 16S rRNA as early as 3 hours post weaning, but we did not see any differences between weaned animals and unweaned controls. Although this data set would benefit from an increased *n*, we have shown that measurement of 16S rRNA is a valid, non-invasive measure of intestinal permeability *in vivo* that has not been previously utilized in pigs.

We also demonstrate an increase in FD4 flux and decrease in TER reflect reduced integrity of tight junctions and increased paracellular permeability, and thus indicate leaky gut. This confirms previous studies that indicate that weaning stress increases intestinal permeability.[2,9,30] Further, here we demonstrate that weaning stress-induced changes in jejunal permeability is mediated by H1R and H2R antagonism. However, histamine receptor antagonism did not prevent weaning-induced decreases in TER. This may be due to intestinal permeability being regulated by tight junctions through two major pathways, the pore pathway, measured by ion transport, and the leak pathway, measured by 16S rRNA and transepithelial flux. The pore pathway transports larger quantities of small ions while the leak pathway transports smaller quantities of large molecules.[31-33] This then would indicate that histamine receptors regulate intestinal permeability through the leak pathway but not the pore pathway.

Antagonism of H2R Decreases SGLT1-Mediated Glucose Transport

It is well established that intestinal epithelial transporters are responsible for nutrient absorption, but have been recently established as key players in the progression of enteric and inflammatory disease.[9,34] Previous studies by Boudry et al., have shown that increased glucose transport in response to early weaning in pigs is due to functional increases in SGLT1-mediated transport and are not due to structural changes within the mucosa.[23,35] Despite glucose being a necessary energy source, studies in humans have indicated that stress-induced hyperglycemia is a marker of an impaired immune system.[36] Here we demonstrate for the first time that weaning-induced increases in SLGT1-mediated glucose transport are attenuated by antagonism of H2R at 24 hours post weaning. H2R may inhibit SGLT1-meidated glucose transport through a GPCR pathway in the epithelial cell. A study by Dyer et al., gave evidence that glucose sensing in the intestine initiates a GPCR/cAMP–PKA-linked pathway, which eventually leads to increased SGLT1.[37] Considering this is the GPCR pathway activated by H2R, it is plausible that H2R antagonism inhibits cAMP-PKA activation and ultimately downregulates SLGT1. However, further studies would be necessary to determine if H2R downregulates SLGT1 expression itself or reduces cAMP-PKA/SGLT1 activity. Recent work in our lab has also shown that changes in glucose transport induced by early weaning stress persist into adulthood, favors GLUT2-mediated transport, and is correlated with increased inflammation.[17] In our study, we did not measure GLUT2-mediated transport in response to weaning or to the downregulation of SGLT1-mediated transport with treatment of famotidine. Future studies would be necessary to determine the role that histamine receptors play in regulating short term GLUT2-mediated glucose

transport in response to weaning stress as well as the effects of early SGLT1 blockade by H2R antagonism on long-term gut function.

Antagonism of H2R Decreases Neural-Evoked Secretion

Numerous studies have indicated that activation of the enteric nervous system has profound effects on intestinal barrier and secretory functions, as well as inflammatory responses. Further, increased neural-secretory activity is a hallmark of enteric diarrheal disorders. Previous studies show that pigs weaned at 18 days of age exhibit upregulation of enteric nervous system activity into adulthood[8] as well as mast cell number and activation[2] compared with pigs weaned at a later age. Here we demonstrate that weaning-induced upregulation of neural-evoked secretory function is mitigated by H2R antagonism at 24 hours post weaning. This indicates that weaning stress increases intestinal secretions that critical in the pathogenesis of enteric disorders and that this is regulated by H2R. Interestingly, we did not see significant responses of neural-evoked secretory activity to weaning stress or histamine receptor antagonism at 3 hours post weaning. This could be due the expression of voltage-gated sodium channels at this timepoint. Expression of voltage-gated sodium channels increases in response to inflammation in humans.[38] It is possible that there are increased expression levels with increased inflammation following weaning stress that allow for greater responses to stimuli at 24 hours that are not seen at 3 hours. However, further studies assessing voltage-gated sodium channel expression are needed. Previous studies have demonstrated that corticotropin releasing factor (CRF) increases secretions in gut and correlates to increases in acetylcholine, which acts through cholinergic neurons.[8] It has also been shown that CRF reduces gastric acid secretions

through a different mechanism than H2R.[39] This would suggest that cholinergic neural-evoked secretory functions in the gut are not affected by H2R. However, the mechanisms regulating gastric acid and neural-evoked secretory functions may be different and assessment of this would require future studies utilizing proton-pump inhibitors. H2R may also affect other classes of neurons, but additional studies would be necessary to determine the full influence of H2R on neural-evoked secretions.

It has been shown that gut bacteria can influence enteric nervous system by stimulating production of neurotransmitters, including serotonin, histamine, and acetylcholine.[40] This is also in line with previous data indicating that pigs that undergo inflammatory challenge show increased levels of intestinal choline acetyltransferse[41] and that serotonergic, cholinergic, and adrenergic neurons are undergoing major developmental changes during postnatal life.[8] To determine if weaning stress and histamine receptors regulate all neural-evoked secretions or specific classes of neurons, future studies may include stimulation with specific neurotransmitters, such as serotonin and acetylcholine. Neural-evoked secretions are one component of epithelial secretory functions. Given that expression of H2R is localized to the epithelium, it may be a regulator of other inducible secretions. Future studies may also utilize forskolin to assess the effect of histamine receptors on cAMP-induced epithelial cell secretory functions.

Conclusion

In summary, the present studies show that weaning stress, an unavoidable early life stressor in pigs, causes dysfunction in epithelial barrier, glucose transport, and neuro-secretory activity in the jejunum and that H2R mediates these critical functions. Given the importance of these functions in enteric diarrheal disorders, these findings implicate

histamine and histamine receptors as potential targets to in mitigating enteric disease development and improving intestinal health and overall swine production performance. Further, given that GI disease risk in humans, including IBS and IBD, is also increased in those that experience early life stress, these data have implications for potential therapeutics for GI health in both production animals and humans. Understanding the mechanisms that drive functional changes in the gut in response to early life stress and how histamine receptors mediate these critical functions provides a novel target in mitigating lifetime disease risk.

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Histological Staining

Thanks to Amy and Jessie at the Michigan State University Investigative Histopathology Laboratory for their assistance in the histological tissue mounts and staining.

Figures

Figure 3.1: Experimental design
Piglets were selected based on body weight and randomly assigned a treatment group. At 18 days of age, piglets were given an injection 30 minutes prior to weaning of either saline (control), desloratadine (2 mg/kg), famotidine (4 mg/kg), or desloratadine + famotidine. At 0, 3, and 24 hours post weaning, whole blood and jejunum samples were harvested. Whole blood was processed for 16S rRNA analysis. Jejunal mucosa was prepared for Ussing chamber analyses for intestinal glucose transport and neural-evoked secretion experiments. Created with BioRender.com.

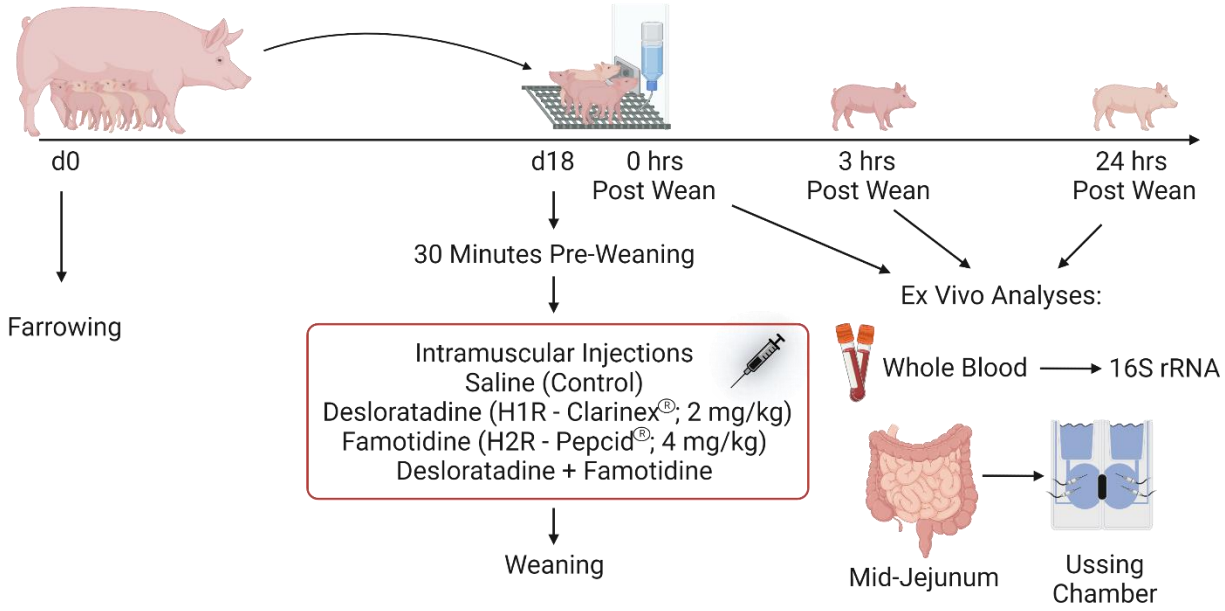


Figure 3.2: H2R but not H1R Antagonist Increases Gastric pH at 3 Hours Post Weaning

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0 and 3 hours post weaning, gastric pH was measured. Data are presented as mean±SEM per animal. *n*=4-6 per treatment group. One-way ANOVA comparison between treatment groups *P*=0.1119. #*P*≤0.1, **P*≤0.05.

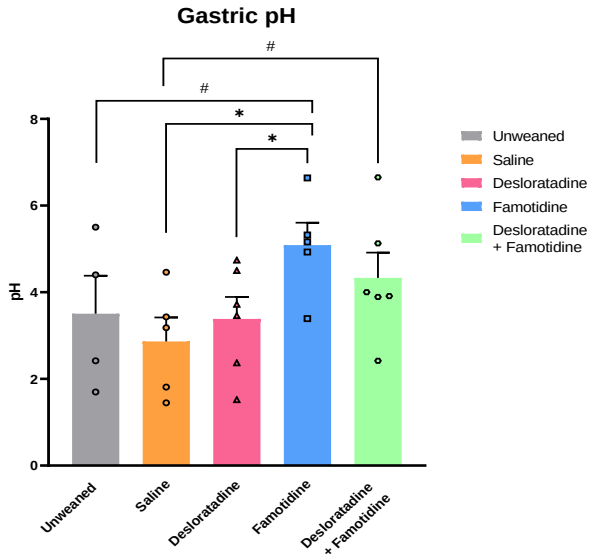


Figure 3.3: Antagonism of H1R and H2R has no Effect on Weaning Stress-Induced Reduction in Jejunal Villus to Crypt Ratio

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mid-jejunum was collected, fixed, and stained with hematoxylin and eosin. Villus lengths and crypt depths were measured across 5 images per animal. Data are presented as mean±SEM per animal. *n*=5-6 per treatment group. One-way ANOVA comparison between treatment groups (A) *P*=0.0126, (B) *P*=0.8145, (C) *P*=0.0570. **P*≤0.05, ***P*≤0.01.

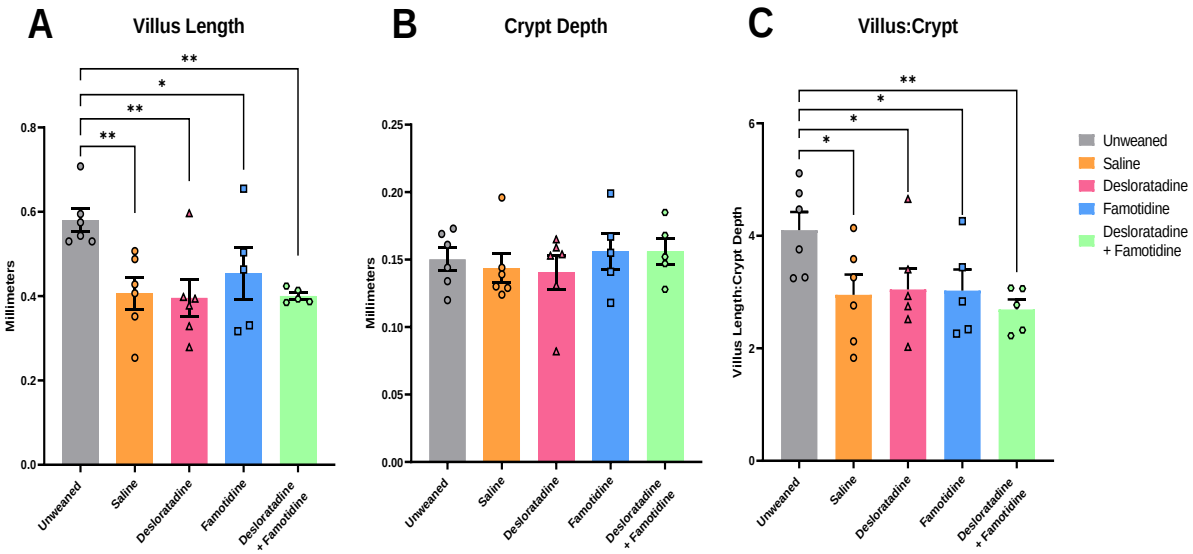


Figure 3.4: Antagonism of Histamine Receptors Decreases Weaning Stress-Induced Transepithelial Permeability *In Vivo* at 3 Hours Post Weaning
Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, plasma samples were collected, cfDNA was isolated, and was amplified via PCR (RT-qPCR) for 16S rRNA. The data are presented as ΔCT in expression relative to control group. *n*=3-6 per treatment group. Two-way ANOVA comparison between timepoints and treatment groups. Treatment Effect - *P*=0.0110, Time Effect - *P*=0.5929, Interaction - *P*=0.7082. **P*≤0.05.

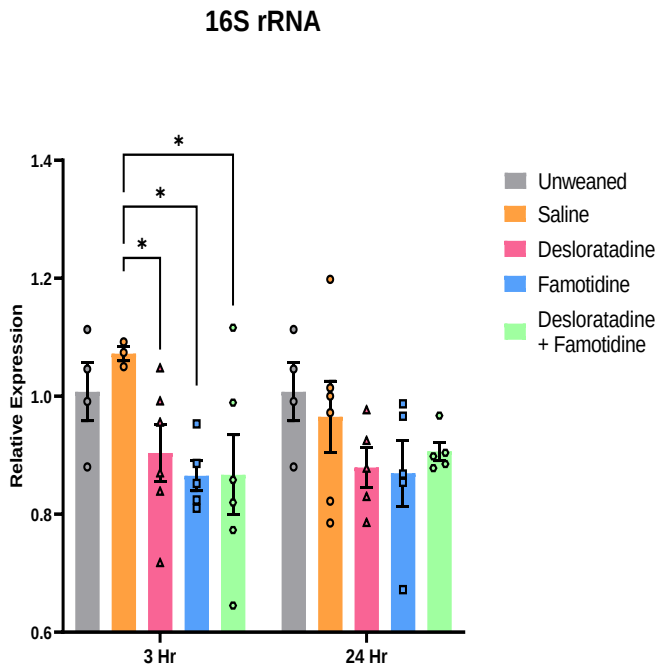


Figure 3.5: Antagonism of H2R Decreases Weaning Stress-Induced Transepithelial Permeability

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mid-jejunum was mounted on Ussing chambers and allowed a 30 minute equilibration period. I_{sc} was recorded over a 10-min period and then averaged to derive the basal I_{sc} (A). Following equilibration period, FD4 was added to assess epithelial permeability over a 60 minute period (B). Data are presented as mean±SEM. *n*=5-6 per treatment group. Two-way ANOVA comparison between timepoints and treatment groups. (A) Treatment Effect - *P*=0.0569, Time Effect - *P*=0.8773, Interaction - *P*=0.7703; (B) Treatment Effect - *P*=0.0006, Time Effect - *P*=0.5067, Interaction - *P*=0.9332. **P*≤0.05, ***P*≤0.01.

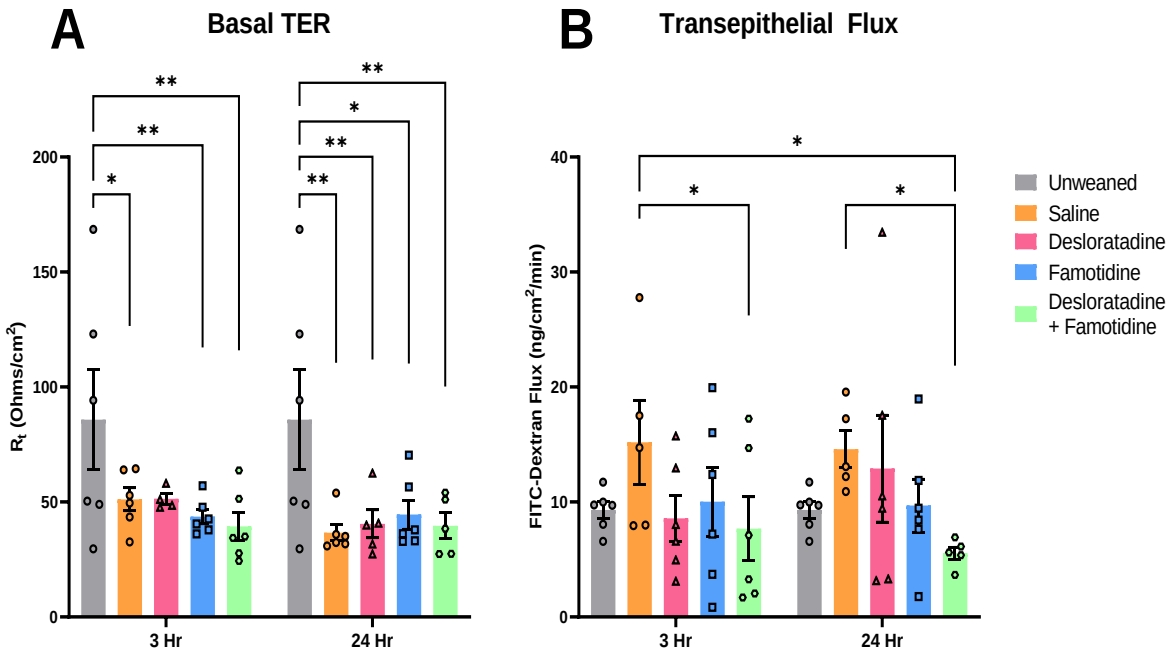


Figure 3.6: H1R Antagonism Increases Basal I_{sc}
Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mid-jejunum was mounted on Ussing chambers and allowed a 30 minute equilibration period. I_{sc} was recorded over a 10-min period and then averaged to derive the basal I_{sc} values for a given animal. Data are presented as mean±SEM. *n*=5-6 per treatment group. Two-way ANOVA comparison between timepoints and treatment groups. Treatment Effect - *P*=0.2423, Time Effect - *P*=0.0106, Interaction - *P*=0.0287. ***P*≤0.01, ****P*≤0.001.

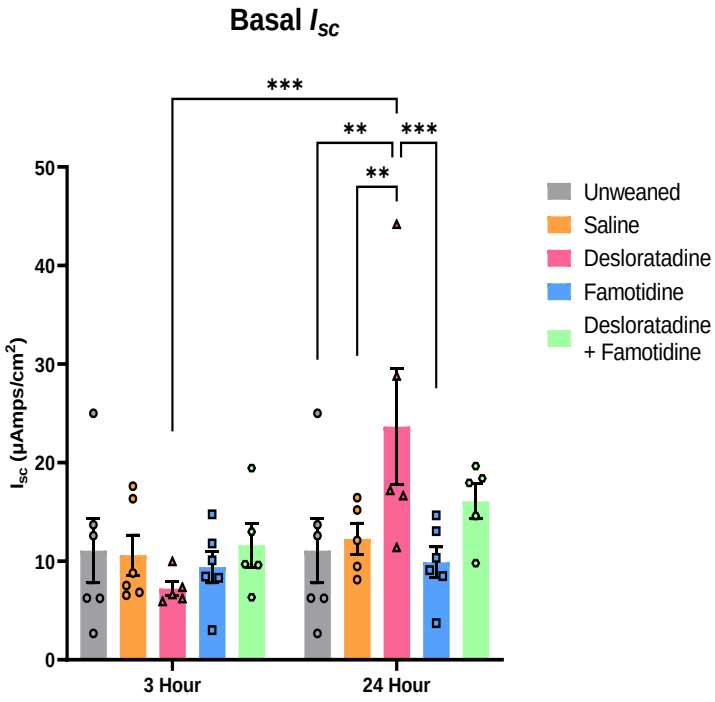


Figure 3.7: Antagonism of H2R Decreases Weaning Stress-Induced SGLT1-Mediated Glucose Transport

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mid-jejunum was mounted on Ussing chambers and glucose was added to assess SGLT1-mediated transport over a 20 minute period. Representative I_{SC} tracings of jejunum isolated at 3 hours (A) and 24 hours (B) post weaning after the addition of glucose (indicated by red arrow). Area under each response curve was then measured (C) to the determine SGLT1-mediated transport. Data are presented as mean ±SEM. *n*=5-6 per treatment group. Two-way ANOVA comparison between timepoints and treatment groups Treatment Effect - *P*=0.0028, Time Effect – *P*<0.0001, Interaction - *P*=0.0045. *$P \leq 0.05$, ***$P \leq 0.001$.

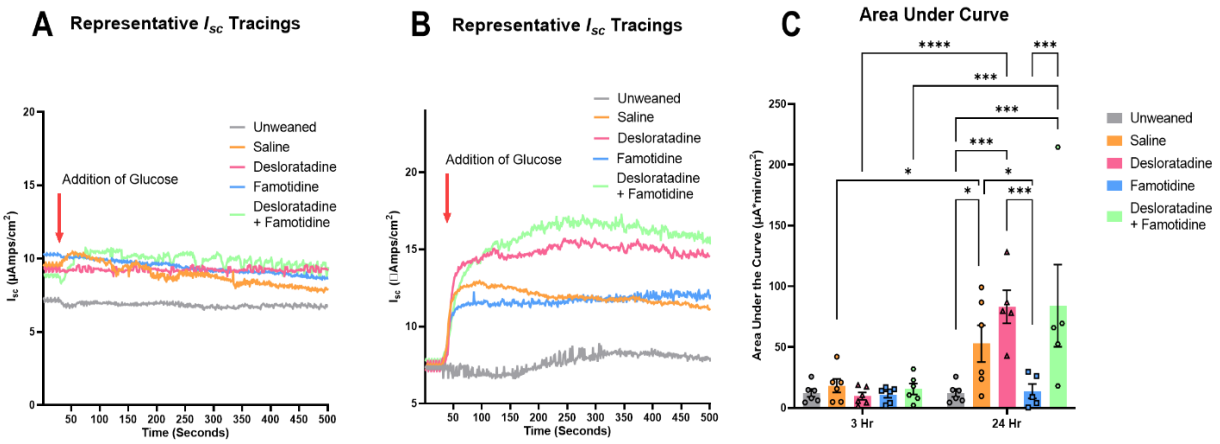
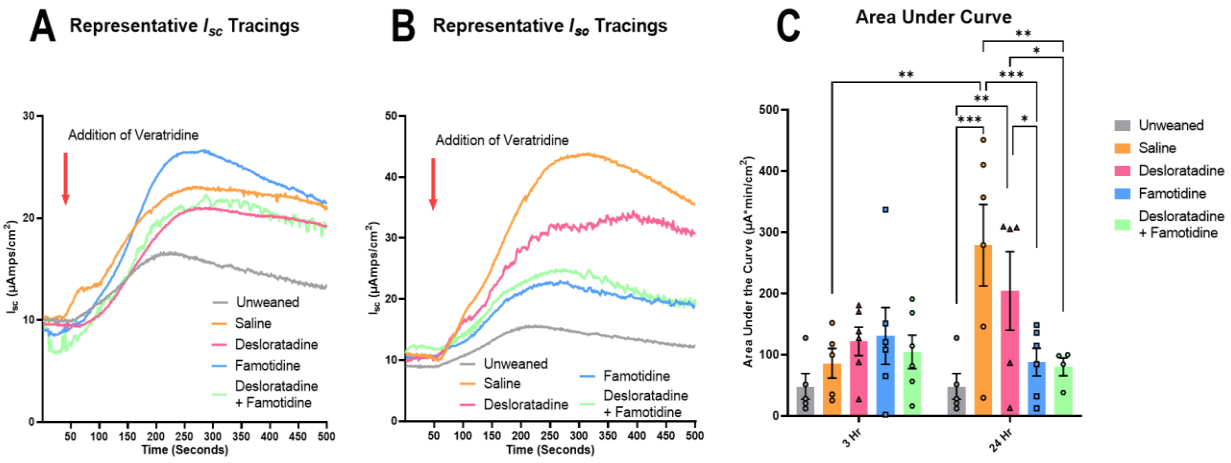


Figure 3.8: Antagonism of H2R Decreases Weaning Stress-Induced Neural-Evoked Secretion

Pigs were injected with saline, desloratadine, famotidine, or desloratadine + famotidine 30 minutes prior to weaning. At 0, 3, and 24 hours post weaning, mid-jejunum was mounted on Ussing chambers and veratridine was added to assess neural-evoked secretion over a 20 minute period. Representative I_{SC} tracings of jejunum isolated at 3 hours (A) and 24 hours (B) post weaning after the addition of veratridine (indicated by red arrow). Area under each response curve was then measured (C) to the determine neural-evoked secretion. Data are presented as mean ±SEM. *n*=5-6 per treatment group. Two-way ANOVA comparison between timepoints and treatment groups Treatment Effect - *P*=0.0110, Time Effect – *P*=0.1031, Interaction - *P*=0.0247. **P*≤0.05, ***P*≤0.01, ****P*≤0.001.



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CHAPTER FOUR: KEY FINDINGS AND FUTURE DIRECTIONS

Overview

Early weaning is a necessary practice in swine production that has been shown to induce increases in GI inflammation, permeability, nutrient transport, and neural-evoked secretions later in life.[1-4] However, the mechanisms regulating inflammatory responses and GI functions in response to weaning stress remain unknown. Here, we have focused on histamine and histamine receptors as regulators of inflammatory responses and changes in gut function in response to weaning stress.

In chapter 2, we investigated the role of histamine receptors in weaning-induced intestinal inflammatory responses. We here show upregulated expression of H1R, localized to the jejunal lamina propria, and of H2R, localized to the jejunal epithelium, in response to weaning stress. We also demonstrate that an interesting downregulation of inflammatory gene expression in response to weaning stress and histamine receptor antagonism. However, we observe that famotidine, a selective H2R antagonist, prevents weaning-induced elevations of jejunal myeloperoxidase and IL1β expression, as well as TGFβ and β-Integrin expression in the mesenteric lymph nodes. These data demonstrate that weaning stress and histamine receptors selectively regulate gene expression related to extracellular matrix, cellular adhesion, and cytokines, but that H2R mediates expression of specific inflammatory proteins.

In chapter 3, we explored the role of histamine receptors in functional gut responses to weaning stress. We demonstrate histamine receptor antagonism prevented weaning-induced increases in plasma 16S rRNA expression, transepithelial flux, SGLT1-mediated glucose transport, and neural-evoked secretion. These data demonstrate that weaning stress induces early functional responses in the gut that are mediated by H2R,

including epithelial permeability, nutrient transport, and neuro-secretory functions. Together, these studies demonstrate histamine receptor specific localization patterns, weaning stress and histamine receptor downregulation of inflammatory genes, and histamine receptor attenuation of weaning stress induced changes in gut function.

Highlight of Key Findings

Histamine Receptors Mediate Select Aspects of the Inflammatory Responses to Weaning Stress

This data identifies histamine and histamine receptors as mediators in the early inflammatory response to weaning stress in pigs. Previous studies have demonstrated early rise in circulating histamine level[5] as well as increases in mast cell numbers and activity within the gut in response to weaning stress.[4] However, by utilizing histamine receptor antagonists, we here were able to demonstrate that histamine receptors regulate specific inflammatory gene and protein expression in both the jejunum and mesenteric lymph nodes at early timepoints post weaning stress, but do not mediate all aspects of extracellular matrix remodeling, cellular trafficking, or cytokine expression. This data provides a foundation to build upon for further characterization of histamine receptor-mediated inflammatory responses.

Establishing Activation of Histamine Receptors as a Regulatory Mechanism of Gut Function in Response to Weaning Stress

This work demonstrates that activation of histamine receptors mediate functional changes in the gut including epithelial barrier integrity (Figure 3.5), nutrient transport (Figure 3.6), and neural-evoked secretory functions (Figure 3.7). As impairment of these functions are all hallmarks of enteric disease progression, determining the mechanism

by which these functions are mediated have profound implications for reducing enteric disease severity or even preventing their onset. Previous work has made evident the impact of early life adversity on long-term GI functions.[1,2,4] However, we here show that these changes occur quickly in response to weaning stress, thus linking histamine receptors to the developmental changes in the gut that persist into adulthood. Moreover, this further implicates histamine and histamine receptors as targets for the development of therapies for enteric diseases.

Study Limitations

Mechanism of Pharmacological H2R Antagonist

H2R antagonists are classically known to decrease gastric acid secretion. Results of our study demonstrated that administration of famotidine was able to reduce gastric acid within 3 hours of weaning stress, which is an indicator of drug activity. A limitation of our study is that we may not be able to tell if the antagonist effect on intestinal inflammation and function is due to its anti-inflammatory properties or its ability to reduce gastric acid. Future experiments could include a proton pump inhibitor to determine if the gastric acid reducing property of the H2R antagonist is causing the changes in intestinal inflammation and permeability. However, proton pump inhibitors have been shown to have off-target effects that include increased intestinal permeability through degradation of tight junctions.[6] Further, parietal cells that secrete gastric acid line the wall of the stomach and not the intestine, it is possible that the change in gastric acid secretion by H2R antagonists does not have an effect on intestinal inflammation or permeability. Another aspect of this limitation is that the effects of the antagonists are systemic, as the antagonists were administered via intramuscular injection. Since histamine

receptors are ubiquitously expressed, this could mean that blockade of histamine receptors in other tissues, such as the brain, could be significantly altering the inflammatory response as a whole and not as being localized to the gut. Future experiments could utilize *ex vivo* or *in vitro* intestinal epithelial models, such Ussing chambers or IPEC-J2 cell lines, to determine the role that histamine receptors play in intestinal specific inflammatory responses.

Inclusion of Male Castrates

A final limitation is that we here utilized male castrates and did not include females. Although it has been previously demonstrated that females have increased duration of diarrhea and persistent intestinal permeability compared to male castrates,[4] male castrates have twice the mortality rate that is seen in females in the nursery phase alone.[7] Further, female mast cells have higher density granules compared to males, thus contain more histamine;[8,9] females also exhibit increased susceptibility to development of functional gastrointestinal disorders.[4] Inclusion of females in future studies would provide additional knowledge of the influence of histamine receptors in weaning-induced intestinal inflammatory responses and permeability, which would aid in developing sex-specific protocols for therapies in swine production and in human health.

Future Directions

The work presented here provides a foundational understanding of histamine receptor-mediated inflammatory responses and gut function in response to weaning stress and serves as a starting point to look at cell-specific and up-stream mechanisms.

Colocalization of Histamine Receptors to Major Cell types of the Intestinal Mucosa and Local Immune Cells

In addition to including females in the data presented here, we would like to identify which cell types express histamine receptors during the acute inflammatory response to weaning stress. Here we determined the localization of H1R and H2R to the jejunal lamina propria and epithelium, respectively (Figure 2.2). However, we did not identify which cell types within these regions showed upregulated expression of these receptors in response to weaning stress. Colocalizing this expression to specific cell types within the intestinal mucosa as well as immune cells will further reveal specific mechanisms of histamine receptor subtypes.

Transcriptomic Analysis of Jejunal Mucosa

Although our study looked at expression of various genes related to the inflammatory response, we saw a major downregulation of many inflammatory genes in response to weaning stress and histamine receptor antagonism (Figure 2.3). Recent studies have assessed the porcine transcriptome, but focused on the ileal region of the gut under basal conditions in adult pigs and populations of lymphocytes that have homogeneity to human lymphocytes.[10] An analysis of the jejunal transcriptome may reveal upstream pathways and other anti-inflammatory targets regulated by weaning stress and histamine receptors.

Long-Term Gut Function and Responses to Immune Challenge

Previous work has indicated that effects of early weaning stress on the gut persist into adulthood.[1,2,4] As the work presented here demonstrates the role of histamine receptors in the short time course post weaning, assessment of long term gut function will also be

necessary in determining the role that histamine receptors play in shaping development for function later in life, as well as in response to subsequent immune challenges. Following the growth progress of animals that receive histamine receptor antagonists prior to weaning and assessing inflammatory and functional responses to a secondary immune challenge, such as exposure to LPS, would reveal the role histamine receptors have in shaping gut function later in life. Understanding both short- and long-term effects of histamine receptor activation during early life is crucial in determining critical timepoints of intervention to prevent enteric disease.

In conclusion, this dissertation provides foundational knowledge of the role of histamine receptors in inflammatory responses and demonstrates that histamine receptors mediate gut functions in response to weaning stress. Future studies should focus on further characterization of the role of histamine receptors in regulating gene expression of early inflammatory responses, as well as in the development of long-term gut function in response to secondary immune challenge. Further, assessment of the transcriptome may identify other anti-inflammatory targets that may be used in the development of targeted therapies to prevent enteric disease in both humans and swine.

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