DIPEPTIDE TRANSPORT IN YUCATAN MINIATURE PIGLET INTESTINE

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Dipeptide transport in *Yucatan miniature* piglet intestine

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Abstract

The PepT1 transporter is getting much attention in modern nutrition due to its universal substrate affinity for di/tripeptides which facilitates efficient transport of amino acids. As such, di/tripeptides may be an important component of amino acid nutrition because they are transported into enterocytes more efficiently than a mixture of free amino acids. However, limited data are available on the contribution of PepT1 to total peptide uptake. We studied the contribution of PepT1-mediated transport versus paracellular movement to total dipeptide uptake in intestinal samples excised from suckling piglets. Using an *in vitro* Ussing chamber model, we determined that PepT1 was responsible for 46% of the glycyl-sarcosine uptake by piglet jejunum and 73% of the glycyl-sarcosine uptake by piglet ileum; these values are lower than previously reported in cell culture models, suggesting that paracellular peptide uptake is quantitatively important in young piglets.

PepT1 and amino acid transport systems both contribute to amino acid uptake by enterocytes, but the contribution of the different routes to overall amino acid absorption has not yet been defined. Furthermore, very little is known about the interaction between free amino acid and peptide uptake at the cellular level. Using an *in vivo* gut loop perfusion model in piglets, we demonstrated that arginine uptake was enhanced by 81% when perfused simultaneously with 20 mM lysyl-lysine, compared to control. In contrast, perfusing loops with equimolar lysyl-glycine did not alter arginine uptake. We speculated that enhanced uptake of arginine was likely due to trans-stimulation of rBAT/$b^{0,+}$ transporter. Dipeptides are taken up by enterocytes via PepT1 and are then

hydrolyzed to release free lysine. High intracellular free lysine trans-stimulates the rBAT/$b^{0,+}$ anti-transporter to enhance arginine uptake. When lysyl-lysine was perfused with an amino peptidase inhibitor (amastatin), the potentiating effect was abolished, suggesting that this trans-stimulation activity was impeded by reducing intracellular hydrolysis of dipeptides. To the best of my knowledge we are the first to demonstrate the interaction between arginine absorption and lysine-containing dipeptides at the cellular level in an *in situ* model.

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Abbreviations

AA- amino acid

AM- amastatin

Arg- arginine

Gly- glycine

LG- lysyl-glycine

LL- lysyl-lysine

Lys- lysine

PepT1- peptide transporter 1

rBAT- basic amino acid transporter

1. Introduction

1.1. Protein digestion overview

Dietary proteins play a critical role for maintaining optimal health. In the period from birth to weaning, dietary protein requirements are high in order to support both maintenance and growth of the individual (Dupont, 2003). According to the World Food and Agricultural Organization, the protein requirement for infants is 2.4 g/kg/d (WHO, 2007). Not only is quantity important, but also the quality of dietary protein is important as it is the only source of essential amino acids, which are crucial for protein synthesis and body homeostasis.

Degradation of dietary proteins begins in the stomach and ends at the small intestinal epithelium (Matthews, 1972). The acidic environment in the stomach denatures the proteins and promotes proteolysis by pepsin. The polypeptides arising from partial protein hydrolysis enter into the small intestine. Thereafter, pancreatic peptidases hydrolyze these polypeptides into small chain oligopeptides (4-6 amino acids) and free amino acids (Freeman *et al.*, 1979). The degradation of dietary protein subsequently continues by the action of hydrolytic enzymes, called brush border membrane peptidases, on the apical surface of the intestinal epithelium. These peptidases split the small chain oligopeptides into di and tripeptides (2 or 3 amino acids) or free amino acids (Caspary, 1992). Analysis of luminal contents after the administration of a protein-containing meal has demonstrated that the end products of protein digestion are mixture of free amino acids and di and tripeptides (Adibi &

Mercer, 1973). These di and tripeptides are taken up by the enterocytes via the hydrogen ion (H^+)-dependent peptide transporter 1 (PepT1), while free amino acids are transported into the cell via a number of different amino acid transporter systems that are present on the apical surface of the enterocytes (Adibi, 1976; Ganapathy & Leibach, 1996).

1.2. Amino acid absorption

The small intestine is the major site of amino acid absorption. There are three major regions in the intestine; proximally to distally, the intestine is divided as duodenum, jejunum and ileum. In addition, the wall of the intestine also consists of three layers of tissues: inner tunica mucosa, middle tunica muscularis and outer tunica serosa (Harrison *et al*, 2004). The inner mucosal layer is folded into finger like projections called villi which are lined by columnar epithelial cells or enterocytes. The apical surface of the enterocytes is organized as microvilli. This structure creates the brush border membrane, which is the active site of free amino acids and di and tripeptide absorption (Barrett, 2006). Both the villi and microvilli structures facilitate absorption by increasing the overall absorptive surface area of the small intestine. Amino acid transporters are present on the apical surface of the small intestine along its entire length, from the duodenum to the ileum; however, the jejunum has the highest amino acid transporter density as reported in the pig, chicken and human (Bröer, 2008).

Free amino acid transport is very complex because of the existence of multiple amino acid carriers with overlapping substrate specificity. For this reason, free amino acid transport activity is commonly referred to as a transport system (Bröer, 2008).

These transport systems differ by the type of substrate, as well as the dependency on Na^+ or Cl^- ions (Dave, 2004; Terada & Inui, 2004). There are five different amino acid transport systems in the brush border membrane: 1) low affinity neutral amino acid transporter system, 2) anionic amino acid transporter system specific for aspartate and glutamate,3) imino acid transporter system for proline and hydroxyproline, 4) transporter system for β-amino acids and anionic amino acids and 5) cationic amino acid transporter system (reviewed by Bröer, 2008).

1.2.1. Neutral amino acid transporter (B^0 amino acid transporter)

The transport of neutral amino acids into enterocytes occurs mainly via the Na^+ - dependent B^0 system. This system is capable of transporting a broad spectrum of neutral and basic amino acids, non-polar amino acids, methionine, glycine, isoleucine and leucine, and aromatic amino acids as well as some metabolites like creatine (Sloan & Mager, 1999; Nakanishi *et al.,* 2001). Mutation of the B^0 system can cause Hartnup's disease, which is characterized by hyperaminoaciduria with photosensitive skin rash, ataxia, and psychotic behavior (Broer *et al.*, 2004).

1.2.2. Anionic amino acid transporter system (X^-_{AG})

The anionic amino acids aspartate and glutamate are taken up by enterocytes via the anionic amino acid transporter system X^-_{AG} (Maenz *et al.,* 1993). System X^-_{AG} is strictly Na^+ dependent (Munck & Munck, 1999) and it is described to be coupled with the inwardly directed electrochemical potential gradients of Na^+ and H^+, and with the outwardly directed gradient of K^+ (Kanai & Hediger, 1992).

1.2.3. Imino acid transport system

The imino acids, proline and hydroxyproline, appear to be transported via Na^+ dependent transport activity (Medow *et al.*, 1986; Stevens & Wright, 1987). Munck (1966) reported two carriers involved in uptake of imino acids into enterocytes. The imino acid system transports the amino acids proline, glycine and sarcosine (Stevens & Wright, 1985). The B^0 system also shares its transporter capacity with imino acids (Munck, 1966). Additionally, the H^+ coupled amino acid transporter 1 (PAT1) has been identified as another imino acid carrier which also shares its transport activity with glycine (Anderson *et al.,* 2004).

1.2.4. β-amino acid transporter system

Taurine and β-alanine are subject to transport via ß-amino acid transport (Liu *et al.,* 1992; Munck & Munck, 1995b). There are two major transporters that have been identified as β-amino acid transporter systems. One is a high affinity Na^+ and Cl^- dependent transporter (Miyamoto *et al.,* 1990); the other is an H^+ dependent low affinity transporter which is shared by β-alanine, taurine, GABA, proline and glycine (Munck & Munck, 1995b).

1.2.5. Cationic amino acid transporter system (rBAT/$b^{0,+}$AT)

Cationic amino acids are taken up into enterocytes by an amino acid transporter system called system rBAT/$b^{0,+}$AT (Palacín, 1994). This cationic amino acid transport system facilitates the Na^+ independent transport of the cationic amino acids arginine, lysine, ornithine and the neutral amino acid cystine (Munck & Munck, 1997).

rBAT/$b^{0,+}$AT consists of two subunits. The heavy subunit rBAT is a type II membrane N-glycoprotein and it belongs to a solute carrier protein (Palacín *et al.,* 2005). rBAT is positioned extracellularly, attaching to the plasma membrane through a single trans-membrane domain (Kanai & Endou, 2001). The light subunit, $b^{0,+}$AT is a highly hydrophobic membrane protein with 12 trans-membrane domains (Palacin *et al.,* 2001). Heavy rBAT and light $b^{0,+}$ subunits are connected by a disulphide bond (Gasol *et al.*, 2004). The rBAT protein is unstable and rapidly degraded when it exists alone (Bauch & Verrey, 2002); on the other hand, the $b^{0,+}$AT subunit is able to function even if expressed alone (Reig *et al.,* 2002). Hagihira *et al.* (1961) have demonstrated mutual inhibition among arginine, cystine, lysine, and ornithine which indicates they share the same transport mechanism (Hagihira *et al.,* 1961). rBAT/$b^{0,+}$AT transports cystine and cationic amino acids with Km values of ~100 μM (Palacín *et al.,* 2005). Stieeger *et al.,* conducted a study using rat renal brush border membrane vesicles, showing inhibition of cationic amino acid transport with high concentrations of phenylalanine and methionine (Stieger *et al.,* 1983). However rBAT/$b^{0,+}$AT has a higher affinity to cationic amino acids than neutral amino acids (Palacin, 1994). Thus, under normal physiological conditions, rBAT/$b^{0,+}$AT transports only cationic amino acids (Palacín *et al.* 2001). An experiment conducted in oocytes using radio-labeled amino acids has shown that rBAT/$b^{0,+}$AT is an obligatory antiporter (Chillaron *et al.,*1996) and as such, it has intracellular and extracellular binding sites. As well, the K_m value for extracellular binding sites is higher compared to intracellular binding sites (Busch *et al.,* 1994). An *in vitro* study conducted in human intestinal epithelial Caco-2 cells demonstrated that 85%

of arginine uptake into the cell occurred via rBAT/$b^{0,+}$AT (Wenzel *et al.,* 2001). A similar kind of study demonstrated that rBAT/$b^{0,+}$AT was responsible for 47% of lysine uptake across the apical surface of human intestinal epithelial Caco-2 cells (Thwaites *et al.,* 1996). Mutation in the cationic amino acid transporter in the intestine and kidney can cause cystinuria (Rajan *et al.,* 1999). Cystinuria is characterized by the inadequate reabsorption of cystine in the proximal convoluted tubules after the filtering of the amino acids by the kidney's glomeruli, resulting in an excessive concentration of this amino acid in the urine which leads to crystals or stones developing in the ureters or bladder (Fjellstedt *et al.,* 2003). Furthermore, malfunction of rBAT/$b^{0,+}$AT can lead to arginine and lysine deficiencies (de Sanctis *et al.,* 2001). Thus, rBAT/$b^{0,+}$AT plays an important role in cationic amino acid uptake.

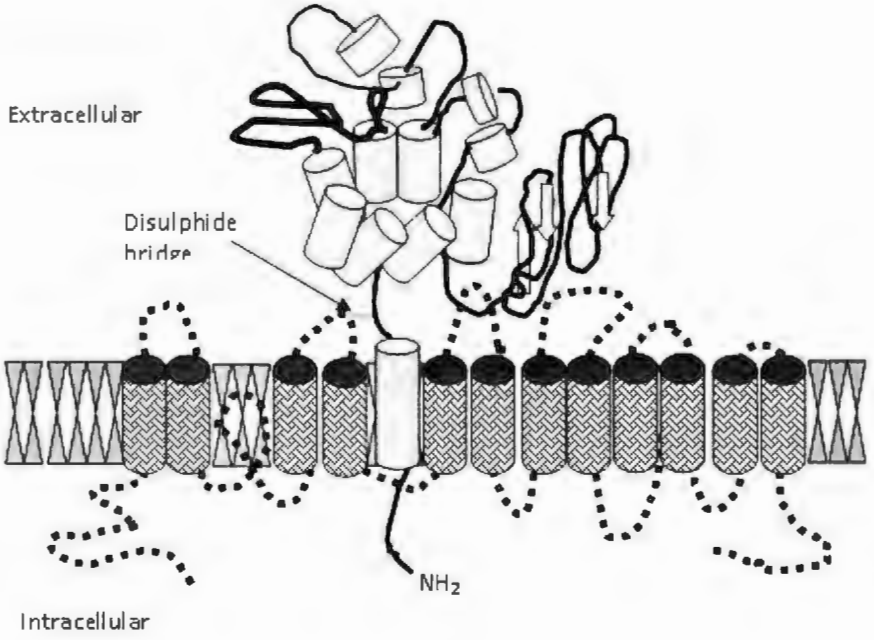


Figure 1-1 Schematic diagram of rBAT/$b^{0,+}$ amino acid transporter. The heavy subunit rBAT is type II membrane glycoproteins with a single trans membrane domain, and the other light subunit $b^{0,+}$, has 12 trans membrane domains, with the NH_2 and COOH termini located intracellularly.

Adapted from Palacin *et al.,* (2005).

1.3. Nutritional importance of small chain oligopeptide absorption

A classic study conducted in human volunteers demonstrated that after a heavy protein meal, a major portion of amino acids were present as small peptides in the gut lumen as opposed to free amino acids (Adibi & Mercer, 1973). This suggests a role for small chain oligopeptides in amino acid uptake. Another human study demonstrated that the transport of amino acids as small chain oligopeptides was more efficient than the transport of the constituent free L-amino acids (Adibi, 1971). Later, similar observations were reported in studies conducted in pigs (Rerat *et al.,* 1992) and rats (Hara *et al.,* 1984). Further evidence of the importance of small chain oligopeptide absorption is their effectiveness as an alternative route to supply specific essential amino acids to the individuals suffering from genetic mutations of free amino acid transporters (Adibi, 1997).

1.4. Transepithelial transport of small chain oligopeptides

Transepithelial transport of small chain oligopeptides (di/tripeptides) occurs through three major pathways: 1) PepT1 mediated transport (Adibi, 1997), 2) passive paracellular movement (McCollum & Webb, 1998) and 3) by cell-penetrating peptides, which act as cargo carriers to take small chain oligopeptides to the cell interior (Sebbage, 2009). However, the majority of studies on transepithelial transport of di and tripeptides have focused on PepT1 mediated trans-cellular transport and passive paracellular movement.

1.5. Passive paracellular movement in di/tripeptides transport

Transepithelial movement of di and tripeptides between epithelial cells is possible via passive paracellular movement. Access to the paracellular route is considered to be quite limited, as it constitutes only 1/10 000 of the total intestinal absorptive surface area (Zhou *et al.,* 1999). Paracellular movement is driven by a concentration gradient, but the size and charge of tight junctions that are present between the epithelial cells also play a part in the regulation of passive paracellular movement (Karczewski & Groot, 2000). A study conducted in sheep reported a high amount of carnosine (beta-alanyl-L-histidine) paracellular movement in omasal epithelial tissue (Matthews & Webb, 1995), even though PepT1 transporter was present in that epithelial tissue (Chen *et al.,* 1999). A similar study conducted in sparrow intestine showed greater paracellular movement of serine-lysine and serine-aspartate compared to receptor mediated transport (Chediack *et al.,* 2006). Another study carried out using human intestinal epithelial Caco-2 cell layers reported passive paracellular movement as the major transepithelial route of transport for the valine-valine diastereomers, L-valine-D-valine, D-valine-L-valine and D-valine-D-valine (Tamura *et al.,* 1996a) although the D-isomer dipeptides are also transported by PepT1(Lister *et al.,* 1995). Tamura *et al.* (1996b) also reported that the major transepithelial transport route for valine-valine-valine stereoisomers is the paracellular route (Tamura *et al.,* 1996b). Furthermore, a study conducted in human intestinal epithelial Caco-2 cell monolayers suggested paracellular movement was the main mechanism for the transport of intact valine-proline-proline across the Caco-2 cell monolayer (Satake *et al.,* 2002). All those

studies listed above have shown higher contribution of paracellular movement in dipeptide transport over PepT1 mediated transport. However some studies have demonstrated higher contribution of PepT1 mediated transport in dipeptide transport over paracellular movements. A study conducted in Caco-2 cell monolayers, which inhibited the PepT1 activity by incubating at 4°C, reported that ~80% of dipeptide transport was via the receptor mediated pathway while ~20% of transport was via passive movement (Scow *et al.,* 2011). Similarly, Chen *et al.,* have reported 70% lower glycyl-sarcosine uptake in PepT1 knockout mice compared to wild type, which suggests that 30% of dipeptide uptake was non-receptor mediated dipeptide transport across the intestine (Chen *et al.,* 2010). A similar observation was reported in another PepT1 knockout mouse study, in which 22% of glycyl-sarcosine uptake was via paracellular movement, and 78% was PepT1 mediated transport (Nässl *et al.,* 2011). Thus, paracellular movement may play an important role in transepithelial transport of di/tripeptides as it contributed to at least 20% of transepithelial transport of dipeptides in the studies above. However PepT1 mediated transcellular transport appears to be the major route of di/tripeptide transport system across the intestinal epithelium in the various models studied.

1.6. Intestinal peptide transporter (PepT1)

Transcellular transport of di/tripeptides occurs through the intestinal peptide transporter (PepT1). PepT1 plays a major role in efficient absorption of dietary protein (Thamotharan *et al.,* 1999). A study conducted in isolated rabbit intestinal brush border

membrane vesicles demonstrated that PepT1 is a H^+-coupled, energy-dependent, Na^+ independent transporter (Terada & Inui, 2004).

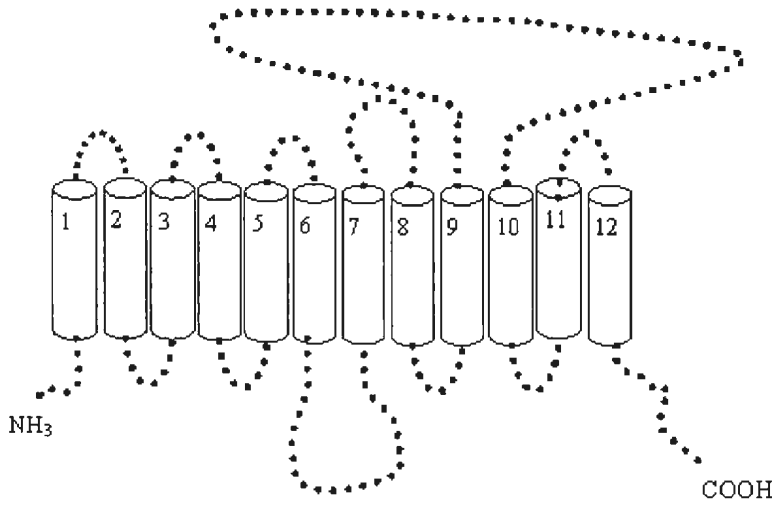


Figure 1-2 Schematic diagram of intestinal peptide transporter (PepT1). It has 12 trans-membrane domains with both amino and carboxyl termini facing the cytoplasmic side.

Adapted from Fei *et al.*, (1994)

1.6.1. PepT1 structure

The PepT1 transporter protein belongs to solute carrier family 15 (SLC15A1) and is encoded by the SLC15A1 gene (Daniel, 2004a). PepT1 mRNA was first isolated from the rabbit and cloned in Xenopus oocytes (Fei *et al.,* 1994). The structure of the PepT1 transporter has been predicted using hydropathy analysis, and the predicted structure has 12 trans-membrane domains with both amino and carboxyl termini facing the cytoplasm side (Figure 1-2) (Urtti *et al.,* 2001; Daniel, 2004a). In terms of energetic efficiency, PepT1 can transport 2 or 3 amino acids into the cell using the same energy expenditure required to transport a single free amino acid via an amino acid transporter (Daniel, 2004).

1.6.2. Ion dependency

An early study that was conducted using brush border membrane vesicles showed that dipeptide transport via PepT1 was Na^+ dependent (Sigrist-Nelson, 1975). Later Ganapathy *et al.,* (1981) reported that dipeptide uptake into brush border membrane vesicles was a Na^+ independent mechanism. Similarly Fei *et al.* (1994) and Pan *et al.* (2001) using rabbit PepT1 cloned Xenopus oocytes, Watanabe *et al.* (2005) using PepT1 expressed in HEK293 cells and Ma *et al.* (2011) using everted gut sacs from mice have confirmed the Na^+ independent activity of PepT1. Further work on the transporter in rabbit brush border membrane vesicles has demonstrated that dipeptides are co-transported into enterocytes along with H^+ ions (Ganapathy & Leibach, 1983).

Di/tripeptide transport through PepT1 causes an inward current as a result of a net transfer of positively charged ions across the membrane during peptide transport (Daniel, 1996). Ganapathy & Leibach (1985) demonstrated enhanced dipeptide uptake with interior-negative membrane potential and inhibited uptake with an interior-positive membrane potential. The authors also suggested that the inward proton gradient in the mammalian small intestine acts as an energy source for the uphill transport of peptides via PepT1 (Ganapathy & Leibach, 1985). A study conducted in guinea pig intestinal epithelial cell layers also has shown enhanced dipeptide uptake when the apical pH is more acidic than intracellular pH (Hayashi & Suzuki, 1998). As a whole, an acidic microenvironment surrounding the apical surface of the enterocytes acts as the driving force for the PepT1 transporter (Daniel & Kottra, 2004b). The combination of a Na^+-H^+ exchanger in the brush-border membrane and a Na^+-K^+-ATPase on the basolateral membrane is likely responsible for the maintenance of the proton gradient in the intestine (Ganapathy & Leibach, 1985; Adibi, 2003).

1.6.3. PepT1 distribution

The PepT1 transporter is primarily expressed in intestinal epithelial cells (Daniel & Kottra, 2004). Within the intestine, PepT1 mRNA expression has been predominantly detected in the small intestine, with different rates of expression corresponding to different regions. Freeman (1995) reported PepT1 mRNA expression in rabbits was highest in the duodenum and decreased along the length of the intestine towards the ileum. In humans, PepT1 transporters are also present in the colon but with lower

expression levels compared to small intestine (Ford *et al.,* 2003). PepT1 has been identified in a variety of species including humans, mice (Groneberg *et al.* 2001), pigs, chickens, sheep, dairy cows (Chen et al. 1999), rats, guinea pigs, rabbits (Adibi, 2003) and black bears (Gilbert *et al.,* 2007). Interestingly, the amount of PepT1 expressed varies within regions of the intestine amongst different species. In chickens, pig, and ruminants, the highest PepT1 mRNA was detected in the duodenum, jejunum, and ileum respectively (Chen *et al.,* 1999). A previous study conducted in our lab demonstrated high PepT1 mRNA expression levels in proximal compared to distal intestine in suckled Yucatan miniature piglets; however, the pattern shifted after weaning, with the greatest expression identified in the distal small intestine (Nosworthy *et al.*, 2012). In contrast, in rats, PepT1 protein expression was greatest in the ileum as compared to jejunum (Tanaka *et al.,* 1998). Gilbert *et al.* (2007) reported PepT1 mRNA levels were high in the mid-region of the black bear small intestine. Apart from the small intestine, PepT1 transporter has also been identified in the omasum and rumen of ruminants (Chen *et al.,* 1999) and in the caecum of the chicken (Chen *et al.,* 1999). Immunohistochemical analyses have shown that PepT1 protein is localized to the brush border membrane of cells lining the villi tips (Ogihara *et al.,* 1999). Localization of PepT1 to the apical side of the enterocyte has been described as a developmental change. A study carried out using mice showed that PepT1 protein at fetal d 18 and at birth was located in the sub-apical cytoplasm, basal cytoplasm, and basolateral membrane and later localized to apical surface of enterocyte (Hussain *et al.,* 2002). In summary, PepT1 distribution

varies along the intestinal length amongst various species and also it varies by developmental stage within a species.

1.6.4. PepT1 transport affinity and substrate specificity

PepT1 has the potential to transport over 400 dipeptides and 8000 tripeptides (Boudry *et al.,* 2010). Daniel (2004a) reviewed this vast substrate specificity of PepT1, and suggested that it is facilitated by a water molecule. She suggested that a water molecule shields the electric charges of amino acid side chains in the PepT1 substrate binding sites and allows both charged, polar as well as large nonpolar substrates to bind at the same site (Daniel, 2004a). Though PepT1 has wide substrate specificity, it has differing affinities for different substrates. Perfusion studies conducted in human volunteers to determine the transport rate of 12 different glycyl-containing dipeptides demonstrated the influence of C-terminal amino acid residues on dipeptide absorption in the intestine. One study demonstrated faster absorption of glycyl dipeptides which contain neutral amino acid in their C-terminus over dipeptides containing cationic amino acids in this position (Steinhardt & Adibi, 1986). Vig *et al.,* (2006) and Pan *et al.,* (2001) have shown that dipeptides which consist of cationic amino acids have lower affinity for PepT1. A study conducted in rabbit PepT1 expressed in Xenopus oocytes suggested that the N-terminus of dipeptides was the primary binding site with PepT1 (Meredith *et al.,* 2000). Moreover, transporter affinity of charged molecules could change depending on the pH of the apical microenvironment (Daniel & Kottra, 2004b).

Studies conducted using glycyl-sarcosine as the model dipeptide have shown that it has similar affinity for PepT1 in multiple species including sheep (0.61 mM), chickens (0.47 mM), pigs (0.94 mM), turkeys (0.69 mM), and humans (1.2 mM) (Gilbert *et al.,* 2008). However, the PepT1 transporter has been reported to have a wide range of substrate affinities both within and amongst species; in sheep the affinity of various peptide substrates ranges from 27 μM to 3.0 mM (Pan *et al.,* 2001), in pigs it is 4 μM to 0.53 mM (Klang *et al.,* 2005) and in humans, 80 μM to 8 mM (Vig *et al.,* 2006). Another study that was conducted using Madin-Darby canine kidney (MDCK) cells in which PepT1 is over expressed, reported that the volume (i.e. size) of dipeptides also determines the transport affinity. These MDCK cells did not transport a tryptophan-tryptophan dipeptide via PepT1 because this dipeptide failed to activate the PepT1 transporter (Vig *et al.,* 2006). The authors suggested that the reduced activity was because of the large total volume of the dipeptide, which is greater than the capacity of the PepT1 binding site (Vig *et al.,* 2006). In summary, PepT1 has broad substrate specificity with a wide range of affinities. The charge of the amino acid in the C-terminus position, and the pH value of the apical microenvironment among other factors, seems to determine the transport affinity.

1.6.5. PepT1 Expression

The composition of the diet, the age of the animal and the pathological and/or nutritional condition have all been identified as regulatory factors of PepT1 expression.

1.6.5.1.Changes of PepT1 expression with diet

The type of nutrients presented to the intestinal absorptive surface changes during the period from birth to post-weaning. At birth, the neonatal diet shifts from amniotic fluid to milk and during weaning the diet changes from highly digestible milk to a solid diet (Pácha, 2000; Gilbert *et al.,* 2008). Such diet changes likely induce changes in PepT1 presence and/or activity in the intestine. Moreover, the protein composition of the diet has also been identified as a regulatory factor for PepT1 expression (Chen *et al.,* 2005). A study conducted in human intestinal epithelial Caco-2 cells which were incubated in dipeptide-rich media, demonstrated a 1.92 fold increase in peptide-induced PepT1 mRNA expression and a 1.64 fold greater uptake of dipeptides, compared to Caco-2 cells incubated with a mixture of free amino acids (Walker *et al.,* 1998). Similarly, Caco-2 cells exposed to glycyl-sarcosine for 24 h had a two-fold increase in glycyl-glutamine uptake and three-fold increase in PepT1 mRNA abundance (Thamotharan *et al.,* 1998). These data suggest a substrate-driven up regulation of PepT1 in intestinal epithelial cells.

1.6.5.2.Changes of PepT1 expression with age

A linear increase in PepT1 mRNA levels in chicken intestine has been observed with age. In rats, PepT1 expression the small intestine was shown to be maximal at 3-5 d after birth; a rapid decline in PepT1 expression followed. Interestingly, again in rats, a rapid increase of PepT1 expression was observed at 24 d after birth, which corresponded to weaning age (Shen *et al.,* 2001). Another study carried out in Tibetan pigs showed a

continuous increase of PepT1 mRNA expression in the duodenum and proximal jejunum from 1 to 14 days of age, followed by a gradual decrease from d 21 to 35 (Wang *et al.*, 2009b).

1.6.5.3.Changes of PepT1 expression under pathological conditions

Expression of PepT1 may also change due to pathological and/or nutritional conditions that an animal experiences. Experiments conducted with tissue taken from food-deprived rats have shown a 2-fold increase in the uptake of dipeptides by isolated brush border membrane vesicles when compared to vesicles from control-fed rats. In the same study, they measured a 3-fold increase in PepT1 protein levels in brush border membrane vesicles from fasted rats (Thamotharan *et al.*, 1999). Furthermore, rats that were food deprived for 4 days showed a distinct increase of PepT1 protein expression in jejunum compared to the control group of chow-fed rats (Ogihara *et al.*, 1999).

Inflammatory bowel disease is a common intestinal disorder that affects approximately 0.5% of Canadians (Bernstein *et al.*, 2006). Higher PepT1 expression was identified in the colon of inflammatory bowel disease patients compared to control patients who did not have colonic mucosal inflammation (Merlin *et al.*, 2001). Similarly, greater expression of PepT1 mRNA was reported in patients with short bowel syndrome compared to control subjects who had intact intestines (Ziegler *et al.*, 2002). In summary, up regulation of PepT1 expression during weaning, fasting and intestinal pathological conditions may represent an intestinal compensatory mechanism to increase amino acid absorption.

1.7. Interaction between peptide and amino acid absorption

Numerous studies have suggested that peptides and free amino acids use different transport mechanisms to enter enterocytes (Cheeseman & Smyth, 1975). However, some studies have reported that interactions occur between free amino acids and peptides during intestinal uptake. A study conducted in monkeys demonstrated an inhibition of glycyl-glycine uptake when provided together with free leucine and methionine (Ganapathy *et al.,* 1979). A similar observation was reported in a study conducted in guinea pigs where they demonstrated an inhibition of glycyl-leucine uptake by leucine and isoleucine (Himukai & Hoshi, 1980). Later, this inhibition was described as a "pseudo-competitive" type of inhibition (Himukai *et al.,* 1982). However, in a study conducted in human volunteers, this effect was not exhibited, and leucine did not inhibit the uptake of glycyl-leucine (Adibi & Soleimanpour, 1974).

In contrast to free amino acids inhibiting peptide uptake, there is evidence that PepT1-mediated uptake of peptides actually increased free amino acid uptake. An *in vitro* study carried out in Caco-2 cells demonstrated enhanced arginine uptake when cells were pre-incubated with dipeptides (Wenzel *et al.,* 2001). Similarly, the uptake of gabapentin (a substrate for cationic amino acid transport system) was also shown to be enhanced by the presence of dipeptide in the incubation media (Nguyen *et al.,* 2007). However, limited studies have been carried out to investigate the interaction between free amino acids and dipeptide uptake; further investigation is required, particularly in *in vivo* models.

1.8. PepT1 mediated trans-stimulation of rBAT/$b^{0,+}$ AT

Cationic amino acid transporter rBATb0,$^{+}$AT accounts for 80% of arginine uptake into the enterocyte (Wenzel *et al.,* 2001). A study conducted in de-folliculated stage VI Xenopus laevis oocytes demonstrated that rBAT$b^{0,+}$AT is an obligatory amino acid transporter. As such, rBAT$b^{0,+}$AT has shown the capability to exchange intracellular for extracellular amino acids under specific conditions, a concept that has been named trans-stimulation (Chillarón *et al.,* 1996). As described previously, rBAT$b^{0,+}$AT has intracellular and extracellular binding sites. The K_m value for the intracellular binding site is higher than that of the extracellular binding site (Busch *et al.*1994). Thus trans-stimulation activity of rBAT$b^{0,+}$AT may occur once intracellular free amino acid concentration reach an elevated level to bind with the intracellular binding site. A study conducted in rabbit renal brush border membrane vesicles to determine Na^+ independent arginine uptake has demonstrated accelerated exchange diffusion of arginine in vesicles that were pre-incubated with lysine and ornithine, but not alanine or proline, suggesting trans-stimulation of Na^+ independent arginine transporter (Hammerman, 1982).

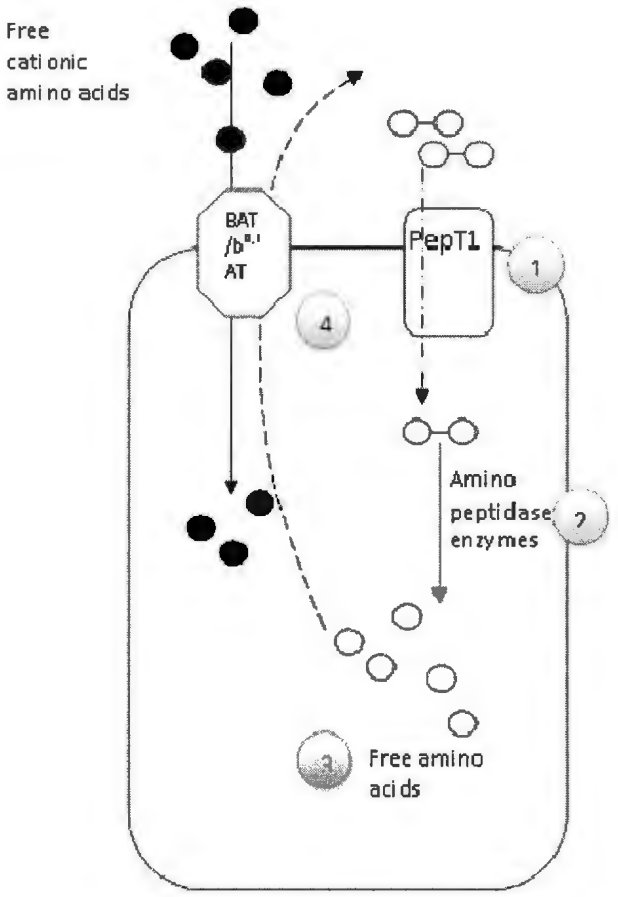


Figure 1-3 Trans-stimulation of rBAT/$b^{0,+}$AT after PepT1 mediated dipeptide uptake into enterocytes. (1) PepT1 mediated uptake of dipeptides into enterocytes; (2) Intracellular hydrolysis of dipeptides into free AA by amino peptidase enzymes; (3) Results in increased intracellular free AA concentration; (4) Exchange of intracellular free AA for extracellular AA (i.e. trans-stimulation).

Wenzel *et al.* (2001) conducted a study using human intestinal Caco-2 cells to investigate the effect of PepT1-mediated dipeptide uptake on the uptake of cationic amino acids. They demonstrated enhanced arginine uptake when the cells were pre-incubated with cationic amino acid-containing dipeptides. The authors suggested that high intracellular free amino acid concentrations were the consequence of the rapid hydrolysis of dipeptides which had been taken up via PepT1; the high intracellular amino acid concentrations triggered the trans-stimulation of rBAT/$b^{0,+}$AT, increasing arginine uptake (Figure 1-3). This effect has been confirmed using hydrolysis-resistant dipeptides and intracellular amino-peptidase inhibitors; and in both these conditions, when intracellular free amino acid concentration was not increased, greater uptake of arginine via rBAT/$b^{0,+}$AT was not observed (Wenzel *et al.*, 2001). Gabapentin is an amino acid-like drug, taken up by enterocytes via rBAT/$b^{0,+}$AT (Nguyen *et al.*, 2007). An *in situ* single-pass rat intestinal perfusion model study demonstrated significantly improved uptake of gabapentin when perfused with dipeptide mixtures, compared to gabapentin alone. The authors concluded that PepT1 mediated uptake of a dipeptides led to trans-stimulation uptake of gabapentin through the transport system rBAT/$b^{0,+}$AT (Nguyen *et al.*, 2007). This concept of a relationship between PepT1 and rBAT/$b^{0,+}$AT represents a new level of understanding of amino acid transport, particularly for the cationic amino acids arginine and lysine; further elucidation of this concept is a primary objective of this thesis.

1.9. Nutritional importance of arginine

The dibasic cationic amino acid arginine was first isolated in crystalline form and subsequently named in 1886 by Schulze and Steiger; in 1895, Hedin showed its presence in animal tissue (Rogers & Visek, 1985). Arginine is a dispensable amino acid in human adults under normal conditions (Tapiero *et al.,* 2002), as it can be synthesized from citrulline in the kidney (Featherston *et al.,* 1973). However, arginine is considered an indispensable amino acid in neonates (Wu *et al.,* 2004a). Arginine becomes indispensable in both neonates and adults if *de novo* synthesis is limited (Hoogenraad *et al.,* 1985) or if the demand for arginine is higher than *de novo* synthesis rates permit, as occurs during growth or in sepsis (Barbul, 1986). Arginine has been identified as the most abundant carrier of nitrogen in mammals as it contains four nitrogen atoms per molecule (Wu *et al.,* 1999b). Arginine is involved in many biochemical reactions in mammals, such as protein synthesis, urea cycle metabolism, polyamine synthesis, nitric oxide (NO) production as well as creatine and agmatine synthesis (Morris, 2006). Thus its metabolism is complex.

1.10. *De novo* arginine synthesis

Arginine can be found in the liver as it is formed via the urea cycle from citrulline (Morris, 2004a). However there is no net arginine synthesis in liver due to high activity of arginase in the hepatocytes which rapidly hydrolyzes arginine to ornithine and urea (Flynn *et al.,* 2002). In adults, *de novo* arginine synthesis involves the intestinal-renal axis. Citrulline is synthesized in intestinal enterocytes from dietary

arginine (Marini, 2012), or from glutamine (Tomlinson *et al.*, 2011a) and proline (Tomlinson *et al.*, 2011a) via pyrroline-5-carboxylate synthase (Blachier *et al.*, 2009). Citrulline is recognized as the major precursor for arginine synthesis in the kidney (Borsook *et al*, 1941; Wu & Morris, 1998). The citrulline that is synthesized in the intestine is released into the portal venous system (Windmueller & Spaeth, 1981); it bypasses the liver and is taken up by the kidney. In the proximal renal tubules, citrulline is converted to arginine by argininosuccinate synthase and argininosuccinate lyase (Morris, 2004b). The newly synthesized arginine is released into the circulation due to low activity of renal arginase, and is used by other body tissues (Bertolo & Burrin, 2008).

Unlike adults, it has been established that in neonatal piglets (Flynn & Wu, 1996) and humans (Tomlinson *et al.*, 2011b) arginine synthesis takes place predominantly in intestinal enterocytes. Studies conducted in pigs have estimated arginine intake from sow's milk to be 0.42 $g \cdot kg^{-1} \cdot d^{-1}$ in one-week-old piglets, but the metabolic arginine requirement for the piglet is estimated to be 1.1 $g \cdot kg^{-1} \cdot d^{-1}$ (Wu *et al.*, 2000). Thus, to meet the entire metabolic arginine requirement, there must be substantial reliance on de novo arginine synthesis in suckling piglets. The amino acid proline has been identified as a major precursor for *de novo* arginine synthesis in neonatal piglets (Brunton *et al.*, 1999) and human neonates (Tomlinson *et al.*, 2011b).

Studies conducted in neonatal pig intestine have demonstrated age related changes in *de novo* arginine synthesis. Enterocytes obtained from 1-d-old piglets exhibited efficient conversion of citrulline produced from its precursors into arginine

(Blachier *et al.,* 1993). On the other hand, more citrulline was released from the small intestine in 7-21-d-old piglets compared to newborn animals. This was likely due to a decrease in activity of argininosuccinate synthase and lyase in the enterocytes as the piglet ages (Wu *et al.,* 1994). Thus, the small intestine transitions from being a major arginine producer to a major citrulline producer as the animal ages.

1.11. Arginine metabolism and function

Arginine has many important metabolic roles (Visek, 1986). As a urea cycle intermediary, arginine plays a major role in ammonia detoxification. Ammonia derived from amino acids, nucleic acids and other nitrogenous compounds, is converted to non-toxic urea in the liver via the urea cycle. Arginine is hydrolyzed to ornithine by the action of arginase to release urea, which also plays an important role in maintaining acid-base balance (Meijer *et al.,* 1990).

Nitric oxide (NO) production is another important metabolic role of arginine (Wu & Morris, 1998). Nitric oxide synthase (NOS) oxidizes the guanidino group of arginine to form NO and citrulline (Marletta, 1989). There are three isoforms of NOS: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS) (Hallemeesch *et al.,* 2002). NO has been identified as a major vascular endothelial relaxation factor. It also acts as mediator of the immune response, a neurotransmitter as well as a widespread signaling molecule (Ignarro, 2002). NO also inhibits platelet aggregation, leukocyte adhesion and superoxide generation (Wu & Meininger, 2000).

Arginine deficiency can lead to reduction of NO production which can result in impaired organ blood flow and growth retardation in young animals (Wu *et al.,* 1999a).

Ornithine, which is derived from arginine, is the immediate precursor for polyamine synthesis. Polyamines act as antioxidants to protect cells from oxidative damage and they have also been found to modulate protein synthesis by stimulating the assembly of 30S ribosomal subunits (Igarashi & Kashiwagi, 2000). As such, polyamines play a key role in cell division, tissue growth and differentiation (Pegg & McCann, 1982).

Creatine is another important metabolite of arginine which acts as a high energy phosphate source for the regeneration of adenosine triphosphate (Visek, 1986). In a review by Wyss & Kaddurah-Daouk (2000), they suggest that creatine has antioxidant, antitumor, antiviral, and antidiabetic effects.

In summary, arginine is a dispensable amino acid in most adult mammals as they are able to synthesize arginine *de novo*. However, in neonates and growing animals, arginine has been recognized as a semi-indispensable amino acid that is in high demand but with limited *de novo* synthesis in situations of growth and weaning.

1.12. Arginine nutrition during growth and weaning

An experiment conducted in weaning piglets has shown that weight gain and efficiency of feed utilization were maximized when arginine was supplied at 0.48% in the diet, which is double the National Research Council recommendations (Southern & Baker, 1983). Another study reported sub-maximal growth in sow-fed piglets compared

to artificially fed animals. Data showed that artificially fed piglets have a potential for growth ≥400 g/d which is 74% greater compared to sow-fed animals (Boyd *et al.,* 1995). Similarly, a study conducted to determine the effect of dietary arginine supplementation at 0.2 and 0.4% of diet in artificially reared piglets demonstrated an improvement of 28% to 66%, respectively, in daily weight gain compared to controls. Final body weights were 15% and 32% higher with the 0.2 and 0.4% supplementation levels compared to piglets fed unsupplemented sow-milk replacer (Kim *et al.,* 2004). Dietary arginine supplementation has also been reported to enhance intestinal development and expression of vascular endothelial growth factor in weaning piglets (Yao *et al.,* 2011). In the muscle, arginine increased mTOR signaling activity in neonatal pigs, which stimulated muscle protein synthesis and promoted weight gain (Yao *et al.,* 2008). Furthermore, arginine enhanced immune status in early-weaned piglets (Tan *et al.,* 2009). Thus, it is interesting that the arginine content in sow milk has been identified as deficient compared to the estimated arginine requirement for optimal piglet growth and metabolism (Wu *et al.,*, 2004b).

Weaning is a stressful event linked with gastrointestinal disorders in piglets and weight gain generally slows during this phase (Moeser *et al.,* 2007). A study conducted in 21-d-old male piglets demonstrated that weaning stress reduced the body weight gain by 15% compared to the sow-fed group. Furthermore, arginine supplementation improved the growth rate of piglets by 5.6% during the stressful weaning period (He *et al.,* 2011).

In summary, dietary arginine supplementation may be beneficial for early growth and especially during weaning due to a low concentration of arginine in sow milk and apparent limited *de novo* arginine synthesis necessary to maximize growth.

1.13. Arginine-lysine antagonism

Though it is not yet a common practice to supplement arginine to pig diets, the addition of lysine to animal feed as a supplement is a common practice amongst poultry and swine farmers. Cereal grains are the major ingredient of animal feed and lysine has been identified as deficient in cereal grain-based diets (Ostrowski, 1978). Lysine is considered the first limiting amino acid in the pig diet, and the second limiting amino acid for poultry (Baker, 2007). Lysine is an indispensable amino acid for mammals and its primary function is as a component of body proteins. However, lysine undergoes obligatory oxidation, despite being limited in the diet (Ball *et al.,* 2007). Lysine is also necessary for carnitine synthesis and lysine deficiency will lead to lipid accumulation in the liver, possibly due to a disruption in the transport of long chain fatty acids into the mitochondria for β-oxidation, which requires carnitine (Ball *et al.,* 2007). Thus, lysine deficiency affects not only protein synthesis, but alters lipid metabolism as well.

As described earlier, the cationic amino acid lysine is taken up into enterocytes by rBAT/$b^{0,+}$ AT. As arginine and lysine share the same transporter system for cellular uptake, lysine-arginine antagonism has been observed in chickens (Kadirvel & Kratzer, 1974), growing dogs (Czarnecki *et al.,* 1985), guinea pigs (O'Dell & Regan, 1963) and pigs (Anderson *et al.,* 1984). A study conducted in chickens demonstrated a clear effect

of excess lysine on arginine transport and metabolism (Nesheim, 1968). And in reverse, excess arginine has shown unfavorable effects on lysine utilization in pigs (Hagemeier *et al.,* 1983). The classic study conducted in dogs demonstrated that excess lysine supplementation depressed the growth by antagonizing arginine uptake; dogs developed arginine deficiency with orotic aciduria, depressed urea formation, hyperammonemia, and emesis (Czarnecki *et al.,* 1985). Chicks also have shown growth retardation and reduced food intake when they were fed a high lysine diet (Austic & Scott, 1975). Young pigs also demonstrated a decreased weight gain and feed intake when arginine was supplemented in excess with a high arginine to lysine ratio. Moreover excess arginine supplementation increased plasma arginine, ornithine and citrulline levels while reducing plasma lysine concentration in pigs (Southern & Baker, 1982).

In summary, excess arginine in the diet has an antagonistic effect on cellular uptake of lysine in growing piglets. Deficiency or excess of arginine or lysine in the diet can cause growth retardation and adverse metabolic effects in young and growing animals. Designing the ideal diet to meet the requirements for both arginine and lysine while avoiding the antagonistic effects is remains an important challenge. Maximizing growth performance of domestic animals, especially pigs and poultry, will help to address the increased global food demand.

The interactions between free amino acids and peptide uptake at the cellular level is not well understood, but the use of dipeptide supplementation, as opposed to free amino acids, may help to address the issue of antagonism between amino acids which compete for the same transporter. Describing trans-stimulation activity of $b^{0,+}$ transporter

will open new doors for the supply of nutritionally important amino acids, particularly during intestinal stress or injury, in both domestic animals and humans.

1.14. Rationale

1.14.1. Growing piglet as the animal model

The piglet is considered a suitable animal model in nutritional research related to human neonates because of similar gastrointestinal tract morphology, physiology and metabolic changes during development (Miller & Ullrey, 1987). Intestinal development of pigs is representative of humans at the fetal and neonatal stages of development (Pácha, 2000). Small intestinal mucosal enzyme activities of 6 week old piglets and young human infants are similar (Shulman *et al,* 1988). Also Shulman *et al.* (1993) have reported similar gross body composition in newborn piglets and preterm infants.

Amino acid transporter systems and oligopeptide transporter PepT1 are expressed in the porcine intestine from birth. The PepT1 transporter was detected in Tibetan piglets measured at 1, 14 and 28 days of age (Wang *et al.,* 2009b). A previous study conducted in our lab also showed the presence of the PepT1 transporter in the small intestine of Yucatan miniature piglets at 2, 7, 14 and 21 days of age (Nosworthy *et al.,* 2012). The cationic amino acid transporter rBAT/$b^{0,+}$AT was reported in the duodenum, jejunum, and ileum of the Tibetan pigs at 7 and 21 days of age (Wang *et al.,* 2009a). Hence, the transporters that we were interested in studying (rBAT/$b^{0,+}$AT and PepT1) are present in the 18-21 day old piglet intestine. In our facility, piglets start weaning at approximately 21 days of age, when they begin to take interes in the grain-

based diet fed to the sows, and is completed at 28 days of age when the piglets are removed from the sow. As mentioned earlier, weaning is a stressful event linked to gastrointestinal changes. In order to develop ideal diets to accommodate weaning, it is important to understand nutrient transport physiology at this stage of development. As such, Yucatan miniature piglets at 18 to 21 days of age were selected as the animal model for the studies described in this thesis.

1.14.2. Ussing chambers as the *in vitro* model

Different techniques have been applied for *in vitro* physiological studies of gastrointestinal epithelia including everted sacs, intestinal rings (Hillgren *et al.* 1995) and Ussing chambers. Ussing chambers provide a valuable and validated method for the measurement of electrolyte, nutrient, and drug transport across epithelial tissues (Clarke, 2009). Modified Ussing chambers provide good working interface using viable intestinal tissue under *in vitro* conditions. Studies have demonstrated that with the provision of supplemental oxygen and other nutrients, intestinal tissue can be kept viable for over 120 min in the Ussing chamber system (Soderholm *et al.,* 2002). Winckler *et al.,*(1999) have reported successful transport studies using the Ussing chamber as an *in vitro* model for pig intestinal mucosal tissues. Thus, the Ussing chamber system was selected as our *in vitro* model to measure PepT1 transport activity.

1.14.3. Glycyl-sarcosine as the model dipeptide

Glycyl-sarcosine was found to be very resistant to hydrolysis by brush-border membrane peptidases in the intestine (Ganapathy *et al.,* 1984). The hydrolysis resistance

characteristic of glycyl-sarcosine ensures that the dipeptide is presented to PepT1 as an intact molecule. As such, it has been used extensively in studies describing dipeptide transport. Pig intestine is capable of energy-dependent, proton-coupled transport of glycyl-sarcosine via PepT1 (Winckler *et al.,* 1999). Thus, glycyl-sarcosine was selected as the model dipeptide for the *in vitro* studies.

1.14.4. Losartan as PepT1 inhibitor

The PepT1 transporter is responsible for the electrogenic transport of dipeptides into intestinal epithelial cells (Adibi, 1997). A number of dipeptide-like drugs competitively inhibit the PepT1 transporter (Sawada *et al.,* 1999; Terada *et al.,* 2000; Knutter *et al.,* 2009). Losartan potassium is among the peptidomimetic drugs, and is an effective drug for treating hypertension. Losartan strongly inhibited PepT1-mediated dipeptide uptake in human Caco-2 cells (Knutter *et al.,* 2009). While losartan has a high-affinity interaction with PepT1, it binds to the transporter but is not transported. By this mechanism, it strongly inhibits PepT1-mediated uptake of dipeptides (Knutter *et al.,* 2009). Thus, losartan was selected for our studies of PepT1 inhibition in the *in vitro* transport studies.

1.14.5. *In vivo* gut loop method as experimental model

The intestinal gut loop method has been recognized as a convenient method for intestinal metabolic studies (Nichols & Bertolo, 2008) as well as in amino acid and dipeptide transport kinetic studies (Nosworthy *et al.*, 2012).

Adegoke *et al.* (1999) successfully validated the continuous perfusion gut loop model to measure first pass metabolism in gut. It was established in our research group by Nichols and Bertolo (2008) to investigate threonine metabolism. Also Ashida *et al.* (2004) and Pan *et al.* (2002) successfully demonstrated the *in vivo* gut loop method as a reliable method for studies of PepT1-mediated transport in rats. Previously in our lab, Nosworthy *et al.* (2012) used the *in vivo* gut loop method for PepT1 transport studies in Yucatan miniature piglets. Multiple loops within one animal allows for the implementation of multiple experimental conditions at one time while using a minimum number of animals. Thus, the intestinal gut loop method was selected as our experimental model to measure the effects of lysine-containing dipeptides on arginine uptake.

1.14.6. Amastatin as amino peptidase inhibitor

Dipeptides undergo rapid hydrolysis after transport via PepT1 into enterocytes. Aminopeptidase enzymes rapidly hydrolyze dietary dipeptides into free amino acids (Newey & Smyth, 1960). Thus, intracellular aminopeptidase inhibitors will interfere with the intracellular hydrolysis of dipeptides (Wenzel *et al.,* 2001). Amastatin and bastatin are two potent aminopeptidase enzyme inhibitors. Bastatin is considered to be a more potent aminopeptidase inhibitor, but it was identified as a strong PepT1 inhibitor as well (Daniel & Adibi, 1994) . In contrast, amastatin does not interact with PepT1 (Daniel & Adibi, 1994). Thus, amastatin was selected as the aminopeptidase inhibitor in this study.

1.15. Objectives and Hypotheses

Study 1:

Objective: To determine the proportion of dipeptide transported by the PepT1-mediated transcellular transport versus paracellular movement in the small intestine under *in vitro* conditions.

Hypothesis: Transepithelial transport of dipeptides consists of passive paracellular movement and PepT1-mediated transcellular transport. Inhibition of PepT1-mediated transport by losartan will inhibit transcellular transport of glycyl-sarcosine and as such, transepithelial movement of glycyl-sarcosine will represent only the passive paracellular avenue of dipeptide uptake.

Study 2:

Objective: To determine whether the presence of lysyl dipeptides enhance the uptake of free L-arginine by the small intestine.

Hypothesis: The presence of dipeptides will enhances the uptake of free arginine into enterocytes through trans-stimulation of the rBAT/$b^{0,+}$ system. The addition of the aminopeptidase inhibitor, amastatin, along with dipeptides will impede the trans-stimulation activity by reducing intracellular hydrolysis of dipeptides.

2. Materials and methods

2.1. Ussing chamber experiment

2.1.1. Animal procedure

Eighteen day old, sow-fed Yucatan miniature piglets were obtained from Animal Care Services, Memorial University of Newfoundland. All procedures were approved by the Institutional Animal Care Committee (Memorial University of Newfoundland), and were in accordance with the guidelines of the Canadian Council on Animal Care. Pigs were anaesthetized using 4%-5% isoflurane (Abbot Laboratories Ltd., Montréal, QC) delivered with 1.5 L/min oxygen. Once the animal was deeply anaesthetized, a mid-line laparotomy incision was made and an intestinal segment was excised from the mid jejunum which was 100 cm distal to the ligament of Treitz. The isolated intestinal section was freed from the mesenteric fixation and the underlying serous membrane was detached. The intestine was rinsed with ice-cold physiological saline (0.9% NaCl) to remove any residual chyme and was placed in ice-cold oxygenated incubation buffer for transport to the laboratory. The composition of the incubation buffer solution (Table 2-1) is the same as that used in the "serosal" side of the Ussing chamber, described below.

2.1.2. Mounting of intestinal tissues on Ussing chamber

Once in the laboratory, the intestine segments were open longitudinally and rinsed with cold saline to remove all luminal debris. A 2-3 cm section taken from the

intestinal segment was allowed to float in oxygenated modified Krebs's buffer solution until it

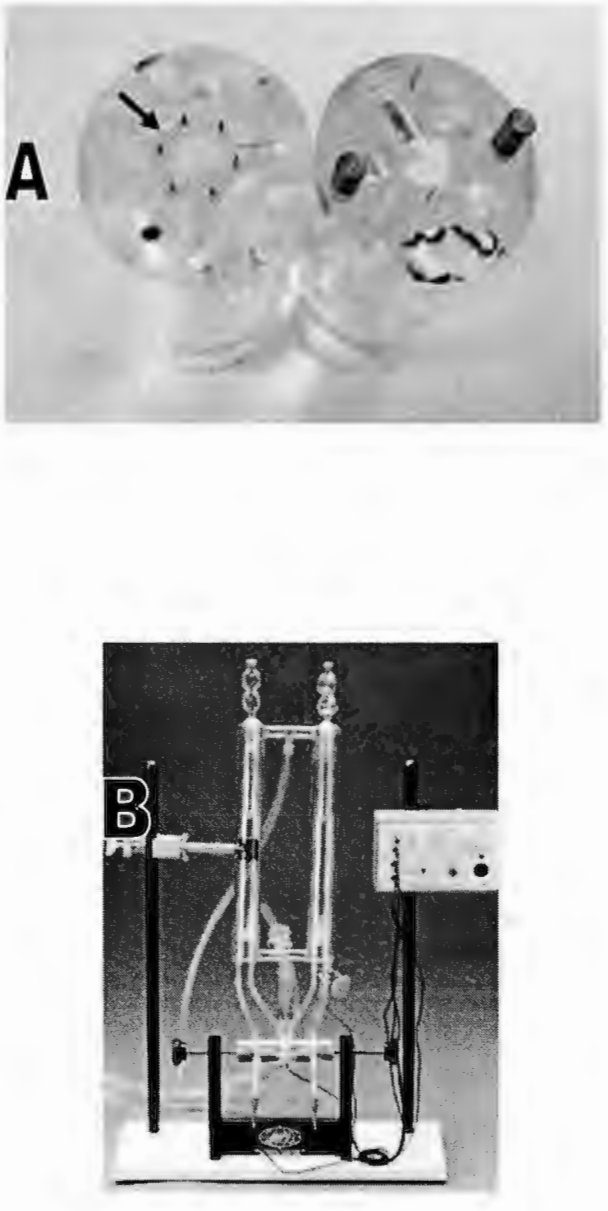


Figure 2-1: **(A)** Ussing chamber compartment and **(B)** Ussing chamber system

spread out completely. Subsequently, it was mounted on a Styrofoam plate and then transferred to the pins on the Ussing chamber port (Figure 2-1a). The other half of the chamber was aligned with the pins and connected to the gas lift system. World Precision Instruments system model CHM6 Ussing chambers with an aperture of 1 cm^2 were used for the experiment (Figure 2-1b).

2.1.3. Ussing chamber transport studies

Tissues mounted in the Ussing chambers were incubated on both luminal and serosal sides with 6 mL of modified Kreb's buffer solution (Winkler *et al.*, 2001) (Table 2-1). The pH was set at 7.4 for the serosal solution and 6.0 for the luminal solution using TRIS buffer. The buffer on the luminal side also contained 5 mM glycyl-sarcosine (Bachem Americas, Torrance, CA) alone or with 0, 1, 5 or 10 mM losartan (Sigma-Aldrich Canada Ltd, Oakville, ON) which was used as the PepT1 inhibitor. The Ussing chamber with glycyl-sarcosine and no inhibitor was used as the control condition. 10 mM glucose was added to the serosal solution as an energy source, and 10 mM mannitol was added to the luminal solution to achieve osmotic balance. The luminal buffer solutions also contained 1 μCi/mL ^{3}H-glycyl-sarcosine and 1 μCi/mL ^{14}C-mannitol. Continuous oxygenation and recirculation of the incubation solutions was provided by the gas lift system and all solutions were maintained at 37°C by using the temperature controlled water jacket. At the initiation of the experiment and at 15 min intervals thereafter, 0.5 mL aliquots were sampled from the serosal buffer. The aliquots were

replaced by an equal volume of fresh buffer solution. The luminal buffer was sampled before and after the experiment. The entire experiment was run for 2 hours.

Table 2-1: Composition of modified Kreb's buffer used as the incubation buffer, and in Ussing chamber experiments

Ingredient	Concentration
NaCl	137 mM
KCl	5.4 mM
$CaCl_2 \cdot 2H_2O$	2.8 mM
$MgSO_4$	1 mM
K_2HPO_4	0.3 mM
NaH_2PO_4	0.3 mM

2.1.4. Measuring glycyl-sarcosine appearance in the serosal buffer

Glycyl-sarcosine transport was determined by ^{3}H-glycyl-sarcosine appearance in the serosal buffer. A 50 µL sample was mixed with 5 mL of ScintiVerse (Fisher Scientific, Fair Lawn, NJ). Disintegrations per minute (DPM) were measured in TriCarb 2810 TR liquid scintillation counter (Perkin Elmer Life and Analytical Sciences, Downers Grove, IL) with a count time of 15 min per sample. Glycyl-sarcosine appearance was determined using the following calculations:

$$Glycyl - sarcosine\ appearance = SRA * DPMt_n$$

$$SRA = \frac{n_0}{DPMt_0}$$

$DPMt_0$: DPM counted in the luminal compartment buffer before the experiment was started

$DPMt_n$: DPM counted in serosal compartment taken after t_n

n_o: total glycyl-sarcosine (nmol) present in the luminal buffer before the experiment was started

SRA: specific radio activity.

2.1.5. Measuring the tissue viability

Viability of the intestinal tissue mounted on the Ussing chambers was assessed using the EK1 electrode kit (WPI, Sarasota, FL) and DVC-1000 voltage/current clamp (WPI, Sarasota, FL). Smaller blue voltage electrodes were plugged into "V1" and "V2" of the DVC-3 preamplifier (WPI, Sarasota, FL) and they were connected to luer ports

located proximal to tissue. The larger red current electrodes were connected with "I1" and "I2" of DVC-3 and connected to luer ports located distally to the intestinal mucosal tissue. Then DVC-3 was connected with DVC-1000 electrode clamp. Voltage gradient between the intestinal mucosal tissues was detected by DVC-1000 electrode clamp. The amplified signals were sent to the computer using the Lab-Trax data acquisition system and were plotted using Lab-Trax data recording software (WPI, Sarasota, FL).

2.2. *In vivo* gut loop study to measure arginine uptake

2.2.1. Animals

Three week old sow-fed Yucatan miniature piglets were obtained from Animal Care Services, Memorial University of Newfoundland and were randomized to one of three treatment groups (n = 5 per group) as described in section 2.4. All procedures were approved by the Institutional Animal Care Committee (Memorial University of Newfoundland), and were in accordance with the guidelines of the Canadian Council on Animal Care. Experimentation was initiated within 2 h of separating the piglets from the sow.

2.2.2. Surgery

Piglets were initially anaesthetized using 5% isoflurane and maintained with 2% isoflurane mixed with O_2 delivered at 1.5 L/min. The ventral abdominal area of the animal was cleaned thoroughly with soap and Povidone-iodine prior to the initiation of surgery. Piglets' body temperature, heart rate, respiration rate and oxygen saturation were monitored throughout the experiment.

A midline incision was made to expose the small intestine. The small intestine was exteriorized and the beginning of jejunum was identified using the ligament of Treitz (end of duodenum) as a landmark. Five or six closed gut loops were positioned along the proximal portion of the jejunum (Adegoke *et al.,* 1999). The first closed gut loop was located 15 cm distal to the ligament of Treitz. An inlet cannula (ID, 1/16 in.;

OD 1/8 in., Watson Marlow, Cornwall, UK) was inserted into the lumen through a small perforation in the intestinal wall and the tubing was secured by tying a suture around the intestine and the tubing. 10 cm was measured distal to the inlet cannula and the same procedure was performed to implant the outlet cannula to form a loop. The remaining loops were created in a similar fashion, placed along the jejunum and separated by 50 cm segments of intestine. Remaining intestine was rinsed with warm saline and placed back into the abdominal cavity. Exposed intestinal segments were moistened with 37°C saline and covered with plastic wrap, in order to prevent dehydration during perfusion. The loops were flushed with warmed PBS (144.6 mM NaCl, 15.9 mM Na_2HPO_4, 1.2 mM NaH_2PO_4. H_2O) to remove any chyme present in the intestinal lumen before starting the perfusion.

2.2.3. Perfusion Procedures

The experimental perfusates (described below in section 2.2.4) were continuously re-circulated through the closed intestinal loops, via the use of a multi-channel peristaltic pump (Watson Marlow, Cornwall, UK) (Figure 2-2). At the beginning of the study, 60 mL of each perfusate was placed in a bottle with the inlet and outlet cannula tubing immersed in the solution, and the bottles were placed in a water bath that was maintained at 37°C. The experimental solutions were randomly assigned to the loops for each animal from the proximal to distal end of jejunum in order to avoid an effect of location. The solutions were perfused for 120 min. The luminal perfusates were sampled (1 mL) every 30 min. At the end of the experiment, the loops were

excised by cautery and flushed with cold 0.9% saline, placed on ice, cut longitudinally and scraped with a microscope slide to remove the mucosa. The mucosa tissues were weighed and immediately flash frozen in liquid nitrogen and stored at -80°C for future analyses.

2.2.4. Amino acid and dipeptide composition of the perfusates

Each perfusate (60 mL) was made up of PBS buffer and contained 10 mM arginine (Ajinomoto North America, Inc, Raleigh, NC). The first perfusion experiment (n=5 pigs) tested the effect of lysyl-lysine (Bachem Americas, Torrance, CA) on arginine uptake in the jejunum. Gut loops were perfused with experimental buffers; each buffer contained 10 mM L-arginine with 10, 20 or 50 mM lysyl-lysine (Bachem Americas, Torrance, CA) or with 20 mM L-lysine (Sigma-Aldrich Canada Ltd, Oakville, ON) (Table 2-2). The objective of the second experiment (n=5 pigs) was to determine the effect of lysyl-glycine on L-arginine uptake. Six gut loops in each piglet were perfused with buffers that contained 10 mM L-arginine along with 10, 20 and 50 mM lysyl-glycine (Bachem Americas, Torrance, CA) or 20 mM L-lysine or 20 mM glycine (Table 2-3). The third experiment (n=5 pigs) assessed the effect of an amino peptidase inhibitor amastatin on L-arginine uptake. Experimental buffers each contained 10 mM L-arginine with 20 mM lysyl-lysine or 50 mM lysyl-glycine with or without 10 μM amastatin (Sigma-Aldrich Canada Ltd, Oakville, ON) (Table 2-4). The gut loops, which were perfused with buffers containing amastatin, were pre-incubated with a 10 μM amastatin solution for 10 min prior to starting the perfusion. In each of the

three experiments, a loop that was perfused with L-arginine as the sole amino acid was used as the control condition. The amino acids and dipeptides were dissolved in PBS buffer which was adjusted to pH 6. To each perfusate, radio-labeled arginine was added in the form of ^{3}H-arginine (2220 kBq or 60 μCi per 60 mL) (American Radiolabeled Chemicals, Inc, St. Louis, MO).

Table 2-2: Experimental conditions (luminal perfusate composition) to determine the effect of lysyl-lysine on arginine uptake.

Ingredient	Perfusate 1	Perfusate 2	Perfusate 3	Perfusate 4	Perfusate 5
Arginine	10 mM	10 mM	10 mM	10 mM	10 mM
Lysyl-lysine	—	10 mM	20 mM	50 mM	—
Lysine	—	—	—	—	20 mM

Table 2-3: Experimental conditions (luminal perfusate composition) to determine the effect of glycyl-lysine on arginine uptake

Ingredient	Perfusate 1	Perfusate 2	Perfusate 3	Perfusate 4	Perfusate 5	Perfusate 6
Arginine	10 mM	10 mM	10 mM	10 mM	10 mM	10 mM
Glycyl-lysine	—	10 mM	20 mM	50 mM	—	—
Lysine	—	—	—	—	20 mM	—
Glycine	—	—	—	—	—	20 mM

Table 2-4: Experimental conditions (luminal perfusate composition) to determine the effect of amino peptidase inhibitor amastatin on arginine uptake.

Ingredient	Perfusate 1	Perfusate 2	Perfusate 3	Perfusate 4	Perfusate 5	Perfusate 6
Arginine	10 mM	10 mM	10 mM	10 mM	10 mM	10 mM
Lysyl-lysine	—	—	20 mM	20 mM	—	—
Glycyl-lysine	—	—	—	—	50 mM	50 mM
Amastatin	—	10 μM	—	10 μM	—	10 μM

2.2.5. Measuring arginine disappearance in luminal perfusates

Arginine uptake was determined by ^{3}H-arginine disappearance from the luminal perfusate. Sampled perfusate (200 µL) was mixed with 5 mL of ScintiVerse (Fisher Scientific, Fair Lawn, NJ). Disintegrations per minute (DPM) were measured in TriCarb 2810 TR liquid scintillation counter (Perkin Elmer Life and Analytical Sciences, Downers Grove, IL) with a count time of 15 min per sample. Arginine disappearance was measured using following calculations

$$Arginine\ disappearance = \frac{DPMt_0 - DPMt_{120}}{\frac{DPMt_0}{n_0}}$$

$$n_0 = \frac{Initial\ arginine\ concentration}{initial\ volume}$$

$DPMt_0$: DPM in luminal perfusate sample before start perfusion

$DPMt_{120}$: DPM in luminal sample taken after 120 min perfusion

n_0: absolute amount of arginine (µmol) present in luminal perfusate before the perfusion was started

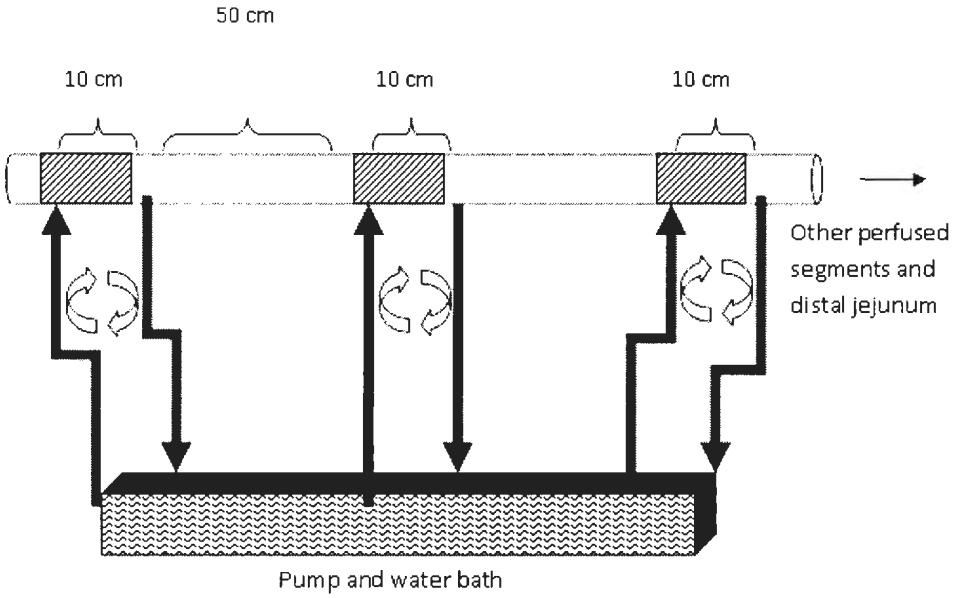


Figure 2-2: Schematic of gut loop model

2.2.6. Amino acid and dipeptide analysis of perfusate samples

Absolute amounts of amino acids and dipeptides in the perfusates were determined using high performance liquid chromatography (HPLC) (Waters, Mississauga, ON) or ultrahigh performance liquid chromatography (UPLC) (Waters, Mississauga, ON).

2.2.6.1. Sample preparation for HPLC analyses

HPLC analyses were performed in order to determine the absolute amounts of free amino acids and intact dipeptides in the luminal perfusates. Samples were diluted 1:10 as the amino acid concentrations were higher than the maximum loading capacity of Waters PicoTag HPLC column. 100 µL of diluted sample was mixed with 20 µL of 2.5 mM norleucine standard (Sigma-Aldrich Canada Ltd, Oakville, ON). Then1 mL of 0.5% trifluoroacetic acid (TFA) (Sigma-Aldrich Canada Ltd, Oakville, ON) in methanol (MeOH) (Fisher Scientific, Whitby, ON) was added to the samples in order to precipitate any protein in the perfusate. Protein-free supernatant was separated after the samples were centrifuged at 4200 x g for 3 min. The supernatant was frozen in liquid nitrogen and vacuum dried on the Thermo Scientific® Digital Series SpeedVac Systems (Thermo Fisher Scientific, Nepean, ON). Subsequently 50 µL of a mixture of triethylamine (TEA) (Sigma Aldrich Canada Ltd, Oakville, ON), MeOH (Fisher Scientific, Whitby, ON) and water in a 2:2:6 ratio were added to each sample. The samples were vacuum dried again using the SpeedVac system. Once dried, the samples were labeled with a mixture of TEA, PITC (Thermo Scientific, USA), MeOH and water

in a 1:1:7:1. 20 µL of the PITC solution was added to each sample, and after an incubation period of 35 minutes at room temperature, the process was stopped by freezing in liquid nitrogen. This derivatization step allows PITC to bind with free amino acids to form phenylthiocarbamyl amino acids. The samples were then dried down again. Prior to HPLC analyses, the samples were re-suspended in 200 µL of sample diluent which was 5 mM Na_2HPO_4 (Sigma Aldrich Canada Ltd, Oakville, ON) titrated to pH 7.4 with 10% H_3PO_4 acid and acetonitrile was added to 10% (Fisher Scientific, Whitby, ON) . Following the addition of the diluent, the samples were vortexed and then centrifuged at 3000 x g for 3 min and the supernatant was transferred to HPLC vials.

2.2.6.2. Sample preparation for UPLC analyses

UPLC analysis was performed after derivatizing the perfusate samples using the Waters AccQ·Tag Amino Acid Analysis Method® (Waters, Mississauga, ON). Samples were diluted 1:10 prior to derivatization. 100 µL of diluted perfusate sample was mixed with 10 µL of 2.5 µM norvaline (Sigma Aldrich Canada Ltd, Oakville, ON) internal standard and vacuum dried using the SpeedVac system. Dried samples were then resuspended in 50 µL of 0.1 N HCl (Fisher Scientific, Whitby, ON). 10 µL of the resuspended sample was mixed with 70 µL of AccQ·Tag ultra-borate buffer (Waters, Mississauga, ON) and 20 µL of AccQ·Tag ultra-reagent (Waters, Mississauga, ON), and was heated for 10 min at 55°C in an oven to facilitate the labeling process of the amino acids with the AccQ•Tag reagent.

2.2.6.3. HPLC analysis of perfusate samples

HPLC sample vials which contained the prepared sample were placed in Waters 717 Plus Auto Sampler system (Waters, Mississauga, ON). A 40 µL aliquot of sample was injected into the reverse phase C18 column which was kept at 46°C to facilitate the separation procedure. The phenylthiocarbamyl amino acids were separated on the column during a 112 min run time, at 1 mL/min and quantified by the UV absorbance at 254 nm. The peaks in the chromatogram for each amino acid were integrated using Breeze software (Waters, Version 3.3, 2002, Waters Corporation, Woburn, MA), and the amino acid concentrations in the plasma were determined by comparing peak areas to the area produced by the internal norleucine standard.

2.2.6.4. UPLC analysis of perfusate samples

UPLC-prepared samples were placed in the Acquity Sample Manager® (Waters, Mississauga, ON). 1 µL of derivatized sample was injected into a 2.1 × 100 mm AccQ·Tag ultra® UPLC column (Waters, Mississauga, ON). The column was kept at 55°C to facilitate the separation procedure. Samples were separated on the column during 9.5 min run time at 0.7 mL/min flow rate. Fluorescence absorbance were quantified at 515 nm, using Acquity FLR detector® (Waters, Mississauga, ON). The peaks in the chromatogram were integrated and analyzed using Empower® software (Waters, Version2, 2009, Milford, MA).

2.2.7. Dipeptide identification

The amino acid elution protocol used in our lab did not provide good separation of dipeptides. Hence, the buffer flow gradient protocol was changed as described in Table 2-4. Once peaks for the dipeptides of interest (lysyl-lysine and lysyl-glycine) were identified on the chromatograph, further standards containing 0.0025, 0.025, 0.25 and 2.5 mM lysyl-lysine and lysyl-glycine standards were analyzed to confirm the identity of the peaks.

2.2.7.1. Fraction collection of HPLC samples

The radioactivity that was associated with arginine was determined by fraction collecting the eluent from the HPLC column that corresponded to the arginine peak; similarly, peaks associated with the urea cycle amino acids (ie ornithine and citrulline) were also collected with subsequent scintillation counting. The eluent for each amino acid was collected into a 7 mL scintillation vial using Waters Fraction Collector II (Waters, Mississauga, ON). The radioactivity in those fractions was determined by liquid scintillation counting using biodegradable scintillant (10 mL) (Fisher Scientific, Whitby, ON).

2.2.7.2. Tissue free amino acid analysis

To determine intracellular free amino acid concentrations, 100 mg of tissue was homogenized with 1 mL of 2% perchloric acid for ~45 seconds. The homogenates were centrifuged at 3000 *g* for 15 min to separate acid soluble free amino acids from protein precipitates. This was repeated 3 times and supernatants were collected and pooled. 50

μL of the internal standard, 25 μM norleucine (Sigma Aldrich, Oakville, Canada) was added to the tissue free supernatant. Subsequently, the supernatant was neutralized with 125 μL of 2 M K_2CO_3 and the samples were centrifuged to separate the supernatant from precipitate. 1 mL of supernatant was vacuum dried and derivatized for HPLC analyses, as described above (Section 2.6.1). However, small changes were made with the amount of derivatization solutions which were used. In the TEA:methanol:water step, 100 μL was used, and for the PITC labeling step, 50 μL was used. In the final step, derivatized samples were re-suspended in 300 μL of sample diluent. Eventually HPLC analyses were performed as described in section 2.6.5.

Table 2-5 Modified buffer flow gradient protocol developed for HPLC dipeptide separation

Standard Amino Acid Separation Gradient						Modified Gradient for Dipeptide Separation				
	Time	Flow	%A	%B	Curve	Time	Flow	%A	%B	Curve
1	0.01	1.0	100.	0.0	6	0.01	1.0	100.0	0.0	6
2	13.5	1.0	97.0	3.0	11	13.5	1.0	97.0	3.0	11
3	24.0	1.0	94.0	6.0	8	24.0	1.0	96.0	4.0	8
4	30.0	1.0	91.0	9.0	5	30.0	1.0	91.0	9.0	5
5	50.0	1.0	66.0	34.0	6	50.0	1.0	66.0	34.0	6
6	66.0	1.0	66.0	34.0	6	66.0	1.0	66.0	34.0	6
7	66.5	1.0	0.0	100.0	6	92.0	1.0	0.0	100.0	6
8	78.5	1.0	0.0	100.0	6	102.0	1.0	0.0	100.0	6
9	79.0	1.0	100.	0.0	6	102.5	1.0	100.0	0.0	6
10	90.0	1.0	100.	0.0	6	112.0	1.0	100.0	0.0	6

3. Results

3.1. PepT1 contribution to the transepithelial transport of glycyl-sarcosine

Transepithelial transport of glycyl-sarcosine in jejunal sections, determined *in vitro* via Ussing chambers, was linear between 15 and 120 min (Figure 3-1). Correspondingly, transepithelial transport of glycyl-sarcosine across the jejunal epithelium with different concentrations of the PepT1 inhibitor losartan was also found to be linear from 15 to 120 min (Figure 3-1). Similarly, in ileal sections mounted in Ussing chambers, glycyl-sarcosine movement was linear between 15 to 120 min (Figure 3-5). The linear relationship of glycyl-sarcosine transport with time indicated the constant movement of glycyl-sarcosine out of the intestinal lumen, which laid the foundation for us to carry out further analyses of glycyl-sarcosine transport through intestinal mucosa.

3.1.1. Glycyl-sarcosine transport across the intestinal sections with differing concentrations of PepT1 inhibitor: preliminary study to determine the ideal PepT1 inhibitor concentration

Glycyl-sarcosine transport rate through the jejunum samples was determined from five different Ussing chamber experiments in intestinal tissues taken from three different animals. The overall mean rate of transport was 3.87 ± 1.45 nmol/cm^2/min (Figure 3-2). The addition of 1 mM of losartan to the luminal buffer reduced the glycyl-sarcosine transport rate significantly to 1.83 ± 0.49 nmol/cm^2/min ($P < 0.05$) (Figure 3-2).

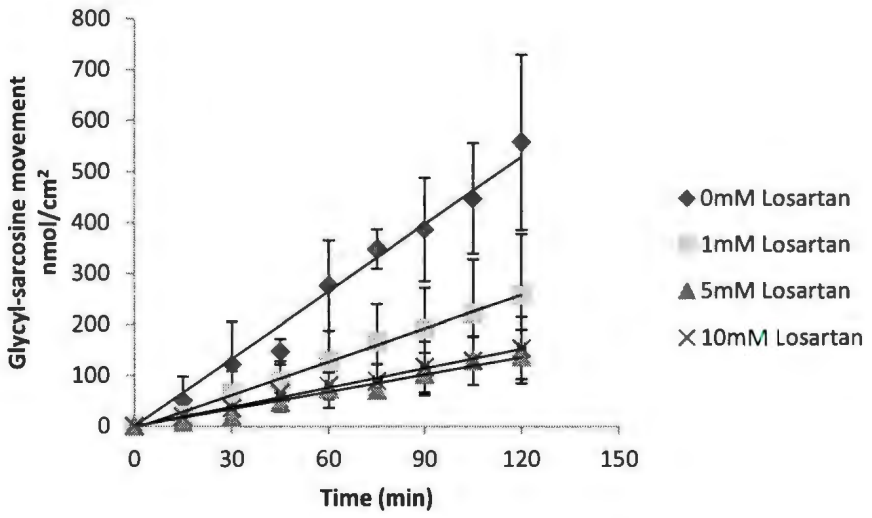


Figure 3-1 **Glycyl-sarcosine movement through neonatal pig jejunum over time**. Values are means ± SD; each data point represents 4 or 5 individual recordings with tissues taken from two piglets. Data are regressed linearly across time, but statistical comparison of concentrations was not performed.

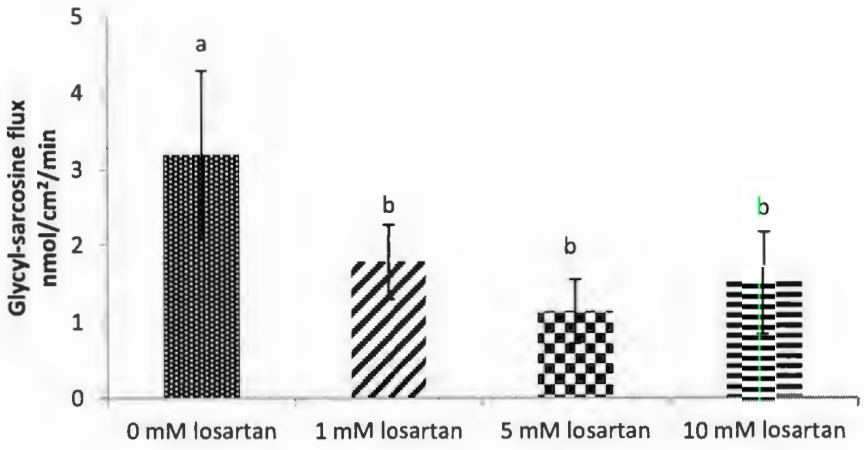


Figure 3-2 Glycyl-sarcosine transport through neonatal pig jejunum with varying concentrations of PepT1 inhibitor (losartan). Values are means ± SD; each bar represents 4 or 5 Ussing chamber experiments, with tissues taken from two piglets. P values were determined by one-way ANOVA. Groups with differing superscripts are significantly different at $P < 0.05$ using Tukey's Multiple Comparison Test.

Further increases in the concentration of losartan to 5 mM and 10 mM reduced the glycyl-sarcosine movement to 1.13 ± 0.42 nmol/cm^2/min and 1.52 ± 0.65 nmol/cm^2/min, respectively (Figure 3-2). These values were significantly lower than the glycyl-sarcosine transport rate without PepT1 inhibitor ($p < 0.05$), but not different ($p > 0.05$) from the 1 mM losartan treatment. Glycyl-sarcosine transport with 5 and 10 mM losartan concentrations were carried out in four Ussing chambers (two per concentration) with tissues obtained from two animals.

3.1.2. Transepithelial transport of glycyl-sarcosine through jejunum with and without PepT1 inhibitor

Transepithelial transport of glycyl-sarcosine was measured in Ussing chambers under standard conditions with and without losartan, a PepT1 inhibitor. A concentration of 1 mM losartan was used based on the study described above that demonstrated no significant further inhibition with higher concentrations. Intestinal tissues were obtained from 5 piglets and each experimental condition was run in duplicate. Glycyl-sarcosine transport rate through jejunum sections without PepT1 inhibitor was 3.31 ± 0.67 nmol/cm^2/min (Figure 3-3). The addition of losartan to the luminal buffer resulted in a glycyl-sarcosine transport rate of 1.78 ± 0.26 nmol/cm^2/min, which was 53% lower than without inhibitor ($p < 0.05$, $n = 5$) (Figure 3-3).

Transepithelial transport of mannitol via piglet jejunum was measured by tracking ^{14}C-mannitol appearance in the basolateral buffer to determine if losartan affected paracellular movement. Transepithelial transport of mannitol without PepT1

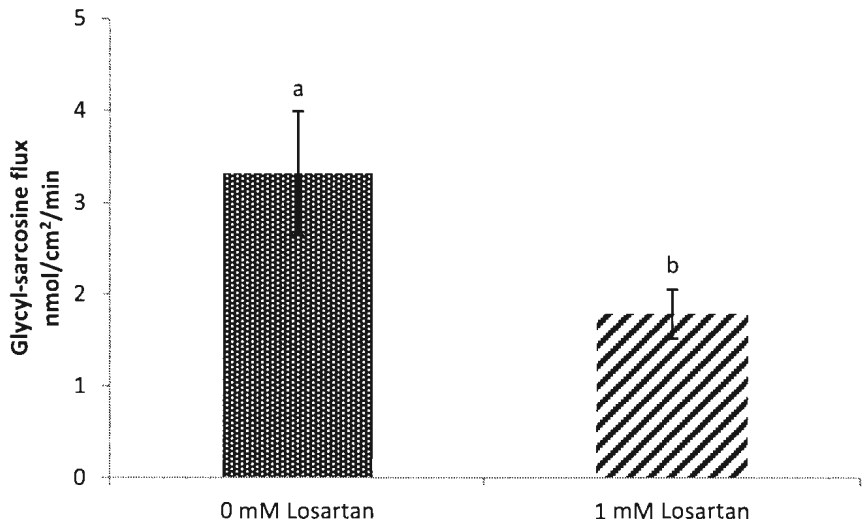


Figure 3-3 Glycyl-sarcosine transport through neonatal jejunum with and without PepT1 inhibitor. Values are means ± SD; n = 5. P values were determined by Student's t-test. Groups with differing superscripts are significantly different at $P < 0.01$.

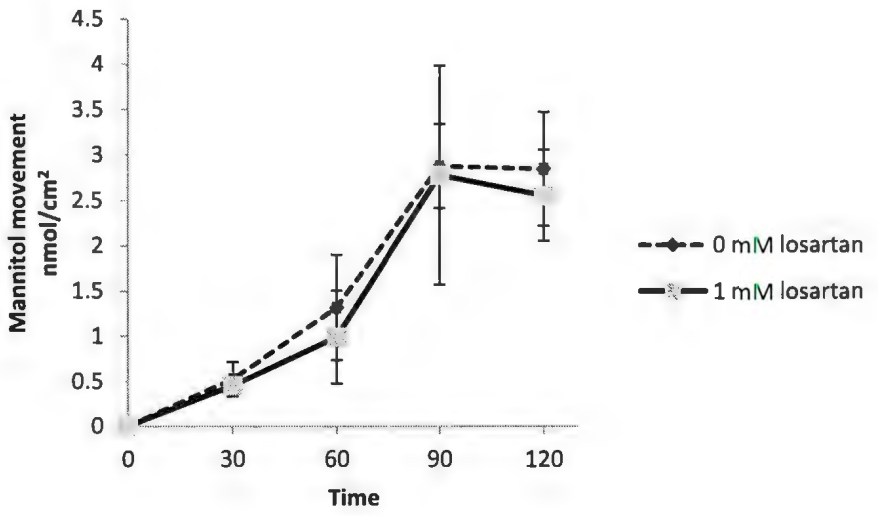


Figure 3-4 Mannitol movement through neonatal pig jejunum against time. P values were determined by repeated measure ANOVA. Values are means ± SD; n = 4.

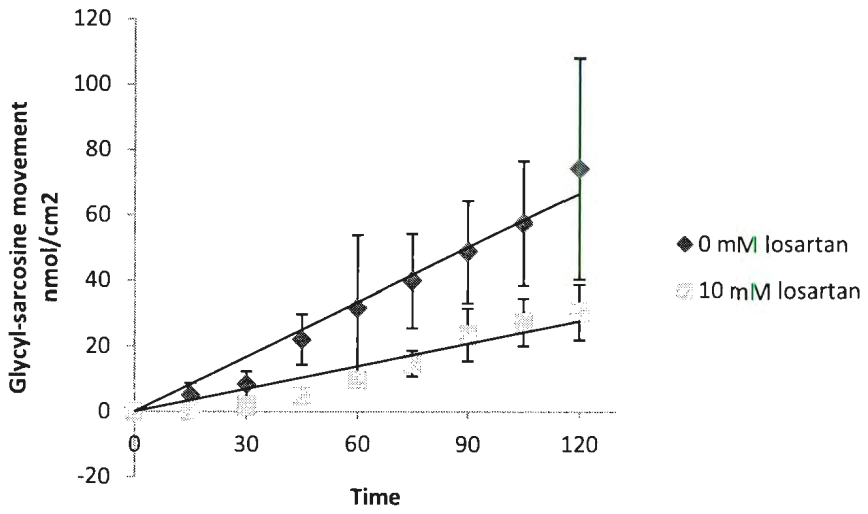


Figure 3-5 Glycyl-sarcosine movement through neonatal pig ileum with time. Values are means ± SD; n = 4.

inhibitor was not significantly different compared to when 1 mM of losartan was present ($p > 0.05$, $n = 3$) (Figure 3-4). Data were acquired from four different Ussing chamber experiments, but tissues were obtained from three animals.

3.1.1. Transepithelial transport of glycyl-sarcosine in the ileum

Glycyl-sarcosine transport was also measured in sections of piglet ileum that were mounted in Ussing chambers, to determine if PepT1 inhibition would have a greater effect in the distal small intestine. Glycyl-sarcosine transport rate through the ileum was 0.56 ± 0.24 nmol/cm^2/min under standard conditions, which was significantly lower ($p < 0.01$) than glycyl-sarcosine transport rate in the jejunum under similar conditions (Figure 3-6). Ileal glycyl-sarcosine transport rate in the presence of 10 mM losartan resulted in significantly lower transport at 0.15 ± 0.10 nmol/cm^2/min compared to when no inhibitor was present ($p < 0.05$, $n = 4$) (Figure 3-7).

3.1.2. Tissue viability

Viability of excised intestinal tissues was determined by measuring transepithelial potential difference. Change with time of transepithelial potential difference after adding 50 mM glucose solution to the luminal buffer was recorded with both control (Figure 3-8A) and with inhibitor (Figure. 3-8B) conditions.

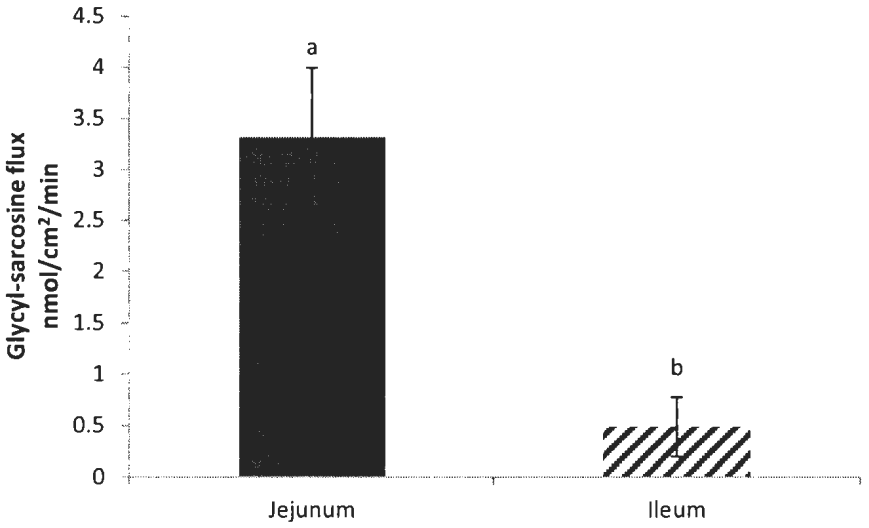


Figure 3-6 Comparison of glycyl-sarcosine transport through jejunum and ileum. Values are means ± SD; n = 4 or 5. P values were determined by Student's t-test. Groups with differing superscripts are significantly different at $P < 0.01$.

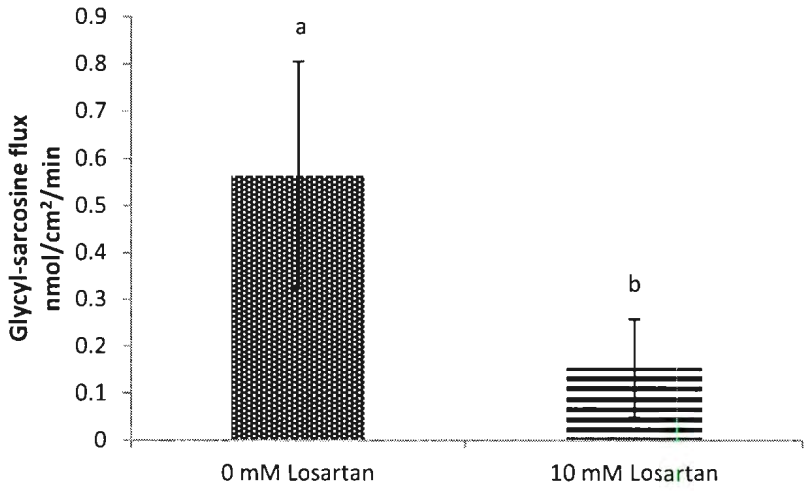


Figure 3-7 Glycyl-sarcosine transport through ileum with and without PepT 1 inhibitor. Values are means ± SD; n = 4. P value was determined by student t-test. Groups with differing superscripts are significantly different at $P < 0.05$.

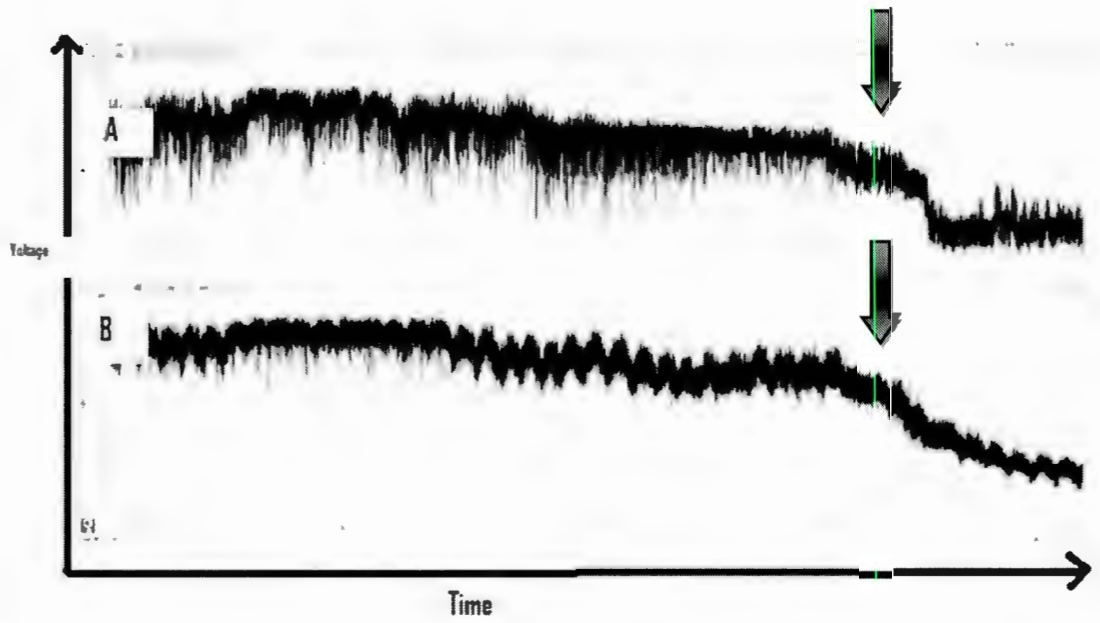


Figure 3-8 **Transepithelial potential difference measured using EK1 electrode kit.** After measuring the glycyl-sarcosine movement over 120 min, 50 mM glucose was added to the luminal buffer (arrow) and the change in the potential difference was measured without PepT1 inhibitor (A) and with PepT1 inhibitor (B).

3.2. Effect of lysine-containing dipeptides on arginine uptake by the rBAT/$b^{0,+}$ system

Arginine uptake in the presence of differing concentrations of lysine-containing dipeptides was measured using the *in vivo* gut loop method. Five 21 d old piglets were assigned to each condition, and 5 or 6 loops were created in each individual animal.

3.2.1. Effect of differing concentrations of lysyl-lysine on arginine uptake

The uptake of arginine into loops of proximal intestine perfused with varying concentrations of lysyl-lysine or 20 mM L-lysine is shown in Figure 3-9. Significantly higher arginine uptake (81% higher than control) was observed when 20 mM lysyl-lysine was added to the perfusate, compared to arginine alone ($p < 0.05$). Arginine uptake with 10 and 50 mM lysyl-lysine (139%, 131% of control, respectively) was not significantly different from the control condition. Moreover, 20 mM L-lysine (120% of control) was also not significantly different.

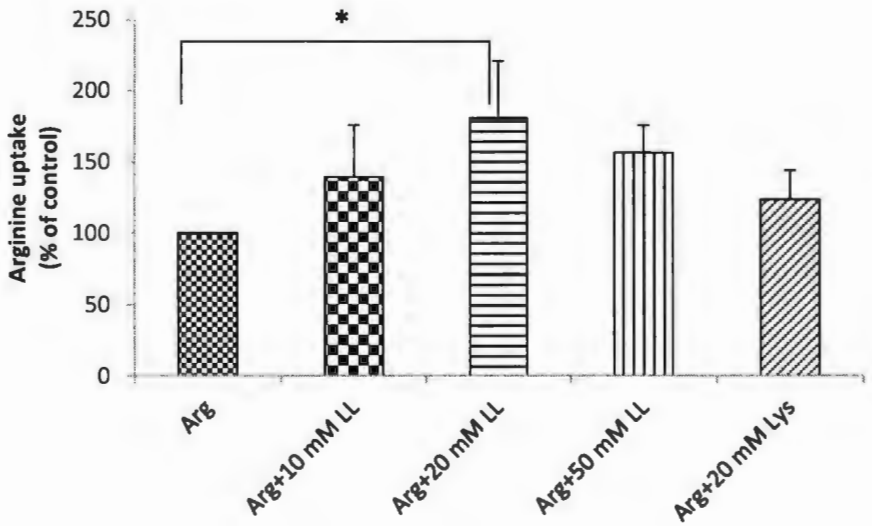


Figure 3-9 Arginine uptake in the presence of lysyl-lysine dipeptide or free lysine. Arginine (Arg) uptake was determined by the disappearance of ^{3}H-arginine + arginine (10 mM) in the absence (control) or presence of 10, 20, 50 mM of lysyl-lysine (LL) or 20 mM L-lysine. Data were expressed as the percentage of baseline, which was arginine disappearance from luminal buffer when perfused alone. Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA (*P <0.05).

3.2.2. Effect of differing concentrations of lysyl-glycine on arginine uptake

Unlike lysyl-lysine, lysyl-glycine did not enhance arginine uptake at any concentration in perfused loops of proximal jejunum compared to arginine alone (Figure 3-10). Arginine uptake with 50 mM lysyl-glycine (124% of control) or 20 mM L-lysine (130% of control) was not significant. However, when 20 mM free glycine was included in the perfusate, arginine uptake was 47% higher than in the control condition ($p < 0.05$) (Figure 3-10).

3.2.3. Effect of the aminopeptidase inhibitor amastatin on arginine uptake

Arginine uptake into the proximal intestine was measured with and without the aminopeptidase inhibitor amastatin under each of the following conditions: arginine alone, arginine with 20 mM lysyl-lysine, and arginine with 50 mM lysyl-glycine (Figure 3-11). Arginine perfused with amastatin did not change arginine uptake in proximal intestine compared to the control. A significantly higher arginine uptake was measured when perfused with 20 mM lysyl-lysine compared to arginine alone ($p < 0.05$), and the effect was abolished when the gut loop was perfused with amastatin and 20 mM lysyl-lysine simultaneously. Arginine uptake with 50 mM lysyl-glycine (181% of control) also was not significantly different (Figure 3-11).

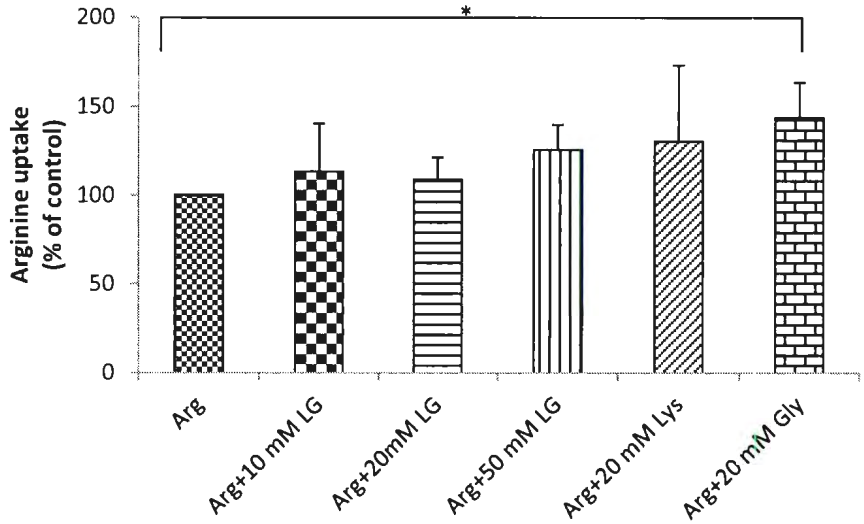


Figure 3-10 Arginine uptake with lysyl-glycine dipeptide. Arginine (Arg) uptake was determined by the disappearance of ^{3}H-arginine + arginine (10mM) in the absence (control) or presence of 10, 20, 50 mM of lysyl-glycine (LG) or 20 mM L-lysine (Lys) or 20 mM glycine (Gly). Data were expressed as the percentage of baseline, which was arginine disappearance from luminal buffer when perfused alone. Values are mean ± SD (n= 4). Data were analyzed by repeated measures ANOVA (* $P < 0.05$).

3.2.4. Free amino acid concentrations in intestinal tissue exposed to varying concentrations of lysyl-lysine and lysyl-glycine

Tissue free amino acid concentrations were measured in intestinal mucosa harvested after the cessation of the perfusion experiments; only the concentrations of arginine, lysine, ornithine and glycine under varying experimental conditions are reported.

3.2.4.1. Tissue free amino acid concentrations in jejunal mucosa following lysyl-lysine perfusion

Mucosal tissue free arginine concentration did not differ in the lysyl-lysine experiment, regardless of the concentration or form of lysine that was delivered in the perfusate (Figure 3-12 A). Tissue free ornithine concentrations were also similar under all experimental conditions (Figure 3-12 B). Compared to the control loop with no lysine in the perfusate, tissue free lysine concentration was significantly higher in intestinal loops exposed to 20 mM lysyl-lysine or 50 mM lysyl-lysine group (0.5 ± 0.2 versus 2.1 ± 0.8 or 2.9 ± 1.1 μmol/g, respectively) (Figure 3-12 C).

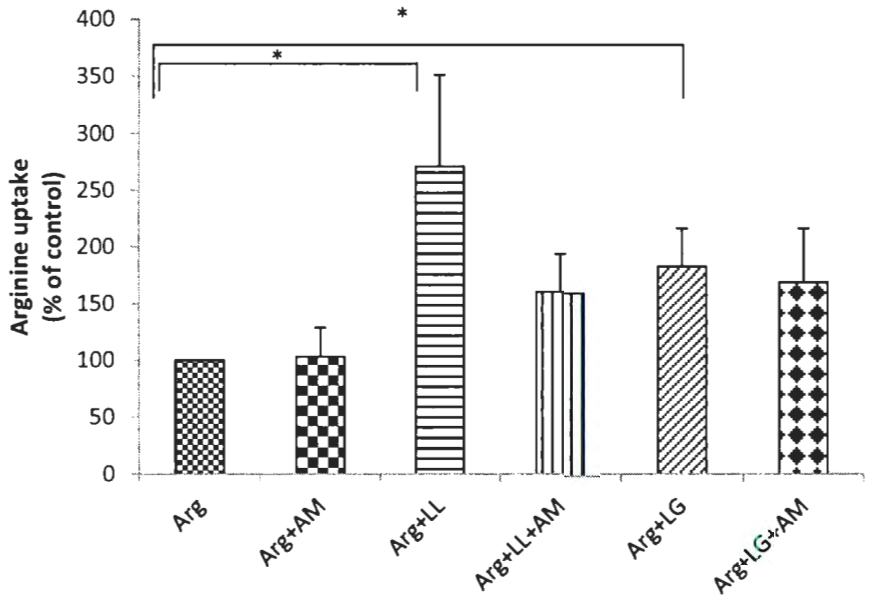


Figure 3-11 Effect of preincubation of small intestine with amino peptidase inhibitor amastatin (AM) on uptake of arginine (Arg). Arg uptake was determined by the disappearance of ^{3}H-arginine (10 mM) in the absence (control) or presence of 20 mM lysyl-lysine (LL) or 50 mM lysyl-glycine (GL) with or without 10 μM amastatin (AM). Data were expressed as the percentage of baseline, which was arginine disappearance from luminal buffer when perfused alone. Values are mean ± SD (n = 5). Data were analyzed by repeated measures ANOVA (*P < 0.05)

3.2.4.2. Tissue free amino acid concentrations in jejunal mucosa following lysyl-glycine perfusion

Mucosal tissue free arginine concentration did not differ in the lysyl-glycine experiment, regardless of the concentrations or form of lysine and glycine that were delivered in the perfusate (Figure 3-13 A). Similarly, tissue free ornithine concentrations were not significantly different under the varying experimental conditions (Figure 3-13 B). Tissue free lysine concentrations rose with increasing lysyl-glycine concentrations in the perfusate, and tissue free lysine concentration was significantly higher in loops perfused with 50 mM lysyl-glycine (7.3 ± 2.7 μmol/g) than in the control loop (1.5 ± 2.2 μmol/g) ($P < 0.05$) (Figure 3-13 C). However, during analysis of standards, the retention time of lysyl-glycine dipeptide peak and the L-lysine peak were found to be very close; as such, when the LG concentrations are high there is a likely possibility that there was overlap of these two peaks and lysine concentrations may not be accurate. Tissue free glycine concentration in the 20 mM glycine-treated loop (11.7 ± 6.9 μmol/g) was significantly higher than that in arginine alone control group (3.4 ± 1.5 μmol/g) (Figure 3-13 D). Tissue free glycine concentration in 10, 20 and 50 mM lysyl-glycine treated loops were not significantly different.

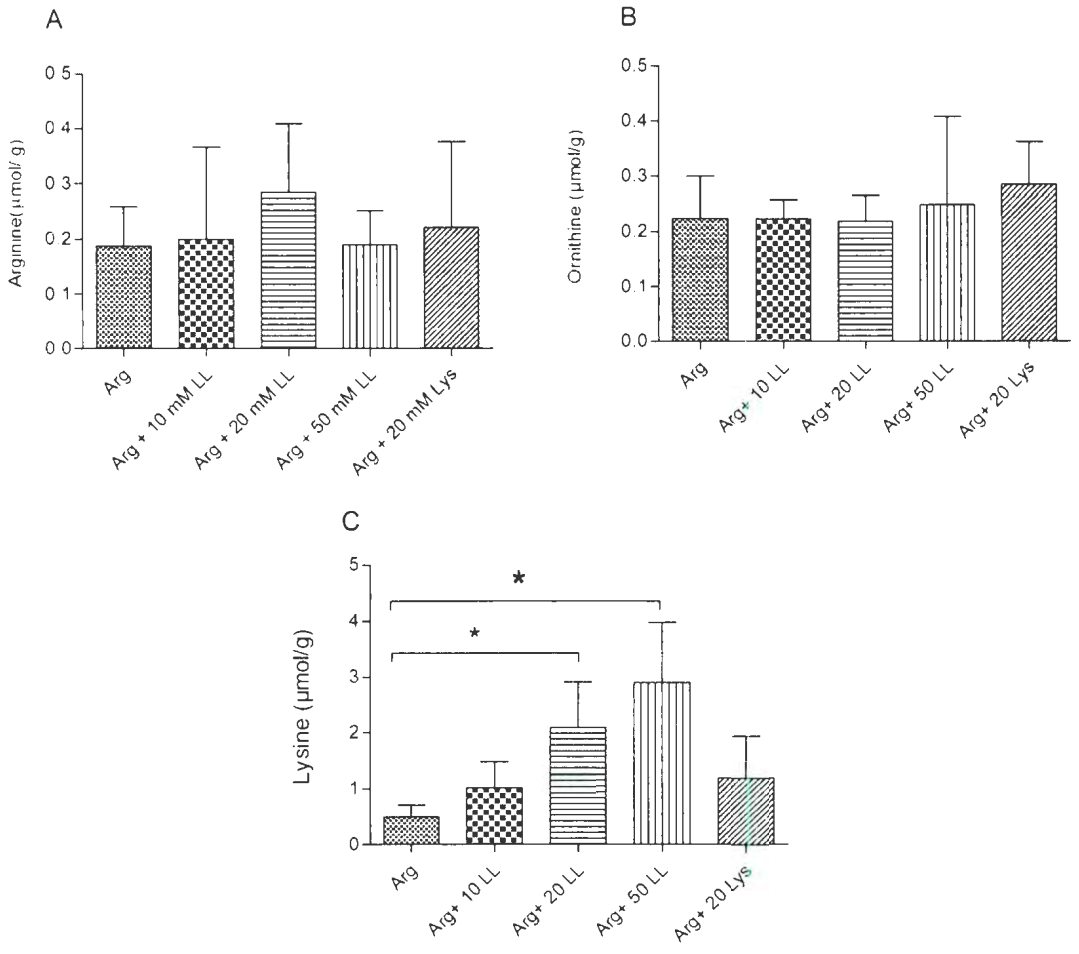


Figure 3-12 Tissue free arginine, ornithine and lysine concentrations in intestinal mucosa treated with differing forms and concentrations of lysine. Intestinal mucosal free amino acid concentrations of arginine (A), ornithine (B) and lysine (C). Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test (* $P < 0.05$).

3.2.4.3. Tissue free amino acid concentrations in jejunal mucosa following lysyl-lysine or lysyl-glycine perfusion with or without amastatin

Similar to previous two experimental conditions, tissue free arginine (Figure 3-14 A) and ornithine (3.14 B) did not exhibit any significant differences in response to changes in the luminal buffer. In the previous two experimental conditions, there was significantly higher tissue free lysine concentration with 20 mM lysyl-lysine or 50 mM lysyl-glycine; however, in this experiment, free lysine concentrations were similar in all experimental groups (Figure 3-14 C).

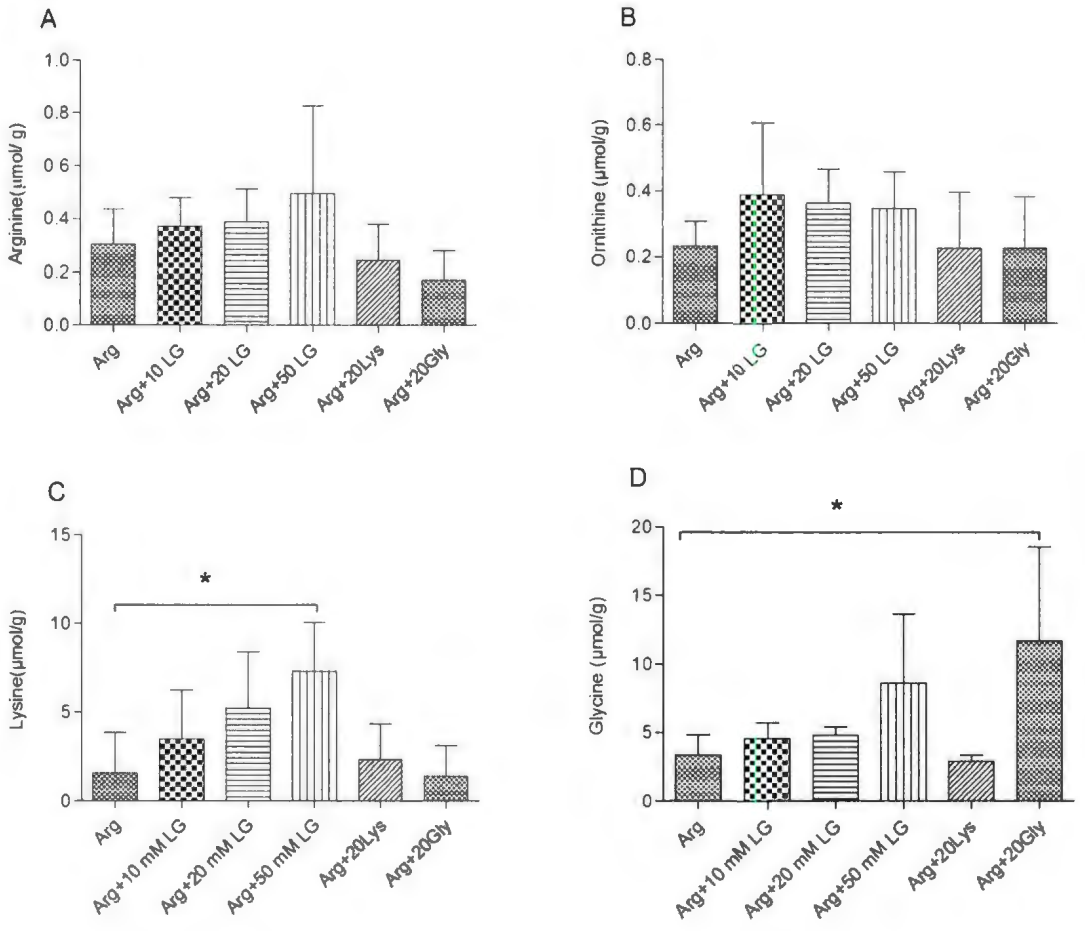


Figure 3-13 Tissue free arginine, ornithine, lysine and glycine concentrations in intestinal mucosa treated with differing forms and concentrations of lysine and glycine. Intestinal mucosal free amino acid concentration of arginine (A), ornithine (B), lysine or lysyl-glycine (C) and glycine (D). Values are means ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test (* $P < 0.05$).

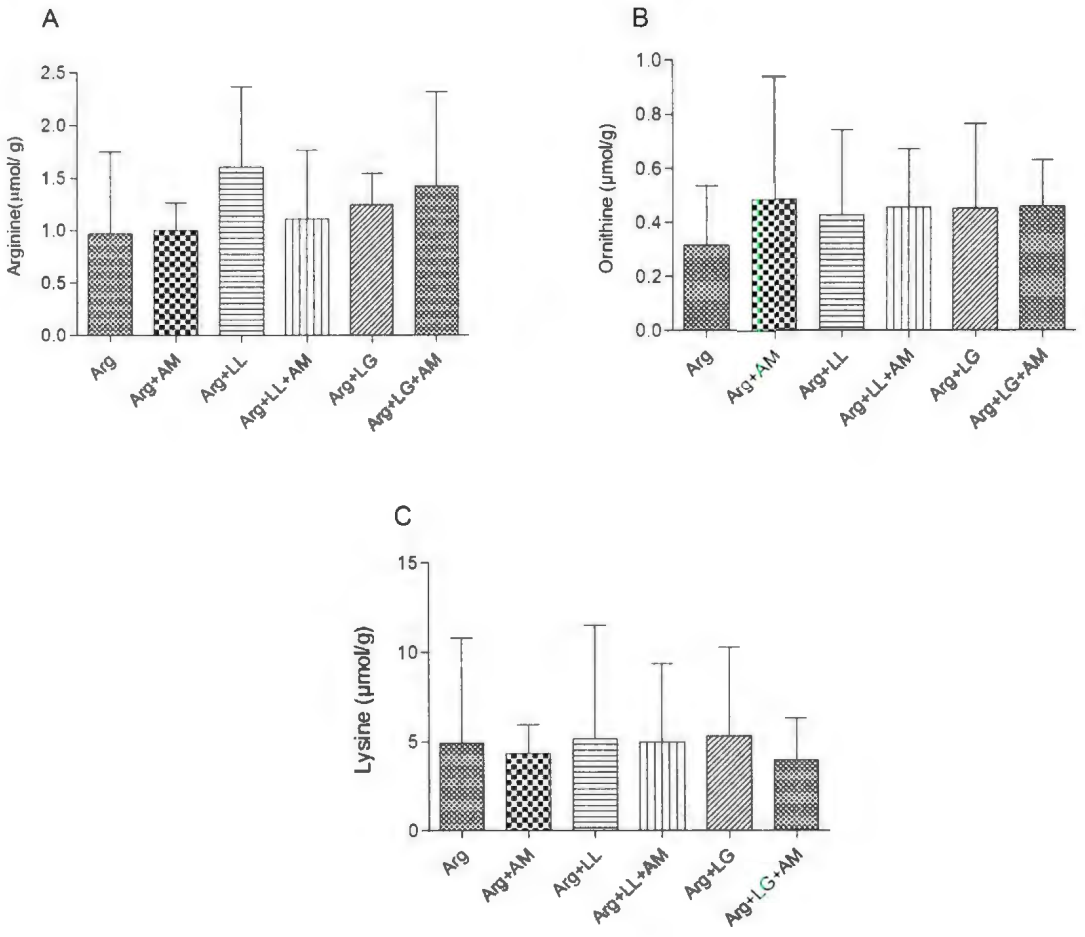


Figure 3-14 Tissue free arginine, ornithine, lysine and glycine concentrations in intestinal mucosa treated with differing forms and concentrations of lysine and glycine, with or without amastatin. Intestinal mucosal free amino acid concentrations of arginine (A), ornithine (B) and lysine or lysyl-glycine (C). Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test.

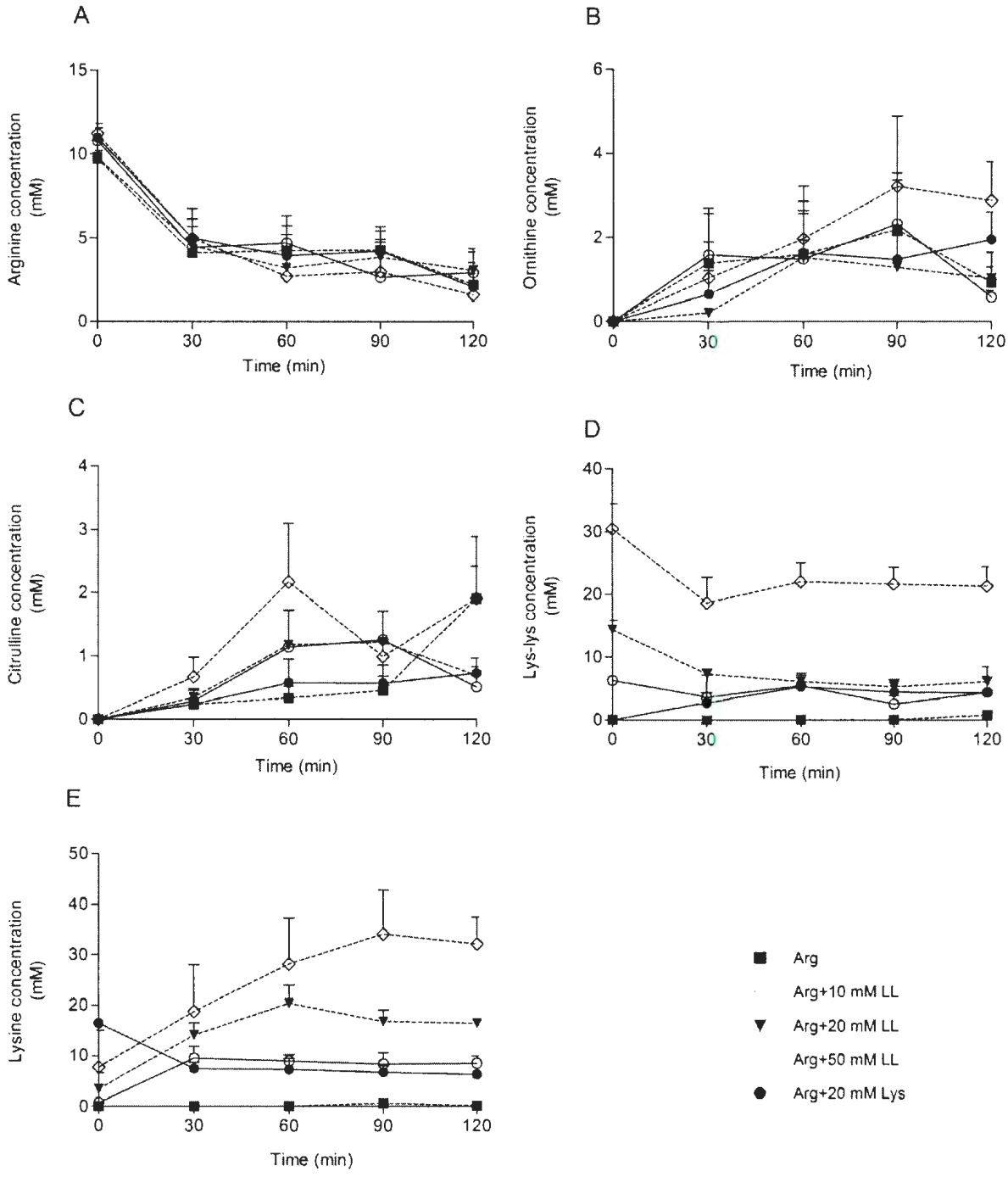
3.2.5. Amino acid concentrations in the luminal perfusates

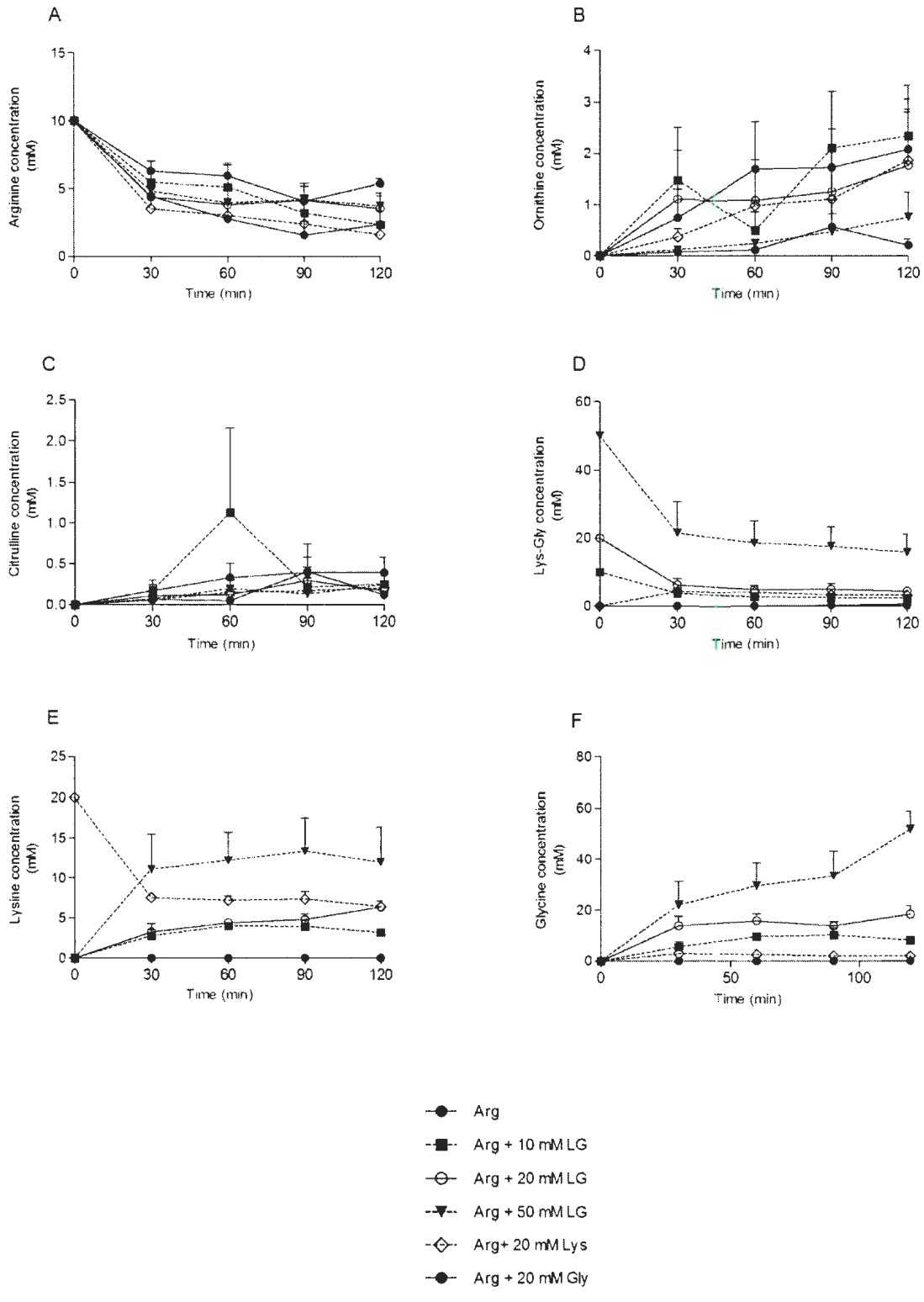
The amino acid concentrations in buffers perfused into the intestinal loops were measured in samples that were collected at 0, 30, 60, 90 and 120 min of the perfusion experiment.

3.2.5.1. Amino acid concentrations in luminal buffers sampled in the lysyl-lysine and lysyl-glycine experiments

The arginine concentration in the perfused buffers declined dramatically (~50%) in the first 30 min in all experimental conditions in both lysyl-lysine (Figure 3-15 A) and lysyl-glycine (Figure 3-16 A) experiments. Subsequently, arginine concentration appeared to plateau or decline only slightly over the next 90 min. Surprisingly, high concentrations of ornithine and citrulline were also found in the luminal buffer samples collected from 30 min onwards in the lysyl-lysine experiment (Figure 3-15 B and C) as well as the lysyl-glycine experiment (Figure 3-16 B and C). Scintillation counting of the fractions collected from citrulline and ornithine peaks demonstrated that the amino acids were radio-labeled with ^{3}H, and thus were at least partially derived from the perfused arginine. Neither ornithine nor citrulline appearance in the luminal buffer differed significantly amongst the various experimental treatments. Lysyl-lysine dipeptide concentrations in perfused buffers also decreased dramatically in first 30 min and appeared to plateau over next 90 min (Figure 3-15 D). Interestingly, the free L-lysine concentration in the lysyl-lysine buffers increased rapidly in first 30 min and afterwards it increased slightly or appeared to plateau (Figure 3-15 E) over next 90 min. Similarly,

lysyl-glycine concentration in perfused buffers declined rapidly in first 30 min and appeared to plateau or decline slightly over the next 90 min (Figure 3-16 D). Another interesting finding was spontaneous hydrolysis of dipeptides in perfusates. Analyses of the buffers sampled prior to the perfusion study demonstrated that 37%, 28% and 39% of the lysyl-lysine underwent spontaneous hydrolysis in the 10, 20 and 50 mM lysyl-lysine solutions, respectively (Figure 3-15 D). However, similar analyses of the lysyl-glycine buffers showed no hydrolysis prior to the start of the perfusion (Figure 3-16 D).


A
Arginine concentration (mM)
Time (min)
B
Ornithine concentration (mM)
Time (min)
C
Citrulline concentration (mM)
Time (min)
D
Lys-lys concentration (mM)
Time (min)
E
Lysine concentration (mM)
Time (min)
Arg
Arg+10 mM LL
Arg+20 mM LL
Arg+50 mM LL
Arg+20 mM Lys


A
Arginine concentration (mM)
Time (min)
B
Ornithine concentration (mM)
Time (min)
C
Citrulline concentration (mM)
Time (min)
D
Lys-Gly concentration (mM)
Time (min)
E
Lysine concentration (mM)
Time (min)
F
Glycine concentration (mM)
Time (min)
Arg
Arg + 10 mM LG
Arg + 20 mM LG
Arg + 50 mM LG
Arg+ 20 mM Lys
Arg + 20 mM Gly

4. Discussion

4.1.Transepithelial transport of dipeptides

The end products of luminal protein digestion are a mixture of free amino acids, di and tripeptides (Adibi & Mercer, 1973). *In vivo* studies in pigs have illustrated that it may be beneficial for absorption when amino acids are presented to the mucosa in the peptide-bound form (Rerat *et al.* 1992). This has also been demonstrated in humans through an *in vivo* experiment during which the absorption rates of amino acids were determined from a mixture of free amino acids or a corresponding mixture of the same amino acids provided as glycyl-dipeptides (Steinhardt & Adibi, 1986). However, the contribution of dipeptides to total amino acid uptake from dietary proteins is still unknown (Daniel, 2004a).

PepT1-mediated transcellular transport (Adibi, 1997) and passive paracellular movement (McCollum & Webb 1998) are recognized as the two major mechanisms for the transepithelial transport of di/tripeptides. In the current study, we investigated the proportion of dipeptide transported by the PepT1 mediated transcellular transport versus paracellular movement in the small intestine of Yucatan miniature piglets under *in vitro* conditions. In the first Ussing chamber study, the rate of transepithelial transport of glycyl-sarcosine was 3.87 ± 1. 50 nmol/cm^2/min. Winkler *et al.* (1999) also used the Ussing chamber system to measure glycyl-sarcosine uptake in pig jejunum, and reported a rate of 62 ± 12.6 nmol/cm^2/10 min. The similarity of these values shows that the

Ussing chamber system can be considered a consistent *in vitro* model to measure transepithelial transport of dipeptides in piglet intestine.

4.1.1. Transepithelial transport of glycyl-sarcosine with different concentrations of PepT1 inhibitors

Our experimental design was based on measuring passive paracellular movement by inhibiting the PepT1-mediated pathway. Therefore, the availability of a potent PepT1 inhibitor (losartan) was essential to the study. In a preliminary study, the transepithelial movement of glycyl-sarcosine across the jejunal epithelium was measured with differing concentrations of losartan in order to determine the concentration that optimally inhibited PepT1. We observed that the addition of 1 mM losartan to the luminal buffer significantly reduced the transepithelial transport of glycyl-sarcosine in piglet jejunum by 52%. Interestingly, further increases in losartan concentration to 5 or 10 mM did not produce a significantly different transport rate compared to 1 mM of inhibitor. However, all losartan concentrations resulted in inhibition of PepT1-mediated transport, and all were significantly different from control group when no losartan was added (Figure 3-2). To the best of my knowledge, we are the first to demonstrate inhibition of PepT1-mediated dipeptide transport by losartan in piglet intestine. Previously, Knutter *et al.,* (2009) reported a strong inhibition of PepT1 mediated uptake of dipeptides into Caco-2 cells with 1 mM of losartan. In that study, Knutter and colleagues measured the cellular uptake of glycyl-sarcosine into Caco-2 cells, which only represents PepT1-mediated transport. However, our model is more physiological than Caco-2 cell lines

which are known to poorly express some metabolic enzymes (Sun *et al.,* 2008). Also, tight junction permeability of Caco-2 cell lines is lower compared to that of human small intestine (Sun *et al.,* 2008). Hence, we conclude that maximum inhibition by losartan on PepT1 transporters is satisfied at 1 mM concentration in piglet jejunum under *in vitro* conditions.

4.1.2. Transepithelial transport of glycyl-sarcosine through jejunum with and without PepT1 inhibitor

It was a surprise to find that the addition of losartan lowered transepithelial transport of glycyl-sarcosine by only 47 to 53%; that is, addition of losartan to the luminal buffer acted to inhibit PepT1-mediated transport such that ~ 50% of the dipeptide movement occurred via the passive paracellular route. Those values for paracellular movement were higher than we expected. However, our estimate of the proportion of dipeptides that were transported via paracellular movement was likely an overestimation, because our PepT1 inhibitor may not have inhibited PepT1 by 100%. Knutter *et al.* (2009) reported that losartan inhibited PepT1 mediated uptake by 80-85% at 1 mM concentration. Thus, ~7-10% of the transport that was attributed to paracellular movement may have actually been due to PepT1-mediated transport, because incomplete inhibition confounded the data. Other than incomplete inhibition of the PepT1 transporter, the *in vitro* Ussing chamber model has its own limitations. One of the main weaknesses is that it is missing the complex physiological system of intestinal mucosa (Clarke, 2009). The excision of tissue from the animal suppresses the action of

any endocrine or nervous stimuli on intestinal mucosa (Clarke, 2009). That would reduce the intestinal tonicity such that the tissue becomes "leakier" than *in vivo*.

Other studies have reported both high and low passive paracellular movement of peptides or peptidomemetic drugs, using a variety of models. In an *in vitro* everted gut sac study, approximately 80% of ampicillin transport occurred via passive paracellular movement (Lafforgue *et al.*, 2008) even though ampicillin is a substrate for PepT1 (Bretschneider *et al.*, 1999). In contrast, a study conducted in Caco-2 cells reported PepT1-mediated transepithelial transport of glycyl-sarcosine to be 69% to 87% of total transport (Scow *et al.*, 2011). However, it has been reported that narrow pores of the tight junctions in Caco-2 cell lines cause an underestimation of the paracellular route contribution (Nagahara *et al.*, 2004), which may have reduced paracellular movement of dipeptides in the Caco-2 model. Two studies conducted in adult PepT1 knockout mice reported 70% (Chen *et al.*, 2010) and 78% (Nässl *et al.*, 2011) lower transepithelial transport of glycyl-sarcosine compared to wild type mice, which suggests a higher contribution of PepT1-mediated transport of dipeptides in mice intestine compared to our *in vitro* model. Data on the importance of PepT1-mediated transport collected from the knockout mouse model are quite different than our findings, and may be due to inter-species variation of PepT1 expression; however, it is more likely related to developmental differences in the models. Intestinal permeability, particularly in the proximal jejunum, is likely very high in 18 day old piglets in contrast to that in adult mice. Further studies are necessary to understand the discrepancy in data on PepT1-mediated transcellular transport of dipeptides from different experimental models.

Mannitol is commonly used as a probe for paracellular permeability in intestinal research *in vitro* (Madara & Stafford, 1989). Unidirectional ^{14}C-mannitol movement was measured in the current study in order to determine if the PepT1 inhibitor losartan impacted paracellular permeability, which would also confound the results. Mannitol transport through piglet jejunum in the presence of 1 mM losartan was not altered (Figure 3-4); thus, paracellular permeability was not affected, and losartan was shown to be a good choice as a PepT1 inhibitor under our experimental conditions.

Tissue viability is an important factor for *in vitro* studies. A considerable change in the potential difference was observed after adding of 50 mM D-glucose to the luminal buffer at the end of the experiment which demonstrated that tissues were still viable (Figure 3-8). The potential difference across the membrane is produced by the active transport of the electrogenic ions (Clarke, 2009). Once glucose is added to the luminal-side buffer, the Na^+ coupled glucose transporter on the apical membrane transports glucose into the enterocyte (Takata, 1996). This event contributes to a high intracellular Na^+ ion concentration, and the Na^+/K^+ ATPase pump in the basolateral membrane actively transports Na^+ to reduce the intracellular Na^+ concentration. The process of transporting Na^+ ions from the luminal to the basolateral side creates the potential difference. In order for that process to happen, the cells need to be viable as the Na^+/K^+ ATPase pump needs cellular energy. Therefore, we can conclude that the intestinal segments used in Ussing chamber experiment were viable at the end of experiment.

4.1.3. Transepithelial transport of glycyl-sarcosine in the ileum with and without PepT1 inhibitor

Paracellular movement in the ileum is known to be lower compared to the jejunum, as reduced inter-cellular space reduces the leakiness of the ileum (Lacombe *et al.*, 2004). We hypothesized that the contribution of the paracellular movement to total dipeptide transport would be lower in ileal compared to jejunal tissue. We observed only 27% paracellular movement in ileum while PepT1-mediated transcellular transport was responsible for 73% of the glycyl-sarcosine transport. Also, contribution of PepT1-mediated transcellular transport in total glycyl-sarcosine transport across the ileal tissue was 20% higher compared to jejunal segments. Therefore, as a proportion of total dipeptide uptake, PepT1-mediated transcellular transport may be of greater importance in the distal compared to the proximal small intestine segments.

4.1.4. Transepithelial transport of glycyl-sarcosine in the jejunum and ileum without PepT1 inhibitor

In the current study we found the overall transepithelial transport of glycyl-sarcosine was significantly lower in the ileum than jejunum under control conditions, and was more affected in the ileum when PepT1 transport was inhibited (Figure 3-7). Ungell *et al.* (1998) have reported that the available surface area of the intestine decreases from the jejunum to the ileum. A reduction in available surface area could cause absorption to drop in the ileum compared to the jejunum. Although PepT1-mediated transcellular transport contributes a greater proportion of total peptide uptake

in the ileum than the jejunum, PepT1 transporters exist in a relatively low concentration in the distal intestine of 18 day old piglets. The piglets we used in the current study had been fed only highly digestible sow milk;PepT1 expression depends highly on substrate exposure (Adibi, 2003). Supporting this argument, Wang *et al.,* (2009b) reported the lowest PepT1 expression in the ileum of 21 day old Tibetan piglets, and Chen *et al.,* (1999) reported lower PepT1 expression in the ileum compared to the jejunum. Hence, without the contribution of paracellular movement and low PepT1 expression in the ileum, total dipeptide transport capacity in the ileum is likely lower compared to the jejunum. Thus, proximal intestine plays a major role in peptide absorption in young neonates.

4.2.Effect of luminal dipeptides on trans-stimulation of rBAT/$b^{0,+}$

Arginine and lysine are cationic amino acids which share the rBAT/$b^{0,+}$ system for intestinal uptake. Lysine is considered the first limiting amino acid in the pig diet (Baker, 2007), and arginine is an indispensable amino acid in both neonates and growing animals. The Na^+-independent rBAT/$b^{0,+}$ system has been described as the major transport mechanism for arginine uptake into enterocytes. Wenzel *et al.*, (2001) reported that 85% of arginine uptake takes place via the rBAT/$b^{0,+}$ system. In the present study we investigated the effect of lysine-containing dipeptides on arginine uptake through rBAT/$b^{0,+}$ transporter.

4.2.1. Arginine uptake with lysyl-lysine dipeptides

To my knowledge, we are the first to demonstrate a positive effect of dipeptides on arginine uptake in an in situ gut loop model. In the presence of 20 mM lysyl-lysine, L-arginine uptake was greater compared to when arginine was perfused alone (Figure 3-9). Enhanced arginine uptake that we measured in response to dipeptides, and likely via the rBAT/$b^{0,+}$ system, is termed trans-stimulation. This process was first described as enhanced arginine uptake by human rBAT injected xenopus oocytes that had elevated intracellular cationic amino acid concentrations (Chillarón *et al.,* 1996). The potential for lysine dipeptides to enhance arginine uptake was described in a study conducted in Caco-2 cells (Wenzel *et al.,* 2001). In that study, cells pre-incubated with 10 mM lysyl-lysine demonstrated enhanced arginine uptake (293.1 ± 5.7%) compared to the controls. In contrast, we did not observe significant enhancement of arginine uptake with 10 mM lysyl-lysine. In our *in vivo* model, intracellular free amino acid concentration generated by 10 mM lysyl-lysine may not have been high enough to trigger trans-stimulation of rBAT/$b^{0,+}$ system. Intracellular free amino acid concentration is the main determinant of trans-stimulation (Chillarón *et al.,* 1996). We measured the tissue-free lysine concentration in the mucosa scraped from the perfused gut sections and found that the concentrations changed in parallel with perfusate lysyl-lysine or free lysine concentrations. The tissue free lysine concentration was lowest in the control condition (arginine alone) and was significantly higher in tissue exposed to 20 mM or 50 mM lysyl-lysine (Figure 3-12 C). Thus, intracellular cationic free amino acid concentration may induce trans-stimulation, as arginine uptake was significantly higher when

perfused with 20 mM lysyl-lysine. However, we also found that the addition of 50 mM lysyl-lysine did not produce any further benefit to arginine uptake compared to 20 mM condition. To follow-up on this result, we measured the amino acid and dipeptide concentrations of the buffers that were sampled during the perfusion studies. Lysyl-lysine when added at 50 mM concentration underwent extensive intraluminal hydrolysis during the perfusion study, such that the free lysine concentration in the perfusate was very high. After 120 min of perfusion, the luminal lysyl-lysine concentration in the buffer had decreased to approximately 21 mM (Figure 3-15 D) and luminal free lysine concentration had increased to 32 mM (Figure 3-15 E). The high free lysine concentration in the luminal buffers may have competed with arginine for the common transporter, resulting in less total uptake of arginine. The appearance of free lysine due to luminal hydrolysis in the 10 mM and 20 mM lysyl-lysine treatments was 8.5 and 16.4 mM, respectively; thus, the lower free lysine concentrations may not have interfered with rBAT/$b^{0,+}$ -mediated arginine uptake. Wenzel et al. (2001) investigated whether the maximum rate of arginine uptake via trans-stimulation was predicted by limitations in dipeptide transport or by intracellular dipeptide hydrolysis. They reported that when Caco-2 cells were exposed to increasing lysyl-lysine concentrations, arginine uptake over time followed a sigmoidal pattern, and the lysyl-lysine EC_{50} value for trans-stimulation was ~ 0.5 mM; maximal trans-stimulation of arginine uptake occurred at ~ 10 mM lysyl-lysine, after which point the rate of arginine uptake plateaued. In our study, the perfusion of gut loops with 50 mM lysyl-lysine may have exceeded the maximal rate of transport that can be stimulated by intracellular lysine. Furthermore, we

found very high free lysine concentration in the buffers during and after the perfusion study with the 50 mM lysyl-lysine condition; thus, it is possible that luminal free lysine may have been taken up by rBAT/$b^{0,+}$, simply exchanging intracellular lysine for extracellular.

In summary, luminal lysyl-lysine appeared to have the capability to enhance arginine uptake by trans-stimulating the rBAT/$b^{0,+}$ system. Trans-stimulation of rBAT/$b^{0,+}$ system seems to be due to increased intracellular free lysine concentration generated by lysyl-lysine hydrolysis.

4.2.2. Arginine uptake with lysyl-glycine dipeptides

In a previous study by others, intracellular free amino acid concentration was found to be a key factor in achieving trans-stimulation of the rBAT/$b^{0,+}$ system (Chillaron *et al.*, 1996). In the present study, lysyl-glycine dipeptide was tested along with lysyl-lysine to further clarify the effect of intracellular cationic amino acid concentration on trans-stimulation of the rBAT/$b^{0,+}$ system. Thwaites *et al.* (1995) reported that glycine was not transported via the rBAT/$b^{0,+}$ system. Thus, we hypothesized that the transport of lysyl-glycine would not enhance arginine uptake to the same extent as lysyl-lysine. Free glycine produced by intracellular hydrolysis of lysyl-glycine should not contribute to enhanced arginine uptake if glycine is not a substrate for counter-transport by the rBAT/$b^{0,+}$ transporter. In the current study, arginine uptake was not significantly higher with 10 (113% of control), 20 (108%) and 50 (125%) mM lysyl-

glycine, compared to the control loops (Figure 3-10). In contrast, Wenzel *et al.*, (2001) reported that Caco-2 cells that were pre-incubated with glycyl-arginine and lysyl-lysine demonstrated an enhanced arginine uptake under both conditions, but arginine uptake was lower when cells were exposed to glycyl-arginine compared to lysyl-lysine. They further demonstrated that glycyl-glycine had no effect on trans-stimulation of the rBAT/$b^{0,+}$ system. It may have been interesting to have included a glycyl-glycine perfusion in our *in vivo* model, to confirm that the system responds only to intracellular concentrations of cationic amino acids.

It was surprising that the 50 mM lysyl-glycine treatment did not produce a significant effect on arginine uptake, as it should result in a similar intracellular free lysine concentration as 20 mM lysyl-lysine. A study conducted in PepT1-expressed MDCK cells demonstrated that the transport affinity for dipeptides via PepT1 is as follows: neutral–neutral > charged–neutral = neutral–charged > acidic–acidic > basic–basic (Vig *et al.*, 2006). For this reason, lysyl-glycine should be taken up into enterocytes more efficiently than lysyl-lysine and increase the tissue free lysine and glycine concentration. We measured the tissue free lysine and glycine concentration of perfused gut loop mucosa and found that lysine concentrations increased with increasing exposure to lysine (as lysyl-glycine). However, the tissue free lysine concentration in the lysyl-glycine perfused mucosa (Figure 3-13C) was much higher than that resulting from 50 mM of lysyl-lysine perfusion (Figure 3-12 C). The apparent high lysine concentration following the glycyl-lysine perfusion may be due to a methodological problem, as there appeared to be inadequate separation between lysyl-glycine and lysine

in the HPLC analysis. We suspect this because tissue free glycine (the other hydrolysis product of lysyl-glycine) did not increase with higher lysyl-glycine treatments (Figure 3-13 D). Another possible factor resulting in low tissue free glycine concentration in lysyl-glycine group is the inefficient hydrolysis of lysyl-glycine in enterocytes. Kottra *et al.* (2009) reported that the amino acid present on the carboxyl terminal affects the hydrolysis rate of dipeptides. They reported that glycine-containing dipeptides are more hydrolysis-resistant than other dipeptides. Accordingly, the high lysine + lysyl-glycine peak determined by HPLC (and the low glycine concentration) could be due to the persistence of intact lysyl-glycine. It follows then, that the inefficient hydrolysis of lysyl-glycine may not have produced a high enough intracellular free lysine concentration to trigger the trans-stimulation of rBAT/$b^{0,+}$. Better separation techniques for lysyl-glycine and lysine are needed to further explore this outcome.

4.2.3. Effect of aminopeptidase inhibitor on trans-stimulation of rBAT/$b^{0,+}$ system

Intracellular hydrolysis is an essential step to increase intracellular free amino acid concentrations in order for dipeptides to induce trans-stimulation of rBAT/$b^{0,+}$ (Wenzel *et al.*, 2001). To initiate trans-stimulation, the intracellular free amino acid concentration may have to reach an elevated level to bind with the intracellular binding site of rBAT/$b^{0,+}$. In the current study, we hypothesized that interfering with dipeptide hydrolysis using an aminopeptidase inhibitor would reduce the intracellular free amino acid concentration, in turn diminishing the favorable effect demonstrated by lysine-

containing dipeptides on arginine uptake. Indeed, we discovered that exposing the gut loops to the peptidase inhibitor amastatin abolished the potentiating effects of 20 mM lysyl-lysine on arginine uptake. Surprisingly, when we repeated a loop with arginine and 50 mM glycyl-lysine (with no inhibitor), arginine uptake was enhanced significantly, in contrast to the first experiment; however, the effect was abolished when arginine and glycyl-lysine were perfused with amastatin (Figure 3-11). These data support our hypothesis that the addition of the aminopeptidase inhibitor amastatin with dipeptides would impede the trans-stimulation activity by reducing intracellular hydrolysis of dipeptides. Wenzel *et al.* (2001) reported a similar observation where the trans-stimulation effect produced by glycyl-arginine was abolished when amastatin was added to the incubation media of Caco-2 cells. Importantly, these authors have also demonstrated that trans-stimulation produced by incubation of cells with free amino acids was not affected by the presence of aminopeptidase inhibitor. Taken together, our findings along with previously reported data clearly demonstrate that intracellular free cationic amino acid concentration is a key factor for trans-stimulation of the rBAT/$b^{0,+}$ system. Furthermore, the intracellular free amino acid concentration generated by PepT1-mediated dipeptide uptake potentiates the effect likely because dipeptides are transported into enterocytes more efficiently and rapidly than free amino acids (Adibi, 1971).

4.2.4. Arginine disappearance with free luminal lysine

The cationic amino acids arginine and lysine enter enterocytes via the rBAT/$b^{0,+}$ system (Van Winkle *et al.,* 1988). Studies conducted in chicks (Austic & Scott, 1975), dogs (Czarnecki *et al.,* 1985) and guinea pigs (O'Dell & Regan, 1963) have reported arginine and lysine antagonism for intestinal uptake as they share the rBAT/$b^{0,+}$ system for cellular uptake. However, the importance of the antagonism between these two cationic amino acids is controversial in pigs, as Edmonds & Baker (1987) did not demonstrate an antagonistic effect on arginine with excess lysine. In the current study we were also unable to observe lysine-arginine antagonism, as 20 mM of free lysine (123-130% of control (Figures 3-9 and 3-10)) did not impair arginine uptake compared to the control situation with no lysine.

We hypothesized that a high concentration of free lysine in the luminal buffers may compete with arginine for uptake by the common transporter; as such, the delivery of lysine as a dipeptide should be more efficient. Conversely, in the current study we did not observe either favorable or negative effect with the free lysine condition. However, high free lysine concentration generated by spontaneous hydrolysis and perhaps brush border hydrolysis during the perfusion of 50 mM lysyl-lysine seemed to negatively influence trans-stimulated arginine uptake. In contrast to our observation, the study conducted in Caco-2 cell culture reported that arginine uptake was enhanced by 246% with 10 mM free lysine (Wenzel *et al.,* 2001). However, they also reported that the potentiating effect of free lysine was significantly lower compared to lysyl-lysine dipeptide. In our study, we observed that arginine uptake was similar when loops were

perfused with free lysine compared to when 10 mM lysyl-lysine was added to the perfusate. Lack of arginine-lysine antagonism in piglet intestine could be due to the uptake of cationic amino acids by alternative transporters. Because of the importance of arginine and lysine to the growing neonate, it is important to further investigate the presence or absence of an antagonistic effect of high lysine on arginine uptake.

4.2.5. Appearance of arginine metabolites in perfused buffers

It was surprising to measure high concentrations of ornithine and citrulline in the perfused buffers. Ornithine and citrulline are major catabolic products of arginine and they are also components of the urea cycle (Wu & Morris, 1998). Scintillation counting of the fractions collected from citrulline and ornithine peaks demonstrated that the amino acids were radio-labeled with ^{3}H. This finding confirms that the ornithine and citrulline in the buffers were derived from the arginine tracer. Arginine hydrolysis by arginase results in the production of urea and ornithine (Wu *et al.*, 1997). Interestingly, a study conducted in developing pig enterocytes reported that arginase activity in intestinal enterocytes is minimal during the first 21 days of life, and then increases (Wu, 1995). We also studied 21 day old piglets, and expected that arginase activity would be low in the intestine, with minimal conversion of arginine to ornithine. I have measured arginase activity in 4 mucosal samples, but could not detect any arginase activity (results not shown). Thus, it will be interesting to investigate the underlying mechanism of this outcome.

4.3. Conclusions

We have established and validated the Ussing chamber model as an *in vitro* experimental model in our laboratory by demonstrating intestinal tissue viability over two hours. To my knowledge, we are also the first to demonstrate inhibition of the PepT1 transporter by losartan in intestinal tissue sampled from piglets.

The results of our investigations on transepithelial transport of dipeptides have demonstrated that passive paracellular movement appears to contribute more to total transepithelial transport than PepT1-mediated transcellular transport in the jejunum of 18 day old piglets. The high proportion of passive paracellular movement in the jejunum is likely due to "leakiness" and perhaps to relatively low expression of the PepT1 transporter in jejunum during the late suckling period. Also, the high transepithelial transport observed in jejunum compared to ileum could be considered developmental adaptation to absorb highly digestible sow milk in the upper gut during the suckling period. However further studies could be done to more carefully explore the high transepithelial rate of transport and high paracellular movement observed in jejunum compared to ileum. The *in vivo* gut loop method would be a better experimental model to measure transport activity in a more physiological situation. Further, contribution of the paracellular and PepT1 mediated transcellular pathways on transepithilial transport of dipeptides should be measured in jejunal and ileal sections of the gut in pre- and post-weaned piglets to determine the developmental changes.

The results of my *in vivo* gut loop study suggested that 20 mM lysyl-lysine was capable of enhancing arginine uptake by trans-stimulating the rBAT/$b^{0,+}$ system. However, the contribution of the rBAT/$b^{0,+}$ system in trans-stimulation has yet to be verified. A study which specifically inhibits the rBAT/$b^{0,+}$ system would be beneficial in determining whether it is involved in the trans-stimulation effect that is enhanced by dipeptides.The high free lysine concentration that appeared in the perfusion buffer both prior to and during the experiment with 50 mM lysyl-lysine may have contributed to a reduced potential effect on arginine uptake compared to 20 mM lysyl-lysine, due to competition of lysine with arginine for rBAT/$b^{0,+}$; however, we do not have enough evidence to conclude that this outcome is the result of competition for the transporter, as arginine uptake was not lower than the control situation. It is possible that free luminal lysine may have exchanged with intracellular lysine via bBAT/$b^{0,+}$ system. Therefore, further experiments are required to understand this scenario. Tissue free amino acid analysis suggested that the absence of trans-stimulation with 50 mM lysyl-glycine may have been due to lack of intracellular hydrolysis of lysyl-glycine. Also, our experiments performed with the amino peptidase inhibitor showed that intracellular hydrolysis of dipeptides is critical step for successful trans-stimulation of the rBAT/b0,+ system.

Although the current study answered our research question about trans-stimulation of rBAT/$b^{0,+}$ system, it also brought to light some important questions requiring further research. We did not observe arginine-lysine antagonism when lysine and arginine were presented to the absorptive surface at the same concentration, but we observed a negative effect on trans-stimulation with higher free luminal lysine

concentration. Lysine is considered the first limiting amino acid in the pig diet and the second limiting amino acid for poultry (Baker, 2007) and arginine is an indispensable amino acid in neonates; thus, it is important to investigate varying antagonistic effects. Another important question arises from the appearance of isotopically labeled ornithine and citrulline in the perfusates. It remains to be determined whether arginase activity was higher in our piglets than previously reported in suckling piglets; furthermore, even with significant arginase activity, we also need to understand more about the transporting mechanism of these arginine metabolites back to the lumen.

To the best of my knowledge, we are the first to demonstrate the interaction between the uptake of arginine and lysine-containing dipeptides at the cellular level in an *in situ* model. Understanding the interrelationships between free amino acids and peptide absorption could be used to enhance the delivery of nutritionally important amino acids, particularly during intestinal stress such as during weaning or infection.

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