Gene Expression of the Intestinal Peptide Transporter 1 and Peroxisome Proliferator-Activated Receptor alpha Following Fasting/Refeeding and Ligand Administration in Chickens

by

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ABSTRACT

The uptake of amino acids is mediated by amino acid transporters and the peptide transporter, PepT1. The nuclear receptor PPARα may play a role in the activation of PepT1. The objectives of this study were to evaluate the effect of a fasting/refeeding regimen and gavaging a PPARα ligand on expression of chicken PepT1 and PPARα. During the fasting/refeeding trial, chicks were placed on a 24h fast and then divided into 3 groups (Continuously Fasted (CF), Refed/Food Withdrawn, and Refed Adlib) and sampled 1, 2, 3, 5 and 7h post-fast. Expression of PepT1 and PPARα increased almost 2-fold post-24h fast ($P < 0.002$). The CF group had highest expression for both genes ($P < 0.0001$) suggesting a coordinate change in PepT1 and PPARα expression during fasting, indicating a possible regulatory role for PPARα of PepT1. Oral gavage trials utilizing PPARα ligand WY- 14643 were conducted with layers and broilers. For the layer trial birds were gavaged daily for 3 d with 50 mg WY-14643/kg in carrier (Ligand) or with just carrier (No Ligand) and sampled on d4. For the broiler trial, birds were gavaged daily with 25 or 50 mg WY-14643/kg or just carrier and sampled on d2 or d3 post gavage. No difference in PepT1 was observed between the Ligand and No Ligand groups. In the layer trial, PPARα decreased 6-fold ($P = 0.005$) in the Ligand group. Broilers treated with WY-14643 showed no effect of ligand. These results indicate a difference between layers and broilers in response to WY-14643.

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STRUCTURE OF THE CHICKEN GASTROINTESTINAL TRACT

From Beak to Gizzard

The material presented in this section can be found in "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001). The process of breaking down the complex organic and inorganic molecules that are found in the diet of a chicken begins with the beak. The primary function of the beak is to gather food; since the bird has no teeth, a reduction in particle size prior to swallowing is low compared to mammals. Within the mouth, the bird can produce anywhere between 15-20 mL of saliva each day. The saliva not only lubricates and softens the feed particles, but it also contains small amounts of amylase, which initiates the breakdown of starches. Feed then moves from the mouth to the crop, via the esophagus. The crop is essentially a pouch in the gullet of a bird and is responsible for moistening and storing feed for later digestion. Being a structure that is uniquely avian, the crop holds more importance within wild populations than with domesticated poultry due to the abundance of food found within commercial operations. Following storage in the crop, feed enters the proventriculus. As it is analogous to the stomach of other monogastrics, the proventriculus is the glandular upper region of the bird's stomach. Secretion of HCl in this region results in a low pH, around 1.5-2. However, the pH in the proventriculus can rise as high as 5 as feed moves through and has a buffering effect. Following the proventriculus, feed moves into the highly muscular, thick-walled region of the stomach called the gizzard. The gizzard is another structure that is unique to avian species. However, since the milling of feed is so commonplace in the commercial industry today as a way to increase the digestibility of feed, the gizzard really has no meaningful use in

domestic poultry. In wild fowl, the gizzard plays an important role in grinding feed, reducing particle size, and mixing feed with the secretions from the proventriculus.

From Small Intestine to Cloaca

From the gizzard, feed then moves into the small intestine where the majority of nutrient absorption occurs. The small intestine is divided into three segments: duodenum, jejunum, and ileum (Figure 1-1). The duodenum extends from the gizzard, forms a loop around the pancreas, and terminates at the bottom of the loop. The jejunum begins at the top of the duodenual loop and ends at Meckel's diverticulum, a remnant of the yolk sac. The ileum starts at Meckel's diverticulum and ends at the ileal-cecal junction. The remaining structures of the gastrointestinal tract include a pair of ceca and the large intestine. Waste products are then eliminated through the cloaca, a cavity common to both the digestive and reproductive systems.

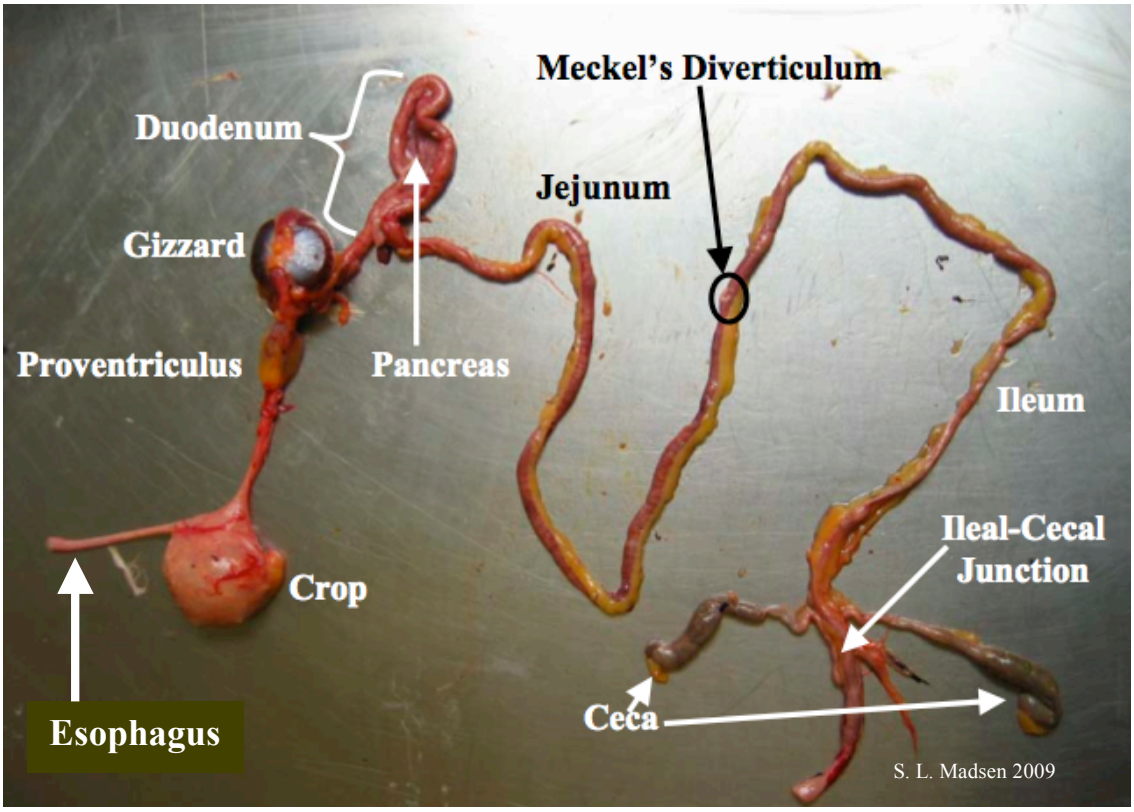


Figure 1-1 Gross anatomy of the chicken gastrointestinal tract

MICROANATOMY OF THE CHICKEN SMALL INTESTINE

Considering the importance of the small intestine in the uptake of nutrients, a close look at its microanatomy is necessary. The following material can be found in "Molecular and Cellular Basis of Digestion" (Desnuelle et al., 1986) and "Textbook of Medical Physiology" (Guyton and Hall, 1996). The small intestine, as with any tubular organ, is made up of four concentric layers: the serosa, the muscularis, the submucosa, and the mucosa which encircles the intestinal lumen. The two inner layers, the submucosa and mucosa, are separated from each other by the muscularis mucosae. The microanatomical structures of the innermost layer have one function: to maximize surface area for nutrient absorption. These structures include the mucosal folds, intestinal villi, microvilli, and specialized cells such as goblet cells.

The absorptive surface of the intestinal mucosa is arranged in many folds called *folds of Kerckring* (Figure 1-2). These folds increase the surface area of the mucosa by about three-fold and they extend in a circular arrangement around most of the intestine, with higher development in the duodenum and jejunum. Covering the surface of the folded mucosa are millions of finger-like projections called villi, which project into the lumen of the intestine and increase absorptive surface area by ten-fold. The villi are composed of two parts: the villus (the finger-like projection) and the crypt. The crypt is an invagination of the mucosa that reaches deep into the muscularis mucosae between adjacent villi.

Along the surface of each villus lies the absorptive epithelial cells of the intestine, called enterocytes. Enterocytes are renewed from the pool of stem cells located in the villus crypts. As these cells migrate up the villus, they undergo morphological and functional maturity, a process that takes 2-6 days, depending on species. Eventually, they reach the villus tip and are released into the lumen.

Enterocytes are polarized columnar cells that have an apical surface that faces the intestinal lumen and a basolateral surface that faces the bloodstream. The apical surface is characterized by the presence of microvilli, which constitutes the brush border membrane. The microvilli increase the absorptive surface area at least twenty-fold. Together with the *folds of Kerckring* and the villi, the microvilli contribute to increasing the absorptive area of the mucosa by almost 1000-fold. Being that the brush border membrane is the location for different nutrient transporter systems and enzymes, it is the site of active nutrient absorption.

A proliferative pool of stem cells within the crypts is a convenient way to produce not only enterocytes, but goblet cells as well. Goblet cells arise from the villus crypt and are part of the epithelium lining the villi. Goblet cells secrete mucus, which functions as a lubricant and helps form a favorable digestive zone. Mucus adheres to the villus epithelium and protects it from any mechanical damage that might arise from the movement of the gut and its contents. Mucus also traps digestive enzymes within its layers and prevents them from being prematurely removed from the intestine via peristalsis. By doing so, the mucus actually places these enzymes in an advantageous position for the digestion of various substrates. The mucus also plays an important role in trapping bacteria and parasites, yet remains permeable enough to allow the passage of vital nutrients.

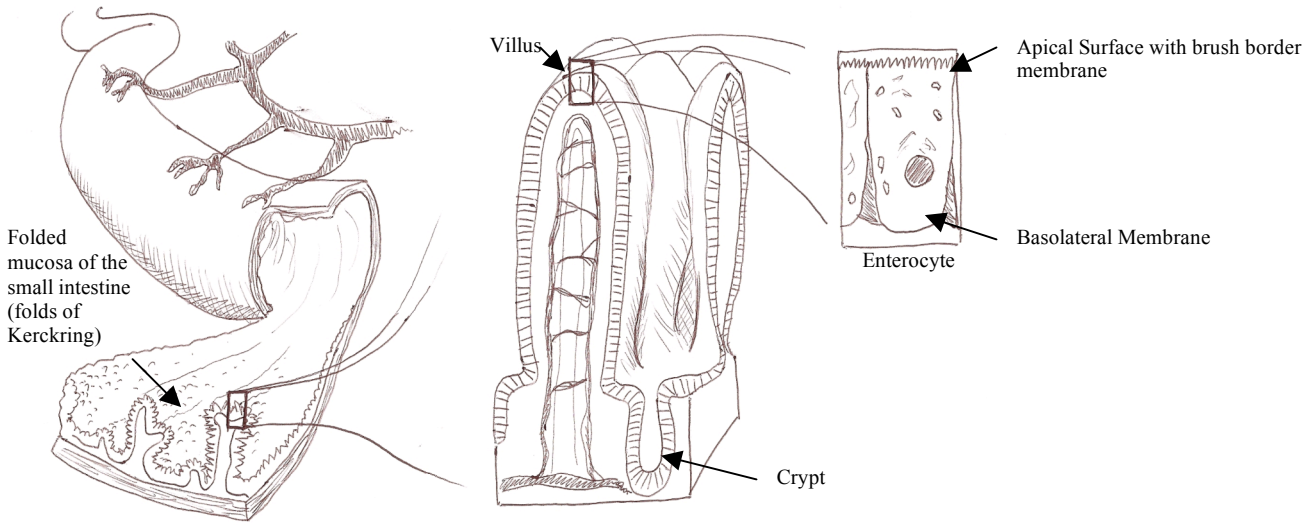


Figure 1-2 Microanatomy of the chicken small intestine

Summary. The digestive tract of the chicken is similar to other monogastrics, with a few uniquely avian structures, such as the crop and gizzard. Of the many parts that make up the digestive system, the small intestine plays the most important role in nutrient absorption. The mucosa of the small intestine is highly folded in order to increase the absorptive surface area. Found along these folds are projections called villi, which in turn are covered with smaller structures called microvilli. The microvilli coat the apical surface of the enterocyte and constitute the brush border membrane. With its thick layer of mucus that traps various digestive enzymes, the brush border membrane is the site of nutrient absorption.

PROTEIN DIGESTION IN THE CHICKEN AND THE BRUSH BORDER MEMBRANE PEPTIDE TRANSPORTER, PEPT1

Introduction

Protein digestion in the chicken is achieved through the secretion of proteolytic enzymes from the proventriculus, duodenum, and pancreas and results in the formation of small peptides and free amino acids. The absorption of the end-products of protein hydrolysis occurs in the duodenum, jejunum and ileum and is accomplished through the H+-dependent, peptide transporter 1 (PepT1). PepT1 is located in the brush border membrane of the enterocyte and due to its low-affinity can assimilate most combinations of di- and tripeptides into the cell. This section reviews the basics of protein digestion in the chicken and the characteristics of the peptide transporter PepT1. Material presented in this section can be found in "Scott's Nutrition of the Chicken" (Leeson and Summers, 2001).

Protein Digestion in the Chicken

There is no significant protein digestion that occurs in the mouth or crop, thus making the proventriculus the first site of meaningful digestion of protein. The gastric mucosa is innervated by the vagus nerve, which immediately becomes stimulated upon the ingestion of feed. Once stimulated, the vagus nerve initiates the release of gastric juice into the proventriculus. The secretions that make up this gastric juice include HCl and pepsinogen, an enzyme precursor that is converted to its active form, pepsin, as the pH declines and as the digesta moves from the proventriculus to the gizzard. Pepsin is capable of hydrolyzing several different peptide linkages, most notably between Leu-Val, Leu-Tyr, and between the aromatic amino acids Phe-Phe and Phe-Tyr. This mixture of partially hydrolyzed proteins and secretions is called chyme, which then passes into the gizzard where it undergoes further proteolysis by pepsin and mixing. The end result of proteolysis within the proventriculus and gizzard is to make peptide molecules available, which will then be susceptible to further digestion by the proteolytic enzymes produced by the intestine.

After mixing in the gizzard, the chyme then enters the duodenum. The chyme is an acidic mixture that causes the pH of the duodenum to decrease. This drop in pH triggers the release of the hormone gastrin, which further causes increased secretions of HCl into the proventriculus, which in turn causes the conversion of pepsinogen to pepsin. Along with the proventriculus, gizzard, and small intestine, the pancreas plays a vital role in proteolysis. The pancreas secretes fluids into the duodenum that contain elastase and various zymogens, such as trypsinogen and chymotrypsinogen. Elastase hydrolyzes elastin molecules to their component peptides in a relatively non-specific manner. Trypsinogen is activated to trypsin in the duodenum by enterokinase, an enzyme secreted by the intestinal mucosa. This activation

process is auto-catalytic, with the newly formed trypsin in turn converting other zymogens to their active forms, including chymotrypsinogen to chymotrypsin. Thus trypsin is central to the development of proper protein degradation. Trypsin, chymotrypsin, and elastase catalyze the hydrolysis of those polypeptides formed from pepsin digestion in the proventriculus and gizzard. Trypsin hydrolyzes peptide bonds that involve amino acids Lys and/or Arg, while chymotrypsin breaks down bonds between aromatic amino acids. The products made by these enzymes include many peptides. The pancreas also produces and secretes exopeptidases that contain carboxypeptidases A and B. These enzymes initiate the hydrolysis of the terminal peptide bonds, thus removing amino acid residues in sequence from the carboxy end of a polypeptide chain. The pancreatic secretions also contain various collagenases that can break down collagen into small peptides. Another proteolytic secretion, erepsin, is secreted from the jejunal wall and can not only break down polypeptides to amino acids and dipeptides, but can also convert nucleic acids to mononucleotides, which are then able to be absorbed.

Following hydrolysis in the duodenum and jejunum, the final product of protein digestion is a combination of small peptides and free amino acids. While up to now it would appear that most protein hydrolysis occurs in the lumen of the small intestine, that is not the case. A majority of peptide digestion occurs very close to the apical surface of the absorptive enterocyte, most notably in the brush border membrane. It is along the brush border membrane where most of the proteolytic enzymes are located. Conveniently, these enzymes are at their highest concentrations in the same area as the various nutrient transport systems. The small peptides and free amino acids produced via hydrolysis in the small intestine are then taken up by the enterocytes by these nutrient transport systems. In the case of peptides, they are absorbed into the cell by the H+-dependent PepT1. A proton-motive force that is provided for by the acidic

unstirred luminal layer of the intestine drives this transporter. PepT1 is a transporter that has low-affinity, meaning it can transport most combinations of di- and tripeptides across the cell membrane, with peptide uptake being the most rapid in the jejunum. Once the peptides have crossed the cell membrane, various peptidases located in the cytoplasm of the enterocyte will hydrolyze some of the peptides to their constituent amino acids. The newly freed amino acids (if not being utilized by the enterocyte) and the remaining peptides can then be transported out of the cell via amino acid and peptide transporters located in the basolateral membrane.

The Intestinal Oligopeptide Transporter, PepT1

Introduction. Prior to the 1970's, studies involving the assimilation of dietary proteins focused mainly on the uptake of free amino acids. The results garnered by early researchers such as Dent and Schilling (1949) and Christensen (1949) generated much enthusiasm. These groups reported no significant concentration of peptides in the portal blood of research animals following a meal containing adequate protein levels. Thus, it was widely regarded that "amino acids enter the animal organism from the intestine almost entirely in the free form" (Christensen, 1964). Prompted by results of their own showing higher luminal concentrations of peptides relative to free amino acids in human subjects, Adibi (1971) demonstrated a more important role for peptides in protein digestion. The 1970's gave rise to a number of studies that showed an increase in peptide concentrations in the blood following their hydrolysis and disappearance from the intestinal lumen (Adibi and Mercer, 1973; Adibi et al., 1975). However, after such a long period where it was thought dietary protein could only be absorbed in free amino acid form, these studies were still met with much skepticism. Validation came when studies used patients with an impaired ability to transport specific single amino acids. Patients suffering from an autosomal recessive metabolic disorder called cystinuria had normal uptake of those amino acids

when they were supplied in peptide form (Hellier et al., 1972; Silk et al., 1975; Leonard et al., 1976). The functionality of the dipeptide transport system was initially characterized in humans (Adibi, 1971; Adibi and Soleimanpour, 1974; Adibi et al., 1975), and has since been found to exist in every researched species. Since this boom in peptide transport research during the 1970's, further research has solidified the physiological importance of the intestinal polypeptide transport system and its efficient role in amino acid assimilation.

The peptide transporter, PepT1, is a member of the Proton-coupled Oligopeptide Transporter (POT) superfamily (Table 1-1), a small gene family that includes peptide transporters in diverse species as well as having ancestral roots going back to fungal, bacterial, and plant transporters. (Botka et al., 2000). PepT1 is capable of transporting most combinations of the 20 amino acids in di- and tripeptide form from the small intestine (Daniel, 2004), as well as a plethora of peptidomimetic drugs such as the β-lactam antibiotics, angiotensin-converting enzyme (ACE) inhibitors for treating hypertension, anti-virals such as valacyclovir, and anti-cancer agents such as bestatin (Meredith and Price, 2006). Fei et al. (1994) reported the first cloning and functional characterization of PepT1 from rabbit small intestine. Following that initial cloning, PepT1 has been cloned in many vertebrate species including human (Liang et al., 1995), rat (Saito et al., 1995), dairy cows (Chen et al., 1999), sheep (Chen et al., 1999; Pan et al., 2001), mouse (Fei et al., 2000), chicken (Chen et al., 2002), zebrafish (Verri et al., 2003), rhesus monkey (Zhang et al., 2004), pig (Klang et al., 2005), turkey (Van et al., 2005), and Atlantic cod (Ronnestad et al., 2007). Recently, Weitz et al. (2007) and Harder et al. (2008) characterized a gene within the *Escherichia coli* (*E. coli*) genome that encodes a protein similar to mammalian PepT1. Called tripeptide permease, this prokaryotic peptide transporter shares characteristics similar to PepT1, such as transporting di- and tripeptides and substrate recognition patterns.

Table 1-1 The proton oligopeptide cotransporter family

Human gene name	Protein	Aliases	Substrates	Transport type/coupling ion	Tissue distribution/cellular expression
SLC15A1	PEPT1	Oligopeptide transporter 1, H+/peptide transporter 1	Di-, and tripeptides	Cotransport, H+	Intestine and kidney apical membrane, lysosomal membrane
SLC15A2	PEPT2	Oligopeptide transporter 2, H+/peptide transporter 2	Di-, and tripeptides	Cotransport, H+	Kidney, lung, brain, mammary gland, bronchial epithelium
SLC15A3	Hptr3	Peptide/ histidine transporter 2, human peptide transporter 3, PHT2	Histidine, di- and tripeptides	Cotransport, H+	Lung, spleen, thymus, brain, liver, adrenal gland, heart
SLC15A4	PTR4	Peptide/ histidine transporter 1, human peptide transporter 4 PHT1	Histidine, di- and tripeptides	Cotransport, H+	Brain, retina, placenta

Structure of PepT1. PepT1 is an integral membrane protein that consists of 12 trans-membrane domains (TMD) with a large extracellular loop between TMD 9 and 10 (Figure 1-3). Studies regarding PepT1 structure have mostly been performed with rabbit (Fei et al., 1994) and human (Liang et al., 1995) PepT1. Both groups predicted the presence of 12 TMDs, which was later confirmed via epitope tagging by Covitz et al. (1998). The gene that encodes rabbit PepT1 (rPepT1) produces a protein with 707 AA (Fei et al., 1994). Human PepT1 (hPepT1) contains 708 AA with 81% identity to rabbit PepT1, with the biggest difference being within the large extracellular loop between TMD 9 and 10 (Meredith, 2009).

The relationships between the regions within the PepT1 protein and their functions have been investigated through a number of approaches, for example the construction of chimeras. Chimeras created from different regions of PepT1 demonstrated the important roles of trans-membrane domains 1-4 and 7-9 in determining substrate binding characteristics and affinity (Doring et al., 1996; Fei et al., 1998; Terada et al., 2000). Another approach, site-directed mutagenesis, has identified amino acid residues that are key for transport and/or controlling transport factors such as binding affinity and transport rate (Meredith and Price, 2006). Bolger et al. (1998) demonstrated a 25% reduction in transport capacity by replacing a tyrosine residue with alanine (Y12A) within the first trans-membrane domain. A highly conserved tyrosine residue within the second trans-membrane domain was replaced with alanine (Y64A) resulting in reduced peptide transport, but when that tyrosine was replaced with phenylalanine (Y64F) a reduction in substrate affinity was observed (Chen et al., 2000). The mutation of another tyrosine (Y91), this time in TMD 3, either to phenylalanine, alanine, or cysteine caused transport rates to decrease by 70-80% (Links et al., 2007). Histidine is another amino acid that has been shown to have an important role in PepT1 activity, particularly the binding of protons. Histidine

57 is located in TMD 2 and replacement with arginine (H57R) resulted in no significant transport activity due to the mutated H57 being nonfunctional (Chen et al., 2000). Further studies have shown that the aromatic compounds adjacent to His57 may be essential to the H^+ binding ability of PepT1 (Chen et al., 2000).

Interestingly, *E. coli* has a H^+ dependent peptide transporter called YdgR that demonstrates many characteristics similar to PepT1 (Weitz et al., 2007). However, YdgR lacks a histidine residue in the area of H57 in mammalian proteins. Yet, it still transports di- and tripeptides using a proton motive force, as well as moves peptidomimetic drugs across the cell membrane. The comparison between mammalian and bacterial peptide transporters proposes the need for further study into the amino acids that have been deemed "functionally important" and necessary for PepT1 activity.

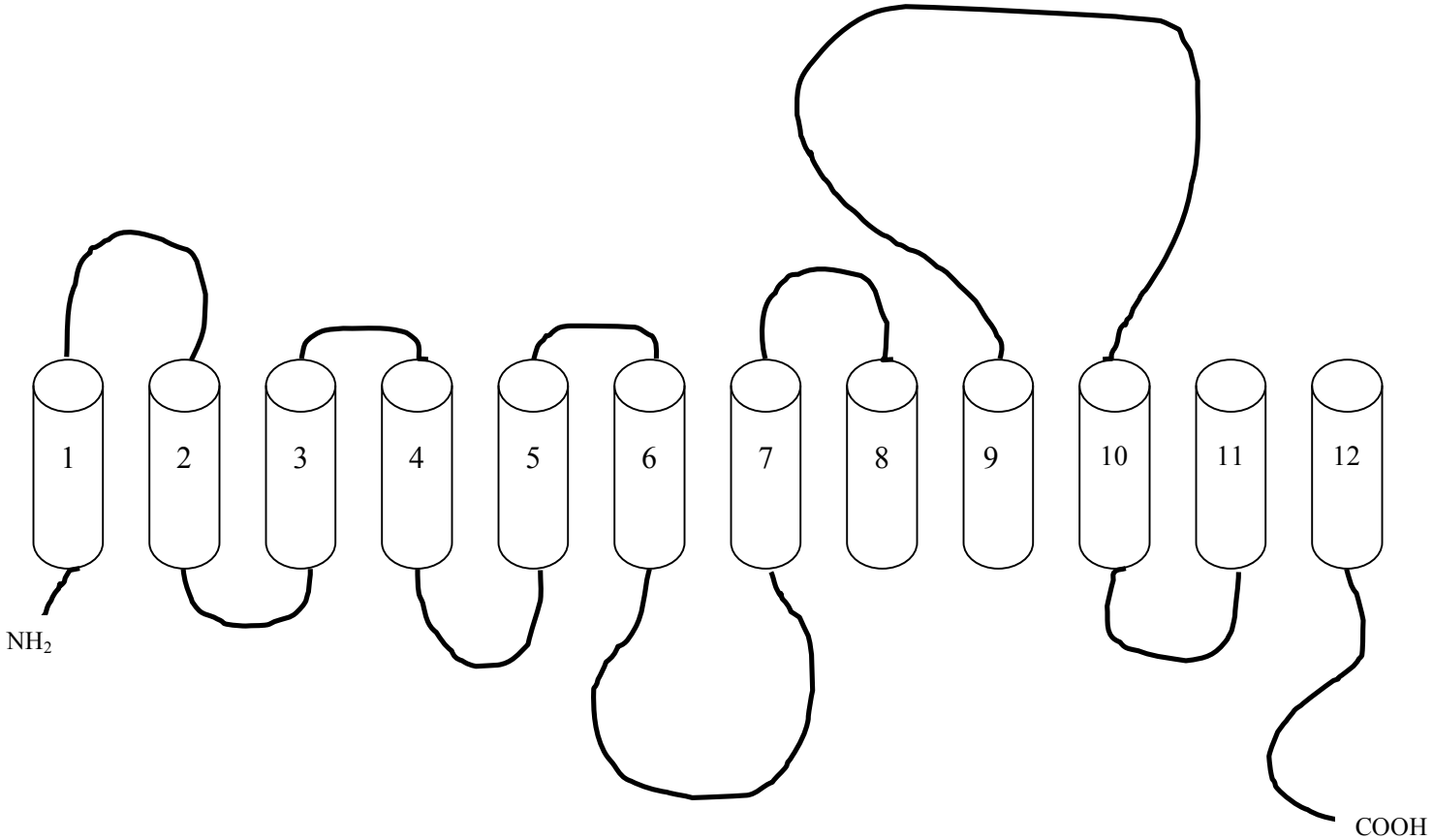


Figure 1-3 Transmembrane domains of PepT1

S. L. Madsen, 2009

Cellular and Tissue Distribution of PepT1. Within the small intestine of rats (Ogihara et al., 1999; Groneberg et al., 2001; Hussain et al., 2002) and mice (Groneberg et al., 2001), PepT1 expression is relegated to the brush border membrane (BBM) of the enterocyte. In rats, the maturity of the enterocyte plays a role in the density of PepT1 protein in the BBM (Ogihara et al., 1999). The enterocyte matures as it moves from the villus crypt to the apical surface. The density of PepT1 increases from villus crypt where there is no PepT1 expression, to the villus tip in mice or rats (Ogihara et al., 1999; Hussain et al., 2002). The cellular location of PepT1 is not only affected by the developmental stage of the enterocyte, but may also be affected by the developmental stage of the animal. In post-natal rats, PepT1 can be found in the cytoplasm beneath the apical membrane of the enterocyte and in the basolateral membrane (Hussain et al., 2002). Such locations could have important implications as the newborn pup transitions from nutrients derived from the bloodstream, to nutrients derived from the intestinal lumen.

While PepT1 has a very specific cellular distribution, its distribution in the tissues is broader. Meredith and Boyd (2000) demonstrated that PepT1 is widely expressed in the small intestine, with some expression seen in the kidney due to its role in reabsorbing proteins from the glomerular filtrate. In ruminants such as dairy cattle and sheep, PepT1 is also expressed in the omasum and the rumen (Chen et al., 1999). Though PepT1 is found in similar tissues across species, its abundance within these tissues under normal dietary conditions is varied. For example, in rabbits (Fei et al., 1994) and pigs (Chen et al., 1999; Klang et al., 2005) PepT1 mRNA is more abundant in the proximal small intestine (duodenum and jejunum). However, in dairy cows and sheep (Chen et al., 1999) and black bears (Gilbert et al., 2007a), the highest abundance of PepT1 mRNA can be found in the distal small intestine. In avians, specifically the chicken, the distribution of PepT1 is greatest in the small intestine and lowest in the kidney and

ceca (Chen et al., 1999, 2002). Within the small intestine, the duodenum has been shown to have the highest expression of PepT1, followed by the jejunum and ileum, respectively (Chen et al., 1999, 2002; Gilbert et al., 2007b). However, in chickens selected for high and low juvenile body weights, PepT1 expression was highest in the ileum in those birds with low body weights (Mott et al., 2008). Ileal upregulation of PepT1 in the low weight birds may be an adaptation to maximize nutrient absorption in times when nutrient availability is low.

Substrate Specificity of PepT1. PepT1 is a low affinity, high capacity transporter that has a broad substrate range. Various competition experiments were used to determine the substrate specificity of PepT1. These experiments determined the level of inhibition that peptides containing more than three amino acids had on the uptake of di- and tripeptides (Leibach and Ganapathy, 1996). Their results showed that substrates of PepT1 only include di- and tripeptides. With 20 naturally occurring amino acids, the PepT1 range could potentially include any of the 400 (20^2) dipeptide or 8000 (20^3) tripeptide combinations as well as many pharmacologically important compounds. With such a wide array of AA combinations, it is astounding that a peptide transporter could be capable of transporting substrates with such variety in structure, molecular size, polarity, net charge, and stereochemistry. A key component of the transport capacity of PepT1 is water. Water plays a critical role in the docking process between substrate and carrier by masking the charges of different amino acid side chains, thus allowing charged and uncharged substrates to be transported at the same site (Daniel 2004).

Potential substrate binding domains in PepT1 (and family member PepT2) were identified through the creation of chimeric proteins and the subsequent observation of substrate affinities (Fei et al., 1998). Chimeras were created by making substitutions within the 12 trans-membrane domains that make up PepT1 and PepT2. One such chimera consisted of the first 9 ½

trans-membrane domains from PepT1, with the remaining domains coming from PepT2. This mutation resulted in a low affinity for Gly-Sar, a common dipeptide used for uptake studies. Low affinity for Gly-Sar is characteristic of PepT1. However, Gly-Sar affinity increased (a characteristic of PepT2) with substitutions of PepT2 within trans-membrane domains 7, 8, 9, and the extracellular loop between domains 9 and 10.

Of the 12 trans-membrane domains making up the PepT1 protein, the extracellular side of the second trans-membrane domain contains a histidine residue that is vital to the function of PepT1 (Daniel and Kottra, 2004). Histidyl residues most likely participate in peptide transport through the binding and translocation of protons (H+). The importance of histidine was supported through studies that looked at the effects of histidine mutations on peptide transport. The replacement of human PepT1 His-57 and PepT2 His-87 with asparagine or glutamate, amino acids that would have the least effect on charge and size, abolished peptide transport (Fei et al., 1997). The idea that His-57 is necessary for proper function of PepT1 was further bolstered when two other conserved histidine residues (His-121 and His-260) were mutated and found to have no inhibition on peptide transport. The possibility that the loss of function of hPepT1 and hPepT2 in the mutants was due to an alteration in protein expression was proven false through immunoblot assays. Western blots showed that the expression levels of the mutants were similar to the wild-type transporters expressed in HeLa cells. Further supporting the importance of histidine, Meredith and Boyd (2000) pretreated renal BBM vesicles with diethylpyrocarbonate (DEPC), a reagent with histidine-modifying properties. They found that pretreatment with DEPC, and the subsequent modification of histidine, resulted in the complete abolishment of peptide transport.

In general, it is the characteristics of the substrates that determine the binding affinity with and subsequent transport by PepT1. For example, Vig et al. (2006) reaffirmed the need for substrates to be larger than a single amino acid yet smaller than a tetrapeptide to be transported by PepT1, indicating size specificity for the PepT1 binding pocket. Greater affinity is found for peptides that contain the L-isomers of AA over those that contain the D-isomer (Leibach and Ganapathy, 1996; Brandsch et al., 2004). Providing ample substrate affinity, the N-terminal amino group of a peptide has been shown to be preferred by PepT1, but not be necessary. Conversely, the C-terminal carboxy group is also not essential and can be replaced by any electrogenic group, although problems can arise due to the size of the group if it is present (Daniel and Kottra, 2004). A minimal requirement regarding the oppositely charged heads of a peptide is that the two heads be separated by a minimum distance of 500 pm and a maximum distance of 635 pm (Doring et al., 1998). Surprisingly a key feature of di- and tripeptides, the amide bond, is not required for specificity (Enjoh et al., 1996; Brandsch et al., 1998; Doring et al., 1998), which if it is present, the trans-configuration is the only accepted form.

Aside from naturally occurring di- and tripeptides, PepT1 is also capable of transporting many peptidomimetic drugs such as the β-lactam antibiotics. The broad substrate capability of PepT1 make it an ideal candidate for transporting many pharmacologically important drugs. However, many of these compounds require some modification in order to increase their bioavailability. For example, antiviral drugs such as acyclovir were reported to be better absorbed when provided with an amino terminal amino acid (Ganapathy et al., 1998) and the absorption of floxuridine (an anti-cancer drug) was improved through the addition of an amino acid ester (Landowski et al., 2005). Even though very high affinities between some pro-drugs and PepT1 were reported, bioavailability assays failed to demonstrate that an increase in

absorption was occurring (Thomsen et al., 2004), thus highlighting the important fact emphasized by Vig et al. (2006) that affinity does not equal absorption. However, with further research, the number of pharmacologically relevant compounds transported by PepT1 will increase.

Transport Mechanism of PepT1. PepT1 is located in the brush border membrane of mature enterocytes and is powered by a proton motive force. By applying an inward proton gradient to rabbit brush border membrane vesicles, Ganapathy et al. (1984) observed an increase in Gly-Sar uptake compared to controls, indicating that such a gradient could be the driving force behind peptide absorption. Voltage-clamp analysis of Xenopus oocytes injected with PepT1 cRNA further supported that transport by PepT1 is an electrogenic process (Fei et al., 1994). In order to maintain the proton gradient, the intracellular H^+ concentration must be less than the extracellular H+ concentration. This gradient is maintained via the Na^+/H^+ exchanger (NHE) located on the apical surface of the enterocyte (Adibi, 1997) (Figure 1-4). The NHE transports 1 cytosolic H^+ into the lumen of the small intestine in exchange for 1 Na^+, which it transports into the enterocyte from the lumen of the small intestine (Adibi, 1997). To maintain the negative intracellular membrane potential needed to drive PepT1, the Na^+ needs to be removed from the cytosol. This is accomplished with the Na^+/K^+ ATPase pump, which is located in the basolateral membrane of the enterocyte and pumps 3 Na^+ out of the cell in exchange for 2 K^+ (Adibi, 1997).

When PepT1 transports di- or tri-peptides across the apical membrane, one or two protons are carried across as well. The proton to peptide ratio for neutral and cationic peptides transported by PepT1 is 1:1; the proton to peptide ratio for anionic peptides is 2:1 (Steel et al., 1997). Steel et al. (1997) further proposed that the second H^+ needed to transport a charged

anionic peptide is there to stabilize the peptide by neutralizing its charge while the other H^+ binds to the transporter.

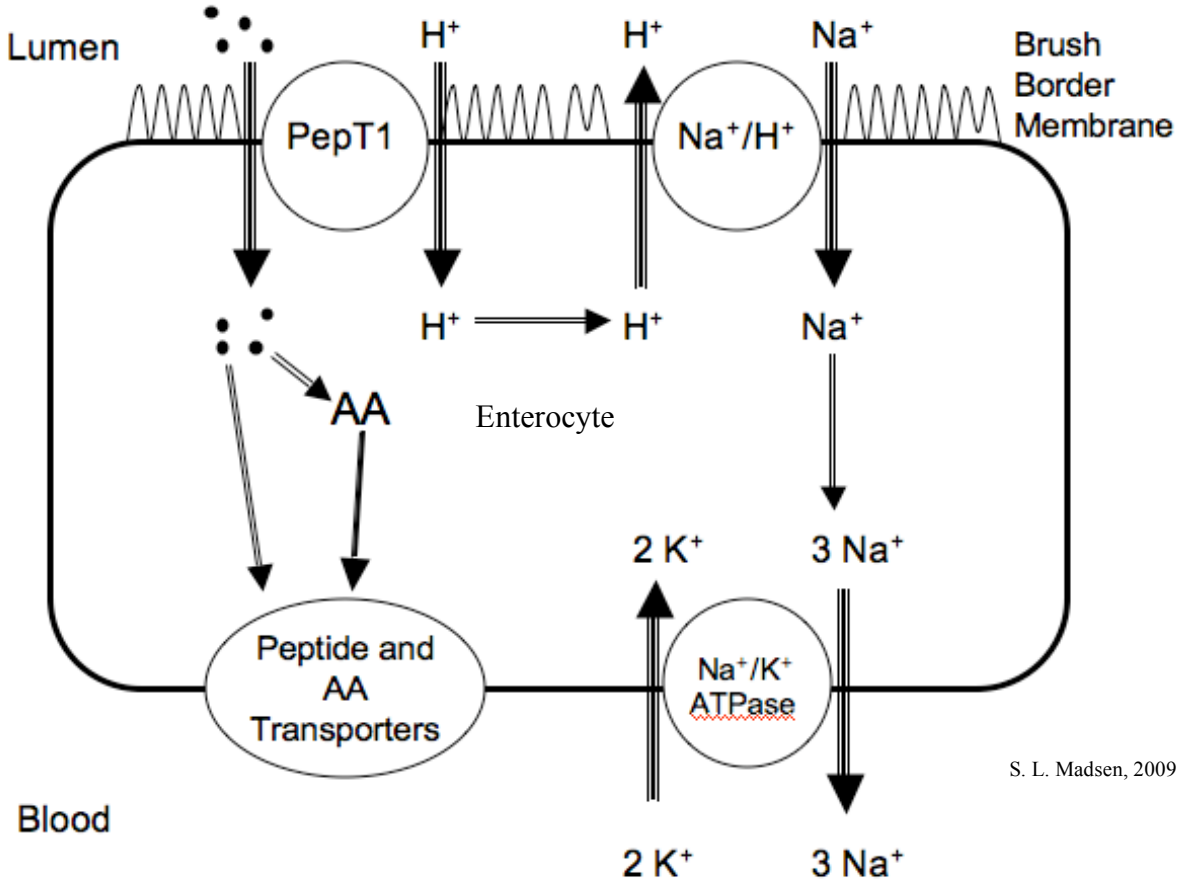


Figure 1-4 Active transport of peptides and H+ by PepT1

Regulation of PepT1. The expression of PepT1 can be regulated by diet, development, hormones, pharmacological agents, and pathological states (Rubio-Aliaga and Daniel, 2007). In regards to diet, PepT1 expression in rats has been shown to increase 2-fold in response to feeding a high-protein diet (Erickson et al., 1995). Conversely, a decrease in expression was noted in rats fed a protein-free diet (Shiraga et al., 1999). Protein quality, or the combination of amino acids required for maximal growth and efficient protein synthesis, also has an effect on transporter expression. Gilbert et al. (2008) demonstrated the importance of protein quality by feeding a high-quality protein source (soybean meal, SBM) and a lower-quality protein source (corn gluten meal, CGM) to Aviagen Line A and B chicks. In this study, PepT1 mRNA levels were greater in those chicks fed the SBM diet at a quantity restricted to that consumed by CGM chicks. These results correspond with an earlier study that showed a positive correlation between crude protein in the diet and PepT1 expression (Chen et al., 2005). Interestingly, while a balanced diet can have regulatory effects on PepT1, so too can fasting. Prolonged fasting consists of three distinct levels of energy depletion (Habold et al., 2007). Phase 1 lasts for only a few hours and is marked by rapid adaptation that includes gut flushing, an increase in fat store mobilization, and a decrease in protein catabolism. During the longer Phase 2, most energy is derived from fats. Finally, in Stage 3, protein catabolism increases while fat stores are increasingly depleted. It is during this final stage that the animal begins to exhibit a more foraging-like behavior in anticipation of a total loss of energy stores (Koubi et al., 1991). Habold et al. (2007) demonstrated a strong increase in PepT1 expression in Stage 2 and Stage 3 fasted male Wistar rats. During a Stage 2 fast, atrophy of the intestinal villi was observed as a way to conserve energy by not supporting the intestinal tract. However, the cytoplasm and the microvilli of the enterocyte were preserved (Habold et al., 2007). This preservation, along with

an increase in PepT1 expression, suggests an optimization of the intestinal lining during fasting in order to absorb nutrients quickly and efficiently when they become available again. In 2006, PepT1 expression was shown to increase in fasting rats along with a concomitant increase in the expression of the peroxisome proliferator-activated receptor alpha (PPARα), a transcription factor that plays a role in the adaptive response to fasting (Shimakura et al., 2006).

The period of time prior to and following birth (or hatch) is incredibly important in terms of the development of the gastrointestinal tract. One important factor in the development of the small intestine is the switch in diet. In mammals, the animal transitions from a prenatal diet derived from the amniotic fluid, to a milk-based diet provided by the mother. In avian species, the chick switches from a pre-hatch diet consisting of lipid-rich yolk to a post-hatch diet rich in carbohydrates and proteins. The developmental changes that occur to adapt to changes in diet affect the expression of many nutrient transporters, including PepT1. The presence of PepT1 during the prenatal or pre-hatch period has been documented in humans, guinea pigs, sheep, rabbits, rats, turkeys, and chickens (Guandalini and Rubino, 1982; Pacha, 2000; Shen et al., 2001; Van et al., 2005; Chen et al., 2005). An increase in PepT1 expression in the days leading up to birth (or hatch), followed by a decrease in expression as the animal ages was a common observation in these studies. For example, intestinal PepT1 levels in the rat increased at birth, with levels reaching their maximum between 3 to 5 days of age followed by a decline and plateau as the animal aged (Shen et al., 2001). A similar trend was observed in turkeys and chickens. Van et al. (2005) reported a 3.3-fold increase in turkey PepT1 from 5 days before hatch to day of hatch, and Chen et al. (2005) reported a 14- to 50-fold increase in chicken PepT1 from embryo day 18 to day of hatch. In rat, turkey, and chicken, maximum PepT1 mRNA levels were reached right before birth or hatch. The increase seen in PepT1 expression just prior to

birth or hatch is just one of the characteristics documented as the intestine adapts to postnatal or post-hatch functions, and becomes the organ solely responsible for nutrient absorption.

Hormones and various pathological states also regulate PepT1. Ashida et al. (2004) induced hyperthyroidism in rats by supplementing the drinking water with 12 mg/L of L-thyroxine (T_4) for 21 days, resulting in a decrease in expression of PepT1 mRNA in the small intestine. A similar study by the same group demonstrated a decrease in PepT1 mRNA levels when human Caco-2 cells were treated with thyroid hormone (3,5,3'-Triido-$_L$-thyronine or T_3) (Ashida et al., 2002). With the many physiological roles of thyroid hormone, any change in its secretion may contribute to various pathological states. In fact, many studies have focused on the effect hyperthyroidism has on the gastrointestinal tract, such as changes in motility (Wegener et al., 1992), structural changes within the small intestine (Hodin et al., 1996), and the absorption of various nutrients (Levin 1969). Hyperthyroidism has also been documented to have an effect on the absorption of pharmacologically relevant compounds (O'Conner and Feely, 1987). With the role PepT1 has on transporting such compounds, understanding the effects that hyperthyroidism has on its expression would be tremendously helpful in treating patients with disorders of the thyroid gland. Conversely, hypothyroidism (via thyroidectomy) resulted in increased PepT1 levels in the kidney of male rats. When these rats were treated with T_3 and T_4, the levels decreased to 33% lower than the controls (Lu and Klaassen, 2006).

Summary. The peptide transporter 1, PepT1, is an integral membrane transporter that, along with apical amino acid transporters, is responsible for the absorption of nitrogen via dietary di- and tri-peptides. Expression of PepT1 is greatest in the small intestine, with the transporter itself localized in the brush border membrane lining the apical surface of the mature enterocyte. Expression of PepT1 mRNA is affected by various factors, including diet,

development, hormones, and pathological state. Chicken PepT1 has been shown to be expressed in all segments of the small intestine, and expression levels have been shown to be affected by developmental age and protein quality.

THE NUCLEAR PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR, PPARα

Introduction

The peroxisome proliferator-activated receptors (PPARs) are members of a distinct subfamily of the nuclear receptor (NR) superfamily. The PPAR subfamily consists of ligand-activated transcription factors that are involved in the regulation of numerous metabolically important genes, such as increasing the number of peroxisomes, a eukaryotic organelle responsible for many oxidative reactions. Three isoforms, each encoded by different genes, exist within this group. They are PPARα, PPARγ, and PPARδ. PPARα was the first isoform in this subfamily to be cloned, thus its α-designation. The name PPAR comes from the ability of the α-isoform to mediate the effects of various peroxisome proliferating compounds, such as hypolipidemic fibrates, certain plasticizers, herbicides, and food flavorings (Reddy and Hashimoto, 2001). Issemann and Green (1990) were the first to report the cloning of PPARα in a mouse model. They detected a high level of PPARα expression in the liver, kidney, and heart with very low levels in the brain and testis. Interestingly, at the time, the expression pattern of PPARα corresponded with the tissue-specific effects of the peroxisome proliferators; thus it was highly expressed in tissues that have a high capacity for fatty acid oxidation. Since 1990, PPARα has been cloned in frog (Dreyer et al., 1992), rat (Gottlicher et al., 1992), human (Sher et

al., 1993), rabbit (Guan et al., 1997), and chicken (Diot and Douaire, 1999) and the location of human PPARα has been mapped on chromosome 22 in the general region 22q12-q13.1 (Sher et al., 1993). PPARα is a nutritional state sensor, thus playing an important role in the adaptive response to fasting by activating the transcription of nutritionally important genes, such as the β-oxidation pathways in the mitochondria and peroxisomes.

PPARγ was first characterized via homology cloning in *Xenopus*, or frog (Dreyer et al., 1992) and then in mice (Zhu et al., 1993). Though it is a member of the PPAR subfamily, it does not share the same peroxisome-proliferating abilities as its predecessor, PPARα. Instead, PPARγ is considered the master transcriptional regulator of adipocyte differentiation (Tontonoz and Spiegelman, 2008) because it is highly expressed in white and brown adipose tissue and controls a large variety of genes implicated in lipid metabolism. Activation of PPARγ results in the conversion of preadipocytes to mature adipocytes by triggering morphological changes and an accumulation of lipids within the mature fat cell (Tontonoz and Spiegelman, 1994). Its role in adipogenesis was further supported when Barroso et al. (1999) and Berger et al. (2000) demonstrated that PPARγ mutants with dominant negative activity inhibited adipogenesis in cultured preadipocytes. This receptor has important pharmacological implications because of its high affinity for the thiazolidinedione (TZD) class of anti-diabetic drugs. Upon activation by the TZDs, PPARγ increases target gene expression that eventually leads to an increase in insulin sensitivity in the peripheral tissues (Lehmann et al., 1995). Because of this action, possible links between not only PPARγ and diabetes, but also between PPARγ and the control of obesity, have been investigated.

At the time that PPARγ was cloned, another member of the PPAR subfamily was identified. PPARδ, along with the α- and γ-isoforms, is expressed in many species including

rodents, humans, fish, and amphibians. Though the biological roles of PPARα and γ are better defined, a growing body of research is establishing a new role for PPARδ in epithelial cell differentiation and survival, as demonstrated by PPARδ-null mice displaying significantly delayed wound healing (Michalik et al., 2001).

The Structure of PPARα. The PPARs are the target of a variety of different synthetic compounds which are used to treat metabolic disorders, such as diabetes and dyslipidemia (Berger and Moller, 2002). Therefore, it is imperative to obtain a thorough understanding of the structure of these receptors. Through nucleotide sequence analysis, Isseman and Green (1990) were able to determine the 468-amino acid sequence and mass (52,400 d) of the mouse PPARα protein. Like other nuclear receptors, PPARs have a modular form that is composed of 6 functional regions (A-F) (Reddy and Hashimoto, 2001). The N-terminal A/B region has a ligand-independent activation function (the AF-1 domain). Within the central section of the receptor, the highly conserved C region contains two type II zinc fingers that enable for sequence specific DNA recognition and dimerization. The C region is also called the DNA-binding domain (DBD) of the receptor. Near the C-terminus of the receptor, the protein is divided into D, E, and F regions. The ligand-binding domain (LBD) is located in region E, and through crystal structures it has been shown to be quite large (~1300 $Å^3$) (Willson et al., 2000). The unusually large size of the LBD has been implicated in the ability of the PPARs to accommodate such a broad range of natural and synthetic ligands. As with the DBD, the LBD is also highly conserved and it functions as a ligand-dependent activation domain (AF-2). Lastly, the F region is relatively small when compared to the other functional regions, however it is considered to play an important role in the interplay between nuclear receptors and their coactivators/corepressors (Glass and Rosenfeld, 2000).

Mechanism of Action of PPARα. Ligand activation of the PPARs causes these receptors to heterodimerize with the retinoid X receptor (RXR) (Miyata et al., 1994) (Figure 1-5). The RXR is another nuclear receptor, which is activated by 9-cis retinoic acid (Allenby et al., 1993). The interaction between these two receptors was discovered through the use of fusion proteins and the yeast two-hybrid system (Miyata et al., 1994). Once bound, the PPAR/RXR complex then binds to PPAR response elements (PPREs) in the promoter region of target genes (Wan et al., 2000). PPREs are typically organized as direct repeats of the sequence AGGTCA, which are separated by one or two nucleotides, and flanked upstream by sequences that are rich in A and T (Lefebvre et al., 2006). The activation of the transcription process relies on five basic steps: receptor/ligand binding; stable binding of the receptor to DNA; removal of corepressors and the recruitment of coactivators; transcription activation; and removal and dissociation of the transcriptional machinery (Lefebvre et al., 2006). Crystallographic studies suggest that the major contribution of the PPARα ligand is to stabilize the receptor, since no major structural reorganization is observed following ligand binding (Duez et al., 2005).

When bound by an agonist, the PPARs will interact with various coactivator proteins in order to initiate transcription (Berger and Moller, 2002). Coactivators of the PPARs interact with the receptors through a conserved LXXLL motif (X being any AA) (Heery et al., 1997). Three main groups of coactivators have been identified (Berger and Moller, 2002). One group, including CBP/p300 and steroid receptor coactivator (SRC)-1, has a histone acetylase ability that allows for the remodeling of the chromatin structure. The second group, with members of the DRIP/TRAP complex (a large macromolecular transcriptional regulator that can interact with the vitamin D_3 receptor (DRIP) or the thyroid hormone receptor (TRAP)) which includes PPAR binding protein (PBP)/TRAP220, forms a bridge between the receptor and the transcriptional

machinery. The third group, which includes PGC-1 and RIP140, still is not well understood. However, it appears to have chromatin-opening abilities in the area of the regulatory region of the target gene (Berger and Moller, 2002).

Ligand activation of PPARα has demonstrated an important role for this receptor in the regulation of cellular uptake and β-oxidation of fatty acids. In regards to cellular uptake of fatty acids, PPARα initiates the expression of proteins that transport fatty acids across the cell membrane, such as fatty acid transport protein (FATP). Following fibrate (a PPARα ligand) treatment in rats, Martin et al. (1997) observed increased FATP mRNA levels in both the liver and the intestine, along with a concomitant increase in fatty acid uptake in these organs. β-oxidation of fatty acids occurs in the mitochondria and/or in the peroxisomes in order to produce acetyl-CoA, which then enters the Krebs cycle. The Krebs cycle (or citric acid cycle) is a vitally important metabolic pathway to aerobic organisms, where carbohydrates, proteins, and fats are broken down into carbon dioxide, water, and ATP. Carnitine palmitoyltransferase I (CPT I) is a key enzyme in the rate limiting step of translocating fatty acids into the inner membrane of the mitochondrion. The CPT I gene is very strongly induced by PPARα ligands and has a functional peroxisome proliferator hormone response element (PPRE) in its 5' flanking region (Brandt et al., 1998). The importance of PPARα in fat metabolism is further supported by Lee et al. (1995) who reported depressed levels of enzymes capable of fatty acid metabolism in mice that had had the LBD of PPARα disrupted. In addition to lower enzyme levels, the null mice did not respond when treated with peroxisome proliferating agents (PPARα ligands). Remarkably, mice that were homozygous for the mutation showed no physical defects and were viable and fertile, thus useful to later investigations into PPARα.

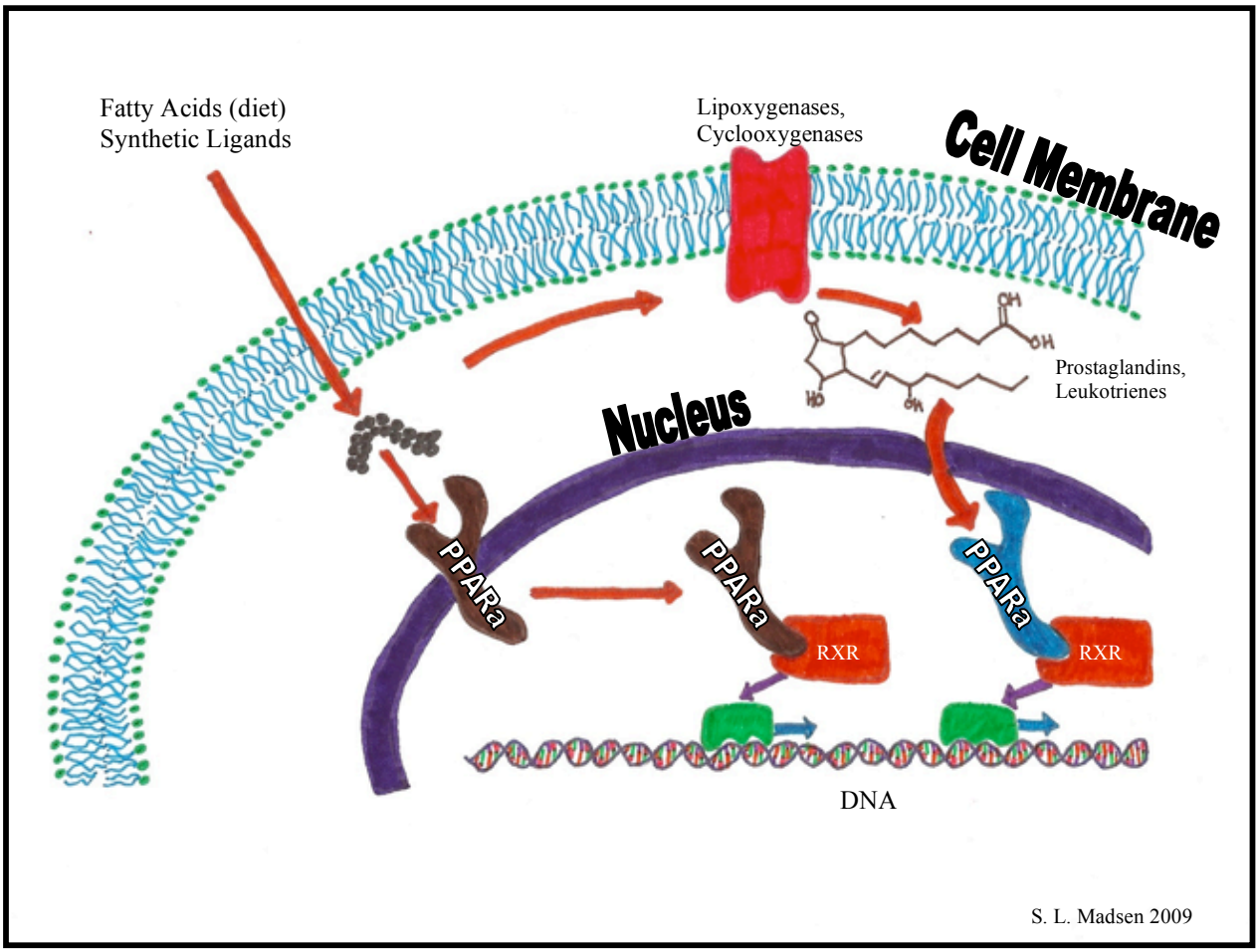


Figure 1-5 Mechanism of action of PPARα

Ligands of PPARα. The LBD of the PPARs is of an unusually large size (Willson, et al., 2000) and can accommodate a structurally diverse class of ligands, both natural and synthetic. The search for "the" natural, endogenous PPARα ligand is ongoing, however many candidates have been proposed. PPARα exerts transcriptional control over the enzymes that are critical to both mitochondrial and peroxisomal FA oxidation pathways. Natural factors capable of inducing these enzymes, therefore, are considered to be natural PPARα ligands. Examples of these natural factors include: long-chain FAs (> C_{20}) and polyunsaturated FAs; phytanic acid (a compound derived from plant chlorophyll); dehydroepiandrosterone (DHEA) produced by the adrenal glands; and various eicosanoids derived from arachidonic acid, such as the prostaglandins (Reddy and Hashimoto, 2001). Any one of these factors is capable of inducing FA β-oxidation via PPARα activation.

The synthetic PPARα ligands are part of a structurally diverse class of compounds whose common feature is the ability to be converted to a carboxylic acid derivative, a possibly important factor in the induction of peroxisome proliferation (Reddy and Hashimoto, 2001). One particular class of chemicals, the hypolipidemic fibrates, have been used for many years in humans to lower plasma triglyceride levels and include clofibrate, gemfibrozil, and WY-14643. The fibrates, when administered long-term, have proven to be potent inducers of hepatic carcinoma in rats and mice (Rao and Reddy, 1996), a characteristic surprisingly not found in humans. Shimakura et al. (2006) proposed a novel function for PPARα in the intestine by feeding the synthetic PPARα ligand WY-14643 to rats, thereby mimicking a fasting state. Following oral administration of WY-14643, induction of PepT1 was observed. The role of PPARα was then confirmed by fasting PPARα-null and wild-type mice. In the knockout mice, the response of PepT1 to fasting was completely abolished, where it was increased in the wild-

type. By utilizing a synthetic PPARα ligand, Shimakura et al. (2006) concluded that PPARα plays a critical role in the induction of PepT1 during a fasting state.

Summary. The peroxisome proliferator-activated receptor alpha, PPARα, is a member of the nuclear receptor superfamily. It is a ligand-activated transcription factor that, when activated, causes the transcription of a number of nutritionally important genes. Its role in the cellular uptake and β-oxidation of fatty acids has been well documented. Numerous ligands, both natural and synthetic, for PPARα exist. The synthetic ligands have been shown to have beneficial therapeutic effects in treating hyperlipidemia in humans; however, these same ligands have also been demonstrated to be carcinogenic when used chronically in mice. The synthetic PPARα ligand WY-14643 has been used in studies to demonstrate a link between PPARα and the induction of PepT1 during fasting situations.

OBJECTIVES

The nutrient transporter PepT1 is located in the small intestine and it is responsible for the absorption of di- and tri-peptides. Expression of this transporter is affected by numerous factors. One of those factors is proposed to be the peroxisome proliferator-activated receptor alpha, or PPARα. PPARα is a nuclear hormone receptor that is activated by free fatty acids, thus playing a role as a nutritional state sensor. Recently, a proposed link between the activation of PPARα (via the synthetic PPARα ligand WY-14643) and the transcription of PepT1 has been suggested. Based on those findings, the gene expression of the nutrient transporter PepT1 and the nuclear hormone receptor PPARα in the chicken small intestine was examined in this thesis, with gene expression assayed by real-time PCR using the relative quantification method. The objective of the first experiment was to examine the effect of a fasting/refeeding regimen on the

expression of these two genes in the chicken small intestine. The objective of the second and third experiments was to determine the effect of feeding a synthetic PPARα ligand (WY-14643) on the gene expression of PepT1 and PPARα in the small intestine of layers and broilers, respectively.

CHAPTER II.

MATERIALS AND METHODS

Animals and Tissue Collection

Feeding Trial. This study was initiated with 93 two-week old Aviagen Line B broilers, as shown in Figure 2-1. The birds were housed in battery cages, with 5-6 birds per cage. The birds were exposed to 24h light and were given free access to feed and water until the start of the experiment. The birds were divided into sixteen groups, with each group being subjected to a specific protocol (Figure 2-1). Samples taken throughout the study include the duodenum, jejunum, and ileum. The small intestine was separated into duodenum (section of tissue beginning at the gizzard and ending at the end of the duodenal loop), jejunum (section of tissue from the end of the duodenal loop to Meckel's diverticulum), and ileum (section of tissue from Meckel's diverticulum to the ileal-cecal juntion). All segments were rinsed in ice cold phosphate buffered saline (PBS) and minced with razor blades. Single aliquots of 20 to 30 mg of tissue were placed in microfuge tubes, frozen on dry ice, and stored at -80 °C. Total RNA was extracted from each sample using the Qiagen RNeasy Kit (Qiagen, Valencia, CA) according to the animal tissue protocol using a homogenizer.

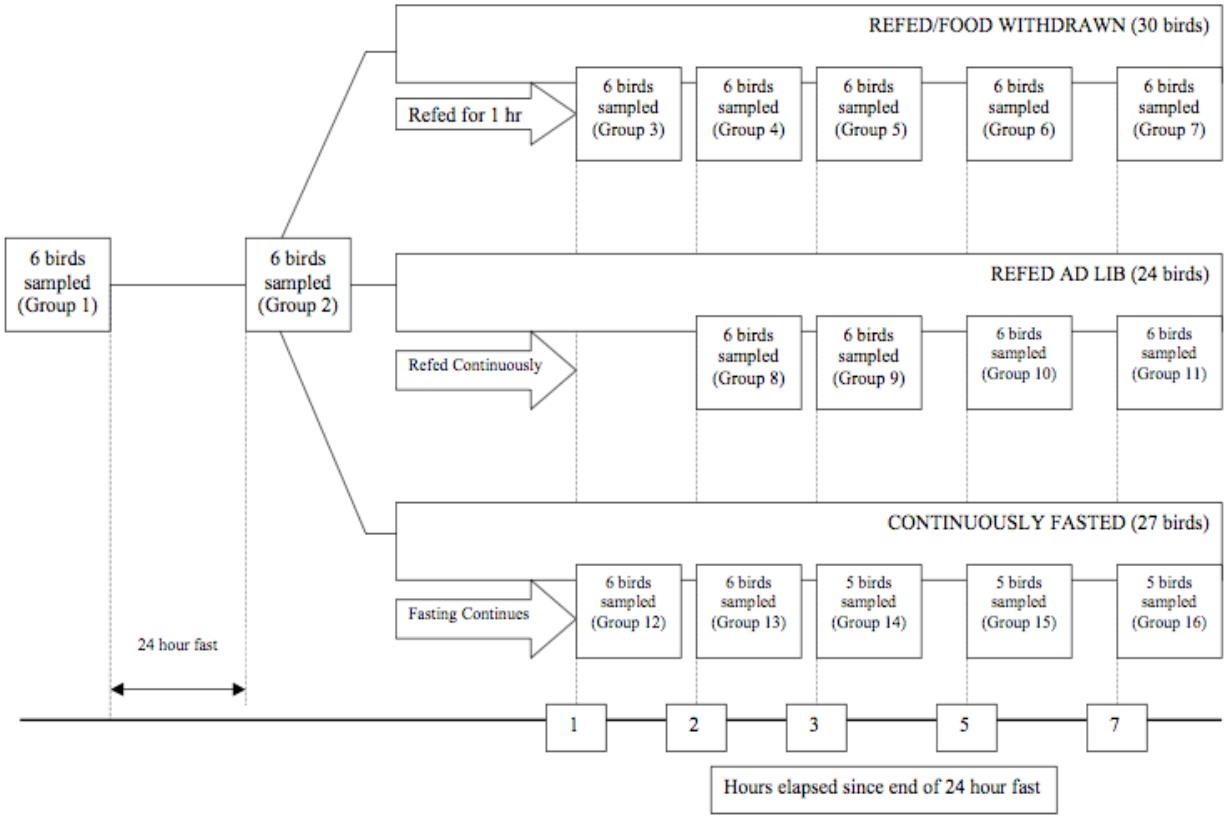


Figure 2-1 Feeding Trial Design. Group 1, consisting of 6 birds, was sampled before the fast for a baseline. The remaining fifteen groups were placed on a 24h fast. After the 24h fast, Group 2, consisting of 6 birds, was sampled in order to determine the effects of fasting on PepT1 and PPARα expression. The remaining fourteen groups of birds were placed on three different feed restrictions that lasted throughout the sampling period. Groups 3 through 7 were refed for 1h. After the 1h refeeding, feed was withdrawn and Group 3 was sampled. Groups 4 through 7 were subsequently sampled 1h, 2h, 4h, and 6h after refeeding. Groups 8 through 11 were refed following the fast and allowed full access to feed throughout the sampling period. Groups 8-11 were sampled 2h, 3h, 5h, and 7h post-refeeding. Groups 12 through 16 were continually fasted throughout the sampling period. These groups were sampled at 1h, 2h, 3h, 5h, and 7h after the end of the initial fast.

Oral Gavage with PPARα Ligand - Leghorns. This feeding trial utilized 19 two-week old White Leghorn layer chicks. The birds were divided into 3 groups and housed in battery cages, with one cage housing 7 birds. The birds were exposed to 24h light and were given free access to food and water throughout the experiment. The 3 groups include the Baseline Group (BL), the +Ligand Group (+L), and the –Ligand Group (-L). The BL group was made up of 6 birds and was sampled on D1 in order to get a baseline expression level for PepT1 and PPARα (Figure 2-2). That same day, the +L (7 birds) and –L (6 birds) groups were gavaged with either 50 mg/kg (based on the average weight of the +L group, which ranged from 0.061 kg on D1 to 0.065 kg on D2) of the PPARα ligand WY-14643 (Cayman Chemical, Ann Arbor, MI) suspended in 1 mL of a 0.5% solution of methyl cellulose or with 1 mL of the carrier alone. This gavage protocol continued once a day for the next 2 days at roughly the same time each day. On D4, the duodenum, jejunum, and ileum were collected from the +L and –L groups. All segments were rinsed in ice cold phosphate buffered saline (PBS), weighed, and minced with razor blades. Whole minced segments were placed in foils, frozen in liquid nitrogen, and then stored at -80 °C. Each sample was manually ground into a fine powder using a mortar and pestle kept cold with liquid nitrogen. Aliquots of 20-30 mg of each sample were used in the RNA extraction process using the Qiagen RNeasy Kit (Qiagen, Valencia, CA).

Sample Baseline/ Gavage	**Gavage**	**Gavage**	**Sample**
D1	**D2**	**D3**	**D4**

Figure 2-2 Gavage Experimental Design – Layers.

Oral Gavage with PPARα Ligand - Broilers. This study was initiated with 30 Aviagen Line B broilers at two weeks of age. The birds were housed in 6 cages in groups of 5 (two treatment groups and one control group). The birds were exposed to 24 h light and given free access to both food and water throughout the study. The treatment groups included those birds receiving either a 50 mg/kg or a 25 mg/kg dose of the PPARα ligand WY-14643 (Cayman Chemical, Ann Arbor, MI) suspended in a 0.5% solution of methyl cellulose. The control group received only the carrier in a dose based on their individual weight. A measurement of baseline expression of PepT1 and PPARα was not performed because no difference was seen between baseline and No Ligand in layers. On D1, each bird was weighed (in kg) and their respective dosage was determined (Figure 2-3). On D1, bird weights ranged from 0.16 kg to 0.23 kg. Each bird was gavaged once with either 50 mg/kg ligand, 25 mg/kg ligand, or just methyl cellulose based on their weight. On D2, the duodenum, jejunum, and ileum were collected from 5 birds from each of the 3 groups. The remaining 15 birds were then gavaged with either 50 mg/kg ligand, 25 mg/kg ligand, or just carrier by weight, with bird weights ranging from 0.19 kg to 0.26 kg. On D3, the remaining 15 birds were sampled following the same procedure as outlined above. Each sample was processed as described for the gavage experiment with layers.

Gavage	**Sample 5 birds/group; Gavage remaining birds**	**Sample remaining birds**
D1	**D2**	**D3**

Figure 2-3 Gavage Experimental Design – Broilers.

Total RNA Extraction

Total RNA was extracted in a similar manner for all three experiments, using the RNeasy Kit according to the animal tissue protocol (Qiagen, Valencia, CA). The aliquots of tissue were removed from -80 °C storage and placed on dry ice until homogenization or manual grinding with a mortar and pestle. With the feeding trial, 600 μL of the RLT solution (2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) diluted 1:100 in buffer RLT (Qiagen, Valencia, CA)) was added to the tissue aliquot prior to homogenization. The sample was then homogenized using a 7 mm tip on a homogenizer (Ultra-Turrax T-25 Basic, Ika, Wilmington, NC) at a speed of 13,500 rpm for 30 s. With both gavaging experiments, the whole segment samples were removed from -80 °C storage, removed from their foil containers, and ground to a fine powder using a mortar and pestle kept cold by liquid nitrogen. Following grinding, 20-30 mg of sample powder was added to a 2 mL tube containing 600 μL of the RLT solution and vortexed for 30 s to ensure proper mixing. Following homogenization or manual grinding, the manufacturer's protocol was then followed and the RNA was eluted by rinsing the column membrane twice with 30 μL of RNase-free water. In the feeding trial, RNA purity and concentration was determined at 260/280 nm using the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Quality of the RNA was examined by gel electrophoresis. Two μg of total RNA were added to 25 μL of RNA sample buffer (0.75 mL deionized formamide (Sigma-Aldrich, St. Louis, MO); 0.15 mL 10X MOPS (200mM MOPS, 50mM NaAc, 10mM EDTA, pH 7.0); 0.24 mL 37% formaldehyde (Sigma-Aldrich, St. Louis, MO); 0.17 mL DEPC water (1 mL Diethylpyrocarbonate (Sigma-Aldrich, St. Louis, MO) per liter ultra-pure water, autoclaved); 0.1 mL glycerol; 8 μL 10% (w/v) bromophenol blue for a final volume of 30 μL. Samples were then incubated in a 65 °C waterbath for 15 min before being separated on a denaturing 1% agarose-formaldehyde gel (1X

MOPS; 1 % (w/v) agarose; 2.2 mol/L formaldehyde). Following gel electrophoresis, the RNA was stored at -80 °C. The two gavaging experiments followed the same RNA extraction protocol as the feeding trial, except RNA purity, concentration, and quality for the broiler gavaging experiment samples were evaluated using the BioAnalyzer (Agilent, Foster City, CA).

Reverse Transcription

All 3 experiments followed the same RT protocol. Complementary DNA (cDNA) was synthesized using the cDNA Archive Kit (Applied Biosystems, Foster City, CA). A 2X reverse transcription master mix was prepared containing, per reaction, 2 μL 10X reverse transcription buffer, 0.8 μL 25X dNTPs, 2 μL 10X random primers, 1 μL Multiscribe reverse transcriptase (50U/μL), and 4.2 μL nuclease free ultra-pure water. Each reaction was conducted in a thin-walled PCR tube and included: 10μL of sample RNA diluted to 0.2 μg/μL and 10 μL of 2X RT master mix. The following reverse transcription reaction was run: 25 °C for 10 min followed by 37 °C for 120 min using a thermocycler. The cDNA was stored at -20 °C.

Quantitative Real-Time PCR

Real-time PCR was performed using an Applied Biosystems 7300 real-time PCR machine using 96-well plates. Complementary DNA was diluted 1:30 in sterile ultra-pure water and 2 μL of diluted cDNA were loaded into each well. Twenty-three microliters of a real-time PCR master mix (per reaction: 12.5 μL 2X SYBR Green Master Mix (Applied Biosystems, Foster City, CA); 0.5 μL of forward primer (5μM) and 0.5 μL of reverse primer (5μM); 9.5 μL sterile ultra-pure water) were added to each well. After loading the plate with samples and master mix, the following PCR reaction was run: 95 °C hold for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The genes analyzed include PepT1 and PPARα. The

endogenous control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used were:

PepT1 forward (5' CCCCTGAGGAGGATCACTGTT 3')

PepT1 reverse (5' CAAAAGAGCAGCAGCAACGA 3')

PPARα forward (5' GGTCCAGGATCTGATGGATCA 3')

PPARα reverse (5' GGAGCTGTGGCAAAATTAATGG 3')

GAPDH forward (5' GCCGTCCTCTCTGGCAAAG 3')

GAPDH reverse (5' TGTAAACCATGTAGTTCA 3')

Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by MWG-BIOTECH, Inc. (Huntsville, AL) Each reaction was run in duplicate.

Real-Time PCR Analysis

The 96-well plates were analyzed using the Auto function of the software program for the 7500 Real-Time PCR machine, which allows analysis of 10 or more plates at a time. Average gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). For all three experiments, the calibrator was the average ΔCt value of all baseline/control duodenum samples.

Statistical Analysis

All data were analyzed using SAS PROC MIXED (SAS Institute, Cary, NC). For the feeding trial, the model included the main effects of time, group, intestinal segment, and all 2- and 3-way interactions. For the gavaging experiment with layers, the model included the main effects of treatment and segment, and all 2- and 3-way interactions. For the gavaging experiment

with broilers, the model included the main effects of age, segment, dose, and all 2- and 3-way interactions. For all experiments, the 3-way interactions were removed from the model as they were determined to be non-significant ($P > 0.05$). Significant effects were further evaluated with Tukey's test for pairwise comparisons.

CHAPTER III.
GENE EXPRESSION OF PEPT1 AND PPARα FOLLOWING A 24 HOUR FAST WITH SUBSEQUENT REFEEDING AND FASTING.

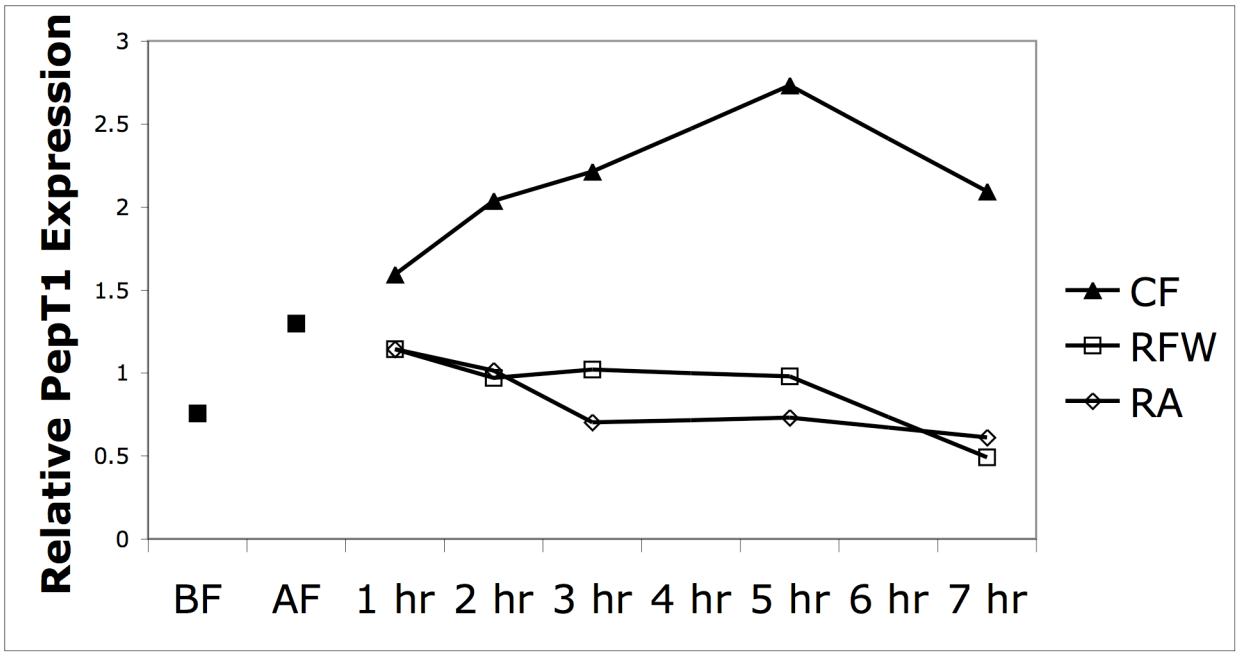
RESULTS

Gene Expression of PepT1 and PPARα

Following the initiation of the 3 feeding groups, a significant time by group interaction was observed for PPARα ($P = 0.002$). The CF group showed numerically higher PPARα levels at all time points (compared to the RFW and RA groups) with a statistically significant peak at 29 h post-fast ($P < 0.0001$) (Figure 3-1). A significant time by group interaction was not observed for PepT1. There was a segment by group interaction for both PepT1 ($P = 0.002$) and PPARα ($P = 0.01$) (Figure 3-2). Within the CF group, PepT1 expression was greatest in the jejunum ($P < 0.001$) and ileum ($P = 0.01$) when compared to the duodenum. No difference was seen in PepT1 expression between jejunum and ileum within the CF group. For PPARα, expression was greatest in the jejunum ($P = 0.03$) when compared to the duodenum, with no difference in expression seen between jejunum and ileum or between duodenum and ileum. Following the initial 24 h fast, both PepT1 ($P = 0.001$) and PPARα ($P < 0.0001$) expression increased almost 2-fold compared to before the fast (Table 3-1); however, there was no difference among the three intestinal segments for either gene, nor was there a segment by time interaction resulting from the initial fast. Following initiation of the 3 feeding groups, a main effect of time was observed for PPARα ($P < 0.001$), but not for PepT1 (Table 3-2). At 5 h post-fast, PPARα expression was higher than at 1 h, 2 h, and 3 h post-fast ($P < 0.03$), but no different than at 7 h. A significant segmental effect was seen for PepT1 ($P = 0.02$). Expression was highest in the jejunum ($P = 0.01$) compared to that in the duodenum, but no difference was seen between the jejunum and ileum or between the duodenum and ileum. A similar main effect of

segment was not observed for PPARα. A main effect of group was found to be significant for both PepT1 ($P < 0.0001$) and PPARα ($P < 0.0001$). The CF group showed the greatest expression (an almost 2-fold increase) for both genes ($P < 0.0001$) when compared to the RFW and RA groups (Figure 3-3).

A.



B.

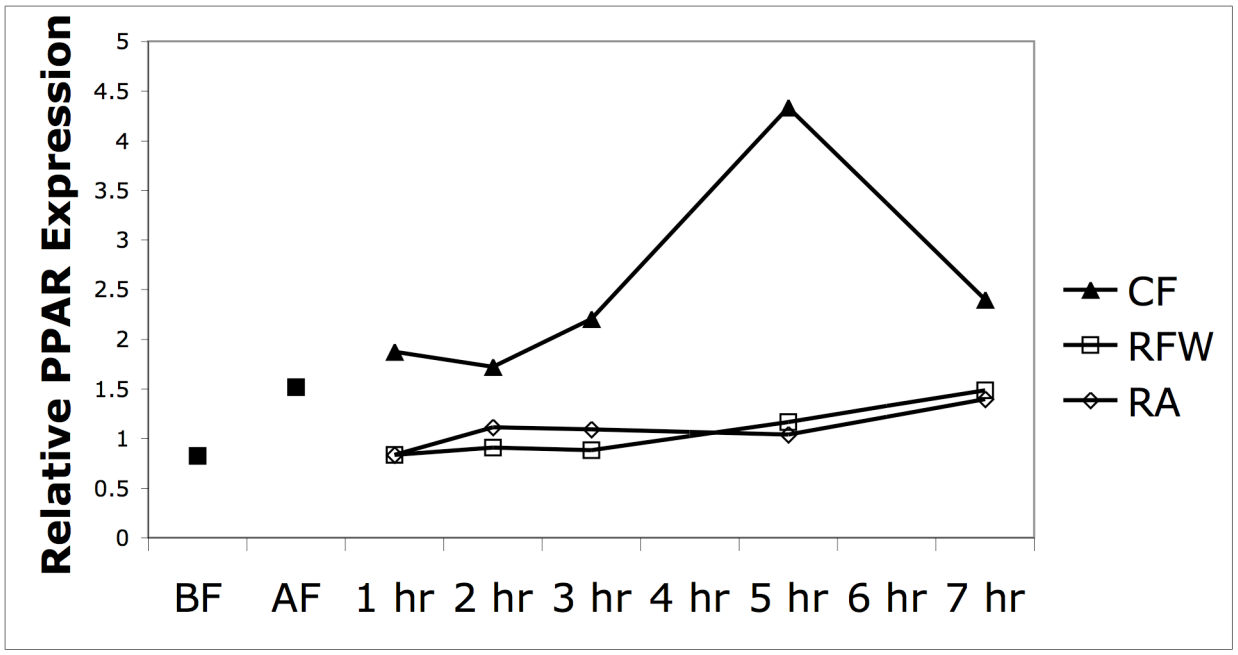
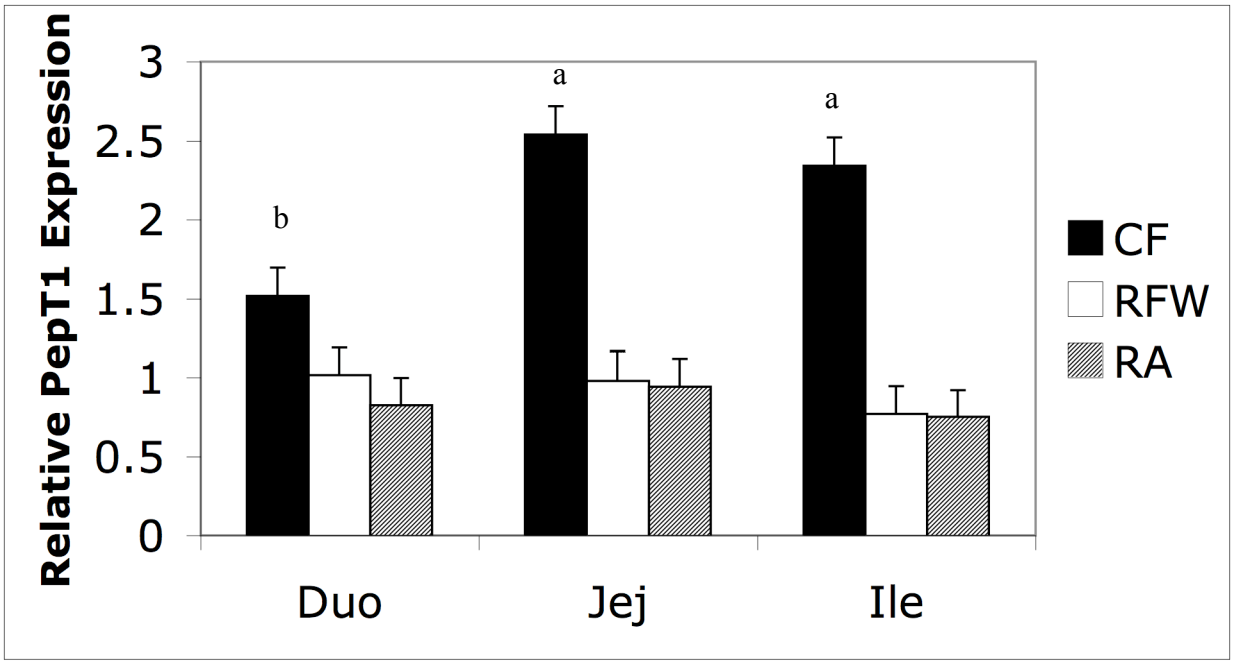


Figure 3-1. Time x Group Interaction for PepT1 (A) and PPARα (B). There was a time by group interaction for PPARα ($P = 0.002$) gene expression in fasted/refed broiler chicks (n = 6) but not for PepT1 ($P = 0.19$). Relative gene expression (2-ΔΔCt) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for Before Fast duodenum as the calibrator. Expression of PPARα in the Continuously Fasted (CF) group peaked at 5 hr ($P = 0.002$). RFW = Refed/Food Withdrawn group, RA = Refed Adlib group, BF = Before 24 h Fast, AF = After 24 h Fast.

A.



B.

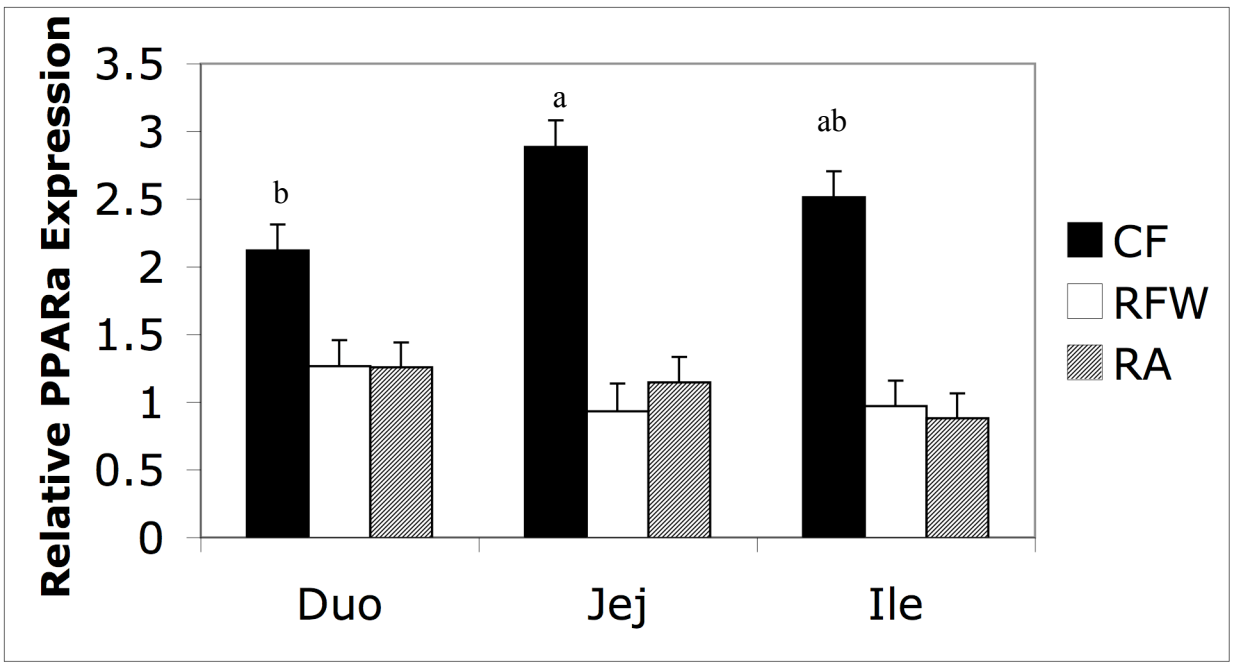


Figure 3-2. Segment x Group Interaction for PepT1 (A) and PPARα (B). There was a segment by group interaction for PepT1 ($P = 0.002$) and PPARα ($P = 0.01$) gene expression in fasted/refed broiler chicks (n = 6). Relative gene expression (2-ΔΔCt) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for Before Fast duodenum as the calibrator. Expression for both genes was highest in the Continuously Fasted (CF) group. RFW=Refed/Food Withdrawn group, RA=Refed Adlib group.

Table 3-1. Relative Expression of PepT1 and PPARα before and after a 24 h fast[1]

	Gene	
Item	PepT1	PPARα
	Relative Gene Expression	
Time		
Before 24 h Fast	0.75[b]	0.82[b]
After 24 h Fast	1.30[a]	1.52[a]
SEM	0.14	0.11
P-value	0.001	<0.0001
Segment		
Duodenum	1.14	1.24
Jejunum	0.99	1.29
Ileum	0.94	0.99
SEM	0.17	0.13
P-value	0.68	0.24
Interaction	Interaction *P*-value	
T x S	0.36	0.17

[a,b] Within a column, means without a common superscript differ.

[1] Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Before Fast duodenum as the calibrator.

Table 3-2. Relative Expression of PepT1 and PPARα following fasting/refeeding[1].

	Gene	
Item	PepT1	PPARα
	Relative Gene Expression	
Segment		
Duodenum	1.11[b]	1.55
Jejunum	1.49[a]	1.66
Ileum	1.29[ab]	1.45
SEM	0.10	0.11
P-value	0.02	0.32
Time Post-fasting		
1 h	1.29	1.18[b]
2 h	1.34	1.25[b]
3 h	1.31	1.39[b]
5 h	1.48	2.18[a]
7 h	1.06	1.76[ab]
SEM	0.16	0.18
P-value	0.50	<0.001
Interaction	Interaction *P*-value	
T x S	0.35	0.77

[a,b] Within a column, means without a common superscript differ.

[1] Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Before Fast duodenum as the calibrator.

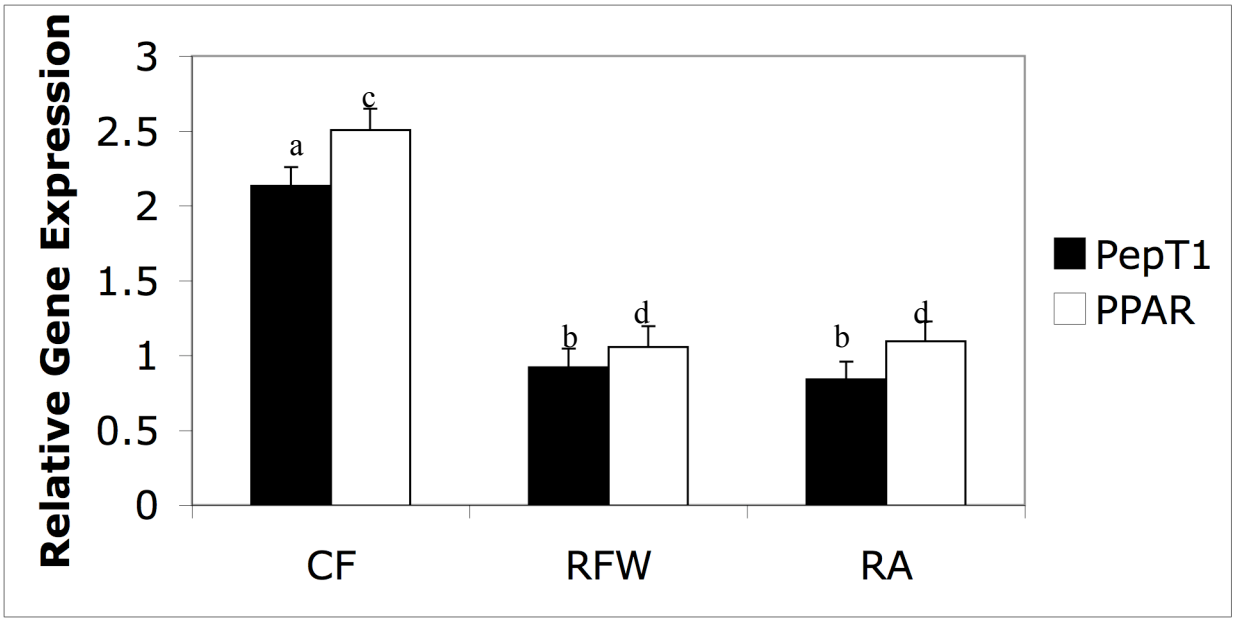


Figure 3-3. Effect of Group on PepT1 and PPARα Expression. There was an effect of group on PepT1 ($P < 0.0001$) and PPARα ($P < 0.0001$) gene expression in fasted/refed broiler chicks (n = 6). Relative gene expression (2-ΔΔCt) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct value for Before Fast duodenum as the calibrator. Gene expression for both genes was greatest in the Continuously Fasted (CF) group when compared to the Refed/Food Withdrawn (RFW) or Refed Adlib (RA) groups.

DISCUSSION

In this study, the expression levels of PepT1 and PPARα mRNA in broiler chicks showed an almost 2-fold increase following a 24 h fast. Expression levels of these two genes remained high within the CF group once the feeding protocol had begun. These findings are in agreement with previous studies that examined the effect of starvation in rats. Thamotharan et al. (1999) found that PepT1 mRNA in rats increased 3-fold after just one day of fasting. Rats subjected to a 4 d fast showed a 179% increase in the expression of PepT1 mRNA (Ihara et al. 2000). Reduced feed intake and total parenteral nutrition (TPN) also affect PepT1 expression levels. Ihara et al. (2000) fed rats at 50% of the intake for controls for 10 d and treated rats with TPN for 10 d and found a 161 and 164% increase in PepT1 expression, respectively. The increase in PepT1 expression during a time of reduced feed intake or starvation suggests the necessity to have ample transporters expressed for when nutrients become available again. However, reduced feed intake and starvation situations do not happen without some change occurring to the mucosal lining of the small intestine, where nutrient absorption occurs. Delayed access to feed for 36 h post-hatch resulted in depressed villus height and decreased crypt depth in addition to decreased growth in all three intestinal segments in chicks (Uni et al. 1998). Silva et al. (2007) fed male broiler chicks at 30% of ad libitum intake from 7 to 14 d and found a decrease in the surface area of the tip of the enterocyte in the small intestine at d 14. In their study where rats were either feed-restricted or TPN-treated, Ihara et al. (2000) reported a reduction in mucosal weight by 41 and 50 %, respectively, despite PepT1 increases of over 160%. Atrophy to the mucosal lining of the small intestine by way of reduced villus height, crypt depth, and overall surface area does not affect PepT1 expression. As these mucosal changes are occurring,

PepT1 expression increases. Though not fully understood, the preservation of PepT1 may help compensate for the loss of functionality of the enterocyte, and thus the mucosa.

Shimakura et al. (2006) concluded that the increases seen in PepT1 expression due to fasting were mediated by PPARα. In PPARα-null mice, the fasting-induced increase in PepT1 expression was abolished; however, in wild-type mice there were significant increases in PepT1 and PPARα expression following a 48 h fast. In this thesis, similar results were obtained following a 24 h fast, suggesting that PepT1 is either directly regulated by PPARα or indirectly regulated via transcription factors that are recruited by PPARα. Shimakura et al. (2006) provided evidence for indirect activation of PepT1 when they demonstrated an increase in the transcription factors SP1 and CDX2 in response to fasting.

Once the feeding protocol began, the CF group had the greatest expression of both PepT1 and PPARα, consistent with previous starvation studies. Within the CF group, expression of both genes was significantly higher in the jejunum compared to ileum and duodenum, with the ileum being numerically higher than the duodenum. Naruhasi et al. (2002) and Howard et al. (2004) demonstrated that starved rats had increased PepT1 expression, with the distal small intestine having the highest levels. By measuring cefadroxil transport, Naruhasi et al. (2002) showed that PepT1 activity levels were highest in the proximal small intestine, despite lower PepT1 gene expression, and activity levels were lowest in the distal small intestine, where gene expression of PepT1 was highest. An increase in PepT1 expression in the mid to distal small intestine compared to duodenum may allow the nitrogen-deficient animal to maximize nitrogen absorption.

An interesting time by group interaction was observed regarding PPARα gene expression in the CF group. Following the initial 24 h fast, PPARα expression remained high (compared to

the RFW and RA groups) in the CF group for the next 7 hours. However, PPARα peaked at 29 h following the initial fast. Why PPARα expression would peak after 29 h of fasting, and not earlier, could be the result of either the depletion of glucose and switch to fat utilization or fasting-related stress. In the case of the former, the carbohydrate stores in the body can supply the necessary energy to allow for normal bodily function for about half a day. Once those stores have been exhausted, the body switches to fat utilization. As fat is increasingly mobilized, the triglycerides that were stored in the adipose tissue are broken down to free fatty acids (FFA) and glycerol. During a starvation situation, FFA increase in the blood in preparation for transport to the liver to be converted to glucose. As stated earlier, FFA have been shown to be natural activators of PPARα. In the case of this thesis, at the 29 h post-fast time point, the peak in PPARα expression could reflect the increase in blood FFA following the natural shift from metabolism of carbohydrates to metabolism of fat. However, a discussion on starvation is not complete without some reference to fasting stress. For any animal, starvation is an incredibly stressful situation that is not without harmful effects. Cortisol, a glucocorticoid, is elevated in times of stress and promotes the mobilization of FFA from adipose tissue (Brindley et al. 1993). This cortisol-mediated shift from glucose to fat utilization requires several hours to become fully functional (Guyton and Hall, 1996) and could explain why PPARα took 29 h to peak following the beginning of the initial fast. Though this peak at 29 h in PPARα expression was significant, a non-significant peak in PepT1 expression was also observed at this time, suggesting that PepT1 was slowly increasing following the induction of PPARα and may have increased more had the experiment been conducted longer.

CHAPTER IV.
EXPRESSION OF PEPT1 AND PPARα FOLLOWING ORAL ADMINISTRATION OF THE SYNTHETIC PPARα LIGAND WY-14643 IN LAYERS AND BROILERS.

RESULTS

Gene Expression of PepT1 and PPARα

The gavage experiment in layers was a preliminary study to assess the effect of administration of WY-14643 on expression of PepT1 and PPARα. Following treatment, there was no difference in PepT1 expression between those birds that received the ligand and those that were treated with only methyl cellulose ($P = 0.18$) (Figure 4-1). However, a significant 6-fold decrease ($P = 0.005$) in PPARα expression was observed in the Ligand group compared to the No Ligand group. The No Ligand group did not differ when compared to Baseline for PepT1 expression ($P = 0.26$) (Figure 4-1). Similarly, PPARα expression in the No Ligand group did not change when compared to Baseline. These results showed that there was no effect of gavaging or exposure to methyl cellulose used as the carrier. No main effect of segment was observed for either gene in layers (Table 4-1), nor were there any significant treatment by segment interactions for either PepT1 or PPARα. However, PepT1 expression showed a general increase in all segments in the Ligand group compared to the No Ligand and Baseline groups (Table 4-2). PPARα expression in all 3 intestinal segments was numerically lower in the Ligand group when compared to the Baseline and No Ligand groups.

Following the preliminary trial in layers, a similar gavage experiment was performed on Aviagen Line B broilers, the same type of bird used in the fasting/refeeding study. Administration of either 25 mg/kg or 50 mg/kg of WY-14643 did not elicit a change in PepT1 or PPARα expression (Table 4-3). While there was no effect of segment seen in the layers, the broilers exhibited a significant segmental effect for PepT1, with expression being highest in the

duodenum ($P = 0.03$), with no difference seen between jejunum and ileum (Figure 4-2). The broilers also had a main effect of segment for PPARα, with expression again being highest in the duodenum ($P = 0.05$) (Figure 4-2), with no difference between jejunum and ileum. Administration of WY-14643 over a 2-day period did not affect PepT1 expression in the broilers; however, PPARα expression was significantly higher in the D3 group compared to the D2 group ($P = 0.01$) (Table 4-3), indicating an effect of age.

Table 4-1 Main effect of segment on relative gene expression of PepT1 and PPARα following administration of the synthetic PPARα ligand, WY-14643, to White Leghorn chicks (n = 6, except for +L Group, where n = 7).

	Gene	
Item	PepT1	PPARα
	Relative Gene Expression	
Segment		
Duodenum	6.62	1.19
Jejunum	7.45	0.74
Ileum	7.22	0.67
SEM	2.18	0.21
P-value	0.96	0.18

Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Baseline duodenum as the calibrator.

Table 4-2 Treatment x Segment interaction for PepT1 and PPARα following administration of the synthetic PPARα, WY-14643, to White Leghorn chicks (n = 6, except for +L Group, where n = 7).

	Gene	
Item	PepT1	PPARα
Segment - Baseline		
Duodenum	1.46	1.28
Jejunum	3.22	1.05
Ileum	1.07	1.29
SEM	3.87	0.37
Segment – No Ligand		
Duodenum	4.81	2.05
Jejunum	6.38	0.97
Ileum	9.71	0.54
SEM	3.87	0.37
Segment – Ligand		
Duodenum	13.59	0.23
Jejunum	12.76	0.20
Ileum	10.90	0.16
SEM	3.58	0.36
Interaction	Interaction *P*-value	
T x S	0.87	0.24

Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the -ΔΔCt method with GAPDH as the endogenous control and the average Ct for Baseline duodenum as the calibrator.

Table 4-3 Relative gene expression of PepT1 and PPARα following administration of the synthetic PPARα ligand, WY-14643, to broiler chicks (n = 5 per sampling).

	Gene	
Item	PepT1	PPARα
	Relative Gene Expression	
Age		
D1	0.85	0.77[b]
D2	0.80	1.00[a]
SEM	0.07	0.06
P-value	0.60	0.01
Dose		
Control	0.84	0.94
25 mg/kg	0.89	0.89
50 mg/kg	0.89	0.83
SEM	0.08	0.08
P-value	0.47	0.62
Interaction	Interaction *P*-value	
S x A	0.90	0.79
A x D	0.80	0.80
S x D	0.80	0.94

Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Control duodenum as the calibrator.
[a,b] Within a column, means without a common superscript differ ($P < 0.01$)

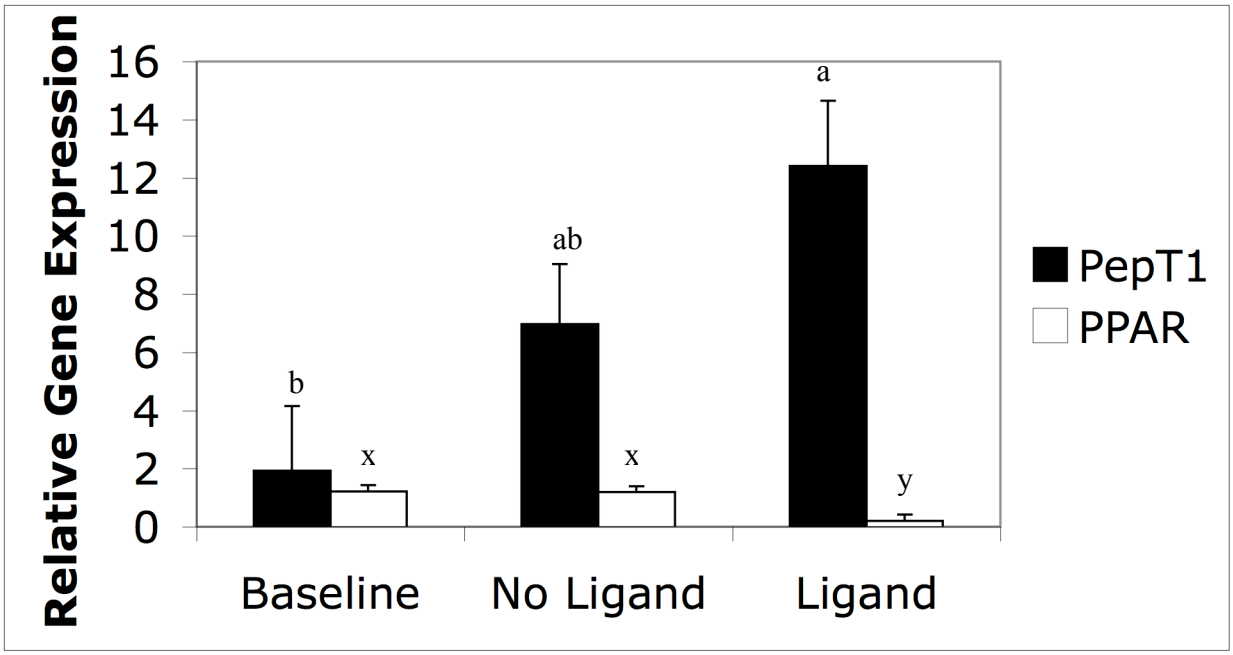


Figure 4-1 Effect of administering the synthetic PPARα ligand, WY-14643, to White Leghorn chicks. There was an effect of ligand on PepT1 and PPARα gene expression (n = 7). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Baseline duodenum as the calibrator. PepT1 expression was highest in the +Ligand group ($P = 0.003$) and PPARα expression was lowest in the +Ligand group ($P = 0.003$) when compared to Baseline.

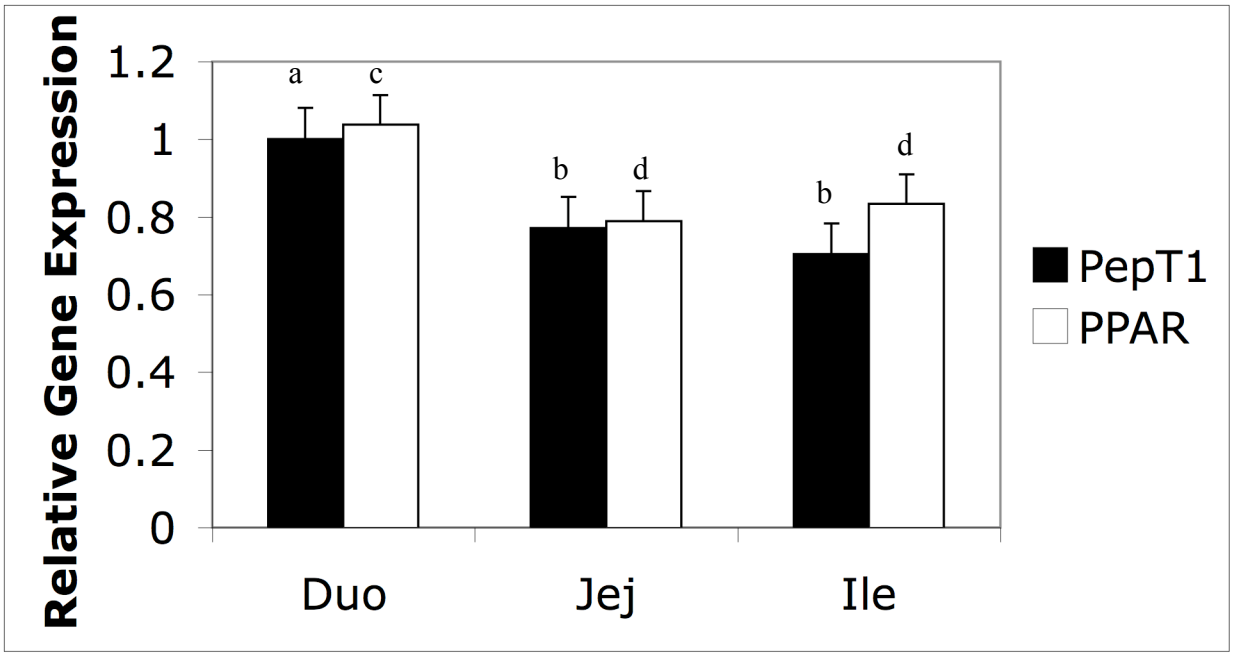


Figure 4-2 Effects of administering the synthetic PPARα ligand, WY-14643, to broiler chicks. There was a main effect of intestinal segment on gene expression of PepT1 and PPARα (n = 5). Relative gene expression ($2^{-\Delta\Delta Ct}$) ± SEM was calculated using the ΔΔCt method with GAPDH as the endogenous control and the average Ct for Control duodenum as the calibrator. Expression of PepT1 and PPARα was highest in the duodenum (P = 0.03 and 0.05, respectively).

DISCUSSION

In this thesis, the effects of WY-14643 on PepT1 and PPARα were determined in layers as part of a preliminary study. Following the initial gavage experiment, the protocol was adjusted for a second experiment in broilers, the same type of bird used in the fasting/refeeding study. In these oral gavage experiments, a few differences were observed between layers and broilers. The most prominent difference was the effect of WY-14643 on PPARα gene expression. Shimakura et al. (2006) showed an increase in PepT1 following WY-14643 gavage (50 mg/kg) in rats. However, they did not report the effects of WY-14643 gavage on PPARα expression. In this thesis, ligand gavage in layers resulted in a 6-fold decrease in PPARα when compared to birds treated with methyl cellulose. This could be a result of saturating the system, and thus creating negative feedback on factors that induce PPARα expression.

With the gavage experiment in broilers, treatment with the ligand did not produce similar results as the layers. Broilers showed no change in PepT1 and PPARα following gavage, regardless of dosage. Similar results were found in a recent study investigating the effects of diets containing 0.1% WY-14643 or 0.01% GW-7647 (a newly developed PPARα agonist) on the regulation of multiple nutrient transporters in the mouse small intestine (Toshitake et al. 2007). By using a genome-wide array approach, they found multiple transporters that were upregulated following treatment with either PPARα ligand; however, PepT1 was not included in that group. The authors concluded that the results obtained by Shimakura et al. (2006) were due to the exceedingly high dose of WY-14643 used to treat rats and human Caco-2 cells. In this thesis, a 25 mg/kg or 50 mg/kg (similar to the Shimakura paper) dose was used to treat the broilers. This was different from the study in layers, where only 50 mg/kg was used. By

including an intermediate dosage of 25 mg/kg, it was thought that a gradual decrease in PPARα expression would be seen in the broilers. However, no change in PPARα expression or PepT1 expression occurred. The lack of response regarding PepT1 and PPARα expression could be due to the high dosages used, possibly causing a negative feedback loop similar to what was seen in the layers. Broilers and layers are such different birds that a putative mechanism involving the regulation of PepT1 by PPARα present in layers may not be inducible in broilers. Such a mechanism in layers could be very important to a bird that has a high feed:gain ratio and that directs a majority of its nutrients towards egg production. However, broilers have been selected for fast growth and high body weights. Because they are so efficient at converting nutrients into body weight gain, it is possible that they are operating at peak nutrient transporter expression levels. These characteristics in broilers could make it difficult to detect any changes in expression.

In order for PPARα to have a role in regulating PepT1, there should be a binding site on the PepT1 gene. The mammalian consensus PPARα binding site has been identified as having the sequence 5' AGG(A/T)CAXAGG(A/T)CA 3' (Kliewer et al. 1992). A search of the chicken PepT1 promoter revealed a potential PPARα binding site (5' AAGTCAGAAGTCA 3') between base-pairs -1718 and -1731. Differences between broilers and layers may lie in their respective genomes or may also arise through modifications that occur during transcription or translation. Such genomic variations and post-transcriptional modifications may be why the two types of birds reacted differently to WY-14643 treatment.

During the gavage trial in layers, no difference was seen in PepT1 or PPARα between the Baseline and No Ligand groups, indicating that there was no effect of gavaging or methyl cellulose on expression. No such effect was seen in broilers either; however, baseline expression

levels for PepT1 and PPARα were not measured in those birds. The timing of both experiments was different, with the layers receiving the treatment for 3 days with sampling on the fourth day. The broilers were treated for the same number of days as the layers, but sampling occurred on D2 and D3. In essence, the layers had a full 24 h longer to react to the last gavage, whereas the broilers did not. If a PPARα mechanism is present in broilers, perhaps that extra time is needed in order for PPARα to decrease as it did in the layers.

CHAPTER V. EPILOGUE

My results have confirmed that PepT1 and PPARα gene expression is induced during a period of continuous fasting in broiler chickens. Refeeding, whether for 1-h or adlib, causes a downregulation in both genes. The coordinate increase in expression of both genes during fasting, or the coordinate decrease after refeeding, is just one more piece of evidence that PepT1 induction is somehow tied to PPARα during a fasting situation. The presence of a direct connection between the two genes cannot be proven based on these results; however, whether it is a direct or an indirect connection, a common trend between the two can be seen. One of the most interesting results of this experiment was the significant peak in PPARα expression in the CF group at 5 h post fast. It is interesting because, at that point, the birds had been fasting for 29 h total. So why the sudden peak in PPARα? Though not significant, PepT1 expression in the CF group followed the same trend as PPARα, with a slight peak at 5 h post fast. Again, the two genes were following a similar trend. To see if the trend observed here is real, it would be interesting to repeat the fasting trial, but carry it out to 48 h or longer so that expression of PepT1 and PPARα could be observed during a prolonged period of starvation. If the peak at 5 h is present in the longer trial, that would suggest that something biologically important is happening at that time, such as my earlier discussion of lipolysis. Carrying the study out to 48 h would also provide the chance to see if PepT1 and PPARα levels stay high, instead of dropping off at 7 h like they did in this study.

The results of the two gavage experiments demonstrate a difference between layers and broilers in terms of activation of PPARα, thus PepT1, via a synthetic ligand. In layers, a difference (though not significant) was seen between the Ligand and No Ligand groups

regarding PepT1 expression, with expression being numerically higher in the birds treated with WY-14643. Between the Ligand and Baseline groups, PepT1 was significantly higher in the Ligand group while PPARα was depressed. This result is at odds with the trend I saw earlier in the fasting/refeeding trial. Because PepT1 was also increased in the No Ligand group (compared to Baseline), I attribute this divergence in expression levels to a combination of saturating the system (hence the decreased PPARα levels). A similar result was not seen in the broilers because baseline expression levels were not measured.

Layers and broilers are two very different birds and further investigation into those differences is warranted. For instance, a comparative study looking at nutrient transporter expression in both types of birds under both normal feeding conditions and fasting conditions would be both interesting and enlightening. Looking at different dosages could be helpful. A dose of 50 mg/kg seemed to have an effect in layers, but it caused PPARα to decrease, so maybe it was too large of a dose. The same dose had no effect in broilers, which could imply one of two things: either the dose was not large enough for the broilers, or broilers have adapted to their maximum transporter expression levels so much that they no longer have a need for the nutritional state sensor afforded by PPARα. Another study could utilize a different ligand, for there are other synthetic PPARα ligands available. Perhaps WY-14643 was not the best choice and maybe there is a better, more species-specific ligand available.

In conclusion, I think this thesis reinforced prior studies regarding PepT1 and PPARα expression following fasting in chickens and answered questions regarding the effects of ligand gavage, something that had not been done in chickens before.

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