The role of peptide transporter 1 (PepT1) in maintaining health in neonates

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A thesis submitted to the School of Graduate Studies at Memorial University of Newfoundland in the degree of Doctor of Philosophy

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Memorial University of Newfoundland

September 2022

St.John's, Newfoundland and Labrador

Abstract

Peptide transporter 1 (PepT1) carries dietary tri-/dipeptides, bacterial peptides and peptidomimetic drugs in the small intestine (SI). The first study in this thesis describes the interaction between the dipeptide lysyl-lysine and arginine uptake from the SI in Yucatan miniature piglets. This study confirmed that arginine uptake was significantly higher when delivered with lysyl-lysine compared to equimolar amount of free lysine. We speculated that more efficient uptake of lysine as a dipeptide led to enhanced arginine uptake through the trans-stimulation of the $b^{0,+}$ transporter. The activity of PepT1 was preserved during the use of parenteral nutrition (PN). As such, the objective of the second study was to determine if there were advantages to providing lysine as dipeptide in neonatal piglets with PN-induced atrophied gut. PN was provided for four days to induce intestinal atrophy and then piglets were switched to enteral diets. When lysine was provided at 75% of requirement, it was found that providing lysyl-lysine during the transition period from PN to enteral feeding improved villus height, mucosal weight, whole-body protein synthesis and reduced the MPO activity compared to free lysine. The addition of glycyl-sarcosine to competitively inhibit lysyl-lysine uptake completely abolished the beneficial effects of lysyl-lysine. Thus, delivering lysine as dipeptide during the high-risk transition period from PN to enteral feeding may serve to enhance gut recovery and avoid complications related to refeeding. Recently, researchers identified that PepT1 influences the secretion of peptide hormones from the enteroendocrine cells, which may affect appetite regulation and glucose homeostasis in adult rodents. The final part of the thesis characterized the role of PepT1 activation on the protein-mediated gut hormone release and glucose kinetics in neonatal

piglets using an in-situ perfusion model. Duodenal casein hydrolysate infusion stimulated the release of incretin hormones GLP-1 and GIP as measured in the portal blood, and the PepT1 inhibitor 4-AMBA hindered this protein-mediated effect on gut hormone release, confirming the involvement of PepT1. Higher baseline plasma insulin and higher insulin response to a glucose challenge strongly suggest that PepT1 activity induces insulin secretion and faster glucose disposal. This mechanism may be advantageous for adults with type 2 diabetes, but in neonates taking in high protein from infant formulas, it contributes to the understanding of the "early protein hypothesis" of childhood obesity.

Overall, these studies shed light on the understanding of the role of PepT1 in the neonatal small intestine and characterize novel influences on neonatal metabolism that may be important in conditions of health and disease.

Acknowledgement

I would first like to thank my supervisor, Dr. Janet Brunton, for giving me this opportunity to be part of her research group and for her support and guidance throughout my program at MUN. The door to Dr. Brunton's office or WebEx meeting room was always open whenever I ran into a trouble spot or had a question about my research. During my study period, she remained very supportive by encouraging, sharing her knowledge and experience, preparing me to grow as a researcher. Secondly, I would like to thank my committee members, Dr. Robert Bertolo and Dr. Fereidoon Shahidi, for their insightful comments, encouragement, and suggestions throughout my PhD program.

I am particularly grateful for the assistance given by Dr. Stefana Egli in using GC/MS at CCART for my analyses. I especially thank Marie Codner and Heather Fifield for their technical assistance to my research. I would also like to acknowledge Donna Hunt for being available for me whenever I needed mental support. I am also thankful to my lab mates Alex, Martin, Julia, and Zack for their help with my animal surgeries whenever I needed it.

I would also like to thank my all-lab mates and the research staff of our lab for their help and encouragement. Finally, I would like to thank my friends who have always stood by me in hard times. I owe my deepest gratitude to them for providing unconditional support and constant encouragement, which helped me to realize my dream. Furthermore, I gratefully acknowledge the Department of Biochemistry, School of Graduate Studies, and NSERC for the financial support to undertake my doctoral research.

Finally, I must express my very profound gratitude to my parents and to my partner Parthi for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

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Ala	Alanine
ANOVA	Analysis of variance
AOM	Azoxymethane
AM	Amastatin
4-AMBA	4-aminomethyl benzoic acid
Arg	Arginine
Asp	Asparagine
BBMV	Brush-border membrane vesicle
BMI	Body mass index
CCK	Cholecystokinin
CD	Crohn's disease
cDNA	Complementary DNA
CP	Crude protein
Dpm	Disintegrations per minute
DPP-IV	Dipeptidyl peptidase IV
DSS	Dextran sodium sulfate
EECs	Enteroendocrine cells
EGP	Endogenous glucose production
fMLP	Formyl-methionyl-leucyl-phenylalanine
GLP-1	Glucagon like peptide-1
Glu	Glutamine
Gly	Glycine

Gly-sar/GS	Glycyl-sarcosine
GIP	Gastric inhibitory peptide,
hGH	Human growth hormone
hPepT1	Human peptide transporter 1
IBD	Inflammatory bowel diseases
IU	International units
IVGTT	Intravenous glucose tolerance test
Ki	Inhibition constant
kDa	Kilodalton
Leu	Leucine
LG	Lysyl-glycine
Lys	Lysine
Lys-lys/LL	Lysyl-lysine
Lys-lys + Gly-sar	lysyl-lysine with glycyl-sarcosine
mRNA	Messenger RNA
MPO	Myeloperoxidase
NHE3	Sodium-hydrogen exchanger 3
NT	Neurotensin
PepT1	Peptide transporter 1
PepT2	Peptide transporter 2
Phe	Phenylalanine
PHT 1	Peptide/histidine transporter 1
PHT 2	Peptide/histidine transporter 2

PN	Parenteral nutrition
Pro	Proline
PYY	Peptide YY
SBS	Short-bowel syndrome
SI	Small intestine
SRA	Specific radioactivity
TMD	Transmembrane domains
TPN	Total parenteral nutrition
Tyr	Tyrosine
Tri-DAP	L-Ala-γ-D-Glu-meso-diaminopimelic acid
UC	Ulcerative colitis
Val	Valine

CHAPTER 1: Introduction

1.1 Introduction to oligopeptide transporters

The proton coupled oligopeptide transporters (POT super family) belong to the solute carrier (SLC) family SLC15A, which consists of four membrane transporters, including intestinal peptide transporter isoform PepT1 (SLC15A1), peptide transporter isoform PepT2 (SLC15A2), and two peptide-histidine transporters PhT1 (SLC15A4) and PhT2 (SLC15A3) (Daniel and Kottra, 2004). PepT1 is a low-affinity, high-capacity transporter and is abundantly expressed in the apical membrane of the small intestine with little or no expression in a healthy colon (Ogihara *et al*., 1996; Groneberg *et al*., 2001; Jappar *et al*., 2010). PepT1 transports dietary derived di- and tripeptides into the enterocytes. PepT1 is also found in renal proximal tubules, bile duct, pancreas, and placenta. PepT2 is considered as a high affinity, low-capacity transporter, which is primarily found in the epithelium of the kidney (Amasheh *et al*., 1997; Smith *et al*., 1998; Shen *et al*., 1999), and is also expressed in other tissues including glial cells, brain, neurons, lung, and mammary gland (Daniel *et al*., 2004; Ruhl *et al*., 2005). Comparatively, in the renal tubules, the high affinity PepT2 expression was higher than PepT1, which facilitates the efficient reabsorption of peptides from the tubular fluid (Shen *et al*., 1999). Expression of PhT1 and PhT2 is found mainly in the immune and nervous systems including lung, spleen, thymus and in the gastrointestinal tract (Yamashita *et al*., 1997; Sakata *et al*., 2001; Bhardwaj *et al*., 2006). However, the functional importance of PhT1 and PhT2 has not been well characterized yet. In summary, there are four types of oligopeptides transporters but only two have been well

characterized. PepT1 plays a major role in the transport of small peptides across the small intestine, and PepT2 is involved in the reabsorption of peptides in the renal tubules.

1.1.1 Introduction to peptide transporter 1 (PepT1)

PepT1 (SLC15A1) is a protein coupled oligopeptide transporter which actively transports a broad range of dipeptides, tripeptides, and a variety of peptide-like drugs. Studies by Ganapathy et al. in 1980s demonstrated that PepT1 is a symporter, and the cotransport process is shared by H^+ and di and tri peptides, but not by amino acid or peptides with more than three amino acid residues (Ganapathy and Leibach, 1985; Ganapathy *et al*., 1987). *In vivo* and *in vitro* studies have shown that the proton gradient is maintained by sodium-proton antiporter activity (NHE-3) in the apical membrane which exports the proton in exchange for an extracellular Na^+ (Ganapathy *et al*., 1985; Kennedy *et al*., 2002). Structural and functional characterization of PepT1 was performed in *Xenopus oocytes* cloned with a rabbit intestinal PepT1 cDNA (Fei *et al*., 1994). The cDNA coding for 707 amino acids suggested that PepT1 has 12 membrane domains with a large hydrophilic loop. Expression of PepT1 increased the uptake of glycyl-sarcosine by 63-fold compared to control oocytes. Furthermore, this study revealed that not only di- and tri-peptides but also peptide derived antibiotics, such as cyclacillin and cephalexin, can be absorbed by intestinal PepT1. The experiment to study the effect of pH on the uptake of glycyl-sarcosine by the oocytes showed that the glycyl-sarcosine uptake was higher between the pH of 5 - 6.5 and drastically reduced when the pH was above 6.5 (Fei *et al*., 1994). In rabbits, northern blot analysis of PepT1 mRNA showed that PepT1 was abundantly expressed in the small

intestine, at a lower level in the kidney and liver and weakly in the brain (Fei *et al*., 1994). The human intestinal PepT1(hPepT1) was characterized using HeLa cells and Xenopus oocytes transfected with hPepT1 cDNA. This study reported that the predicted hPepT1 protein consists of 708 amino acids with a molecular size of 78.8 kDa. Hydropathy analysis showed that hPepT1 also consists of 12 putative transmembrane domains (TMD) with a long hydrophilic loop between the TMD 9 and 10 on the extracellular side. The amino acid sequence of hPepT1 has high degree of homology (81% identity and 92% similarity) with rabbit intestinal PepT1 and distantly similar to yeast and *L. lactis*. The chromosomal localization analysis showed that gene for the PepT1 is present on the chromosome 13 q33-→q34 and consists of 23 exons (Liang *et al*., 1995).

Structure of PepT1 and positional importance of some amino acid residues were investigated using epitope mapping and point mutations respectively, which would predict the binding affinity of substrates. Epitope analysis confirmed that PepT1 has 12 TMD with an extracellular loop between TMD 9 and 10 of approximately 200 amino acids in length. Both amino and carboxy termini were oriented in the cytosolic side (Covitz *et al*., 1998). This extracellular loop between TMD 9 and 10 has only 21% identity between PepT1 and PepT2. However, Doring *et al.* (1996) reported that this loop is not a primary determinant for the differential transporter properties of PePT1 and PepT2. The hPepT1 has 50% overall sequence identity and 70% similarity to high affinity isoform PepT2. PepT1 with single point mutations and functional analysis of chimers of PepT1 and PepT2, have found that first 4 TMD regions and the 7 to 9 domains play crucial role in determining the affinity of the substrates (Doring *et al*., 1996). Studies have shown that histidyl residues of peptide

transporter proteins are essential for the function of peptide transporters. The site-directed mutation of a histidine residue (H57) to glutamine on the interface of the extracellular side of the second TMD completely abolished the PepT1 transporter activity. Furthermore, the histidine residue at 121 (H121) also influenced the substrate binding as well (Kato *et al*., 1989; Terada *et al*., 1996; Fei *et al*., 1997; Chen *et al*., 2000). Treatment of brush border membrane vesicles derived from rabbit kidneys and small intestines with diethlypyrocarbonate (DEPC), which is a histidine-modifying agent, inactivated the transport process (Miyamoto *et al*., 1986; Kramer *et al*., 1988). Later studies in the cell culture model transfected with either rat PepT1 or PepT2 investigated the interaction of antibiotics with histidyl residues and showed that the α-amino group of dipeptides and specific antibiotics interacted with the histidine residues; whether it was interaction with histidine 57 or 121 was unknown (Terada *et al*., 1998). Steel *et al*. (1997) have reported that both residues were important, one for proton coupling and the other for peptide coupling. However, mutation of H111 and H260 did not affect the transport of peptides (Terada *et al*., 1996). The mutation of tyrosine residue (Y56) to phenylalanine reduced the affinity of charged dipeptides. Furthermore, the tyrosine residue Y64 in the central region of the second TMD and Y167 in the fifth TMD appear to be involved in PepT1 activity (Yeung *et al*., 1998; Chen *et al*., 2000).

PepT1 transporter is abundantly expressed in the small intestine and transports dipeptides, tripeptides, and peptide-mimic drugs. In humans, hPepT1 mRNA was detected

in the liver, kidney, pancreas, and very low levels in the placenta. However, the functional properties of peptide transport are well characterized only in the intestine and kidney.

1.2 PepT1 distribution

The PepT1 transporter is abundantly expressed along the small intestine and in negligible concentrations in a healthy colon. It is also expressed in kidney proximal tubules, pancreas, and placenta (Daniel and Kottra, 2004). Ogihara *et al.* (1996) reported that in the intestine, PepT1 was abundant at the tip of the villus and reduced from tip to base. Furthermore, PepT1 expression has been reported in a variety of species, including humans (Terada *et al*., 2005), mice (Groneberg *et al*. 2001), pigs (Wang *et al*., 2009), chickens, sheep, cows (Chen *et al*. 1999), rats (Miyamoto *et al*., 1996), guinea pigs, and rabbits (Adibi, 2003). However, the concentration of the transporter protein varies depending on the location in the intestine, developmental age, and species. Studies in humans have shown the greatest expression in the duodenum and jejunum (proximal small intestine) (Herrera-Ruiz *et al*., 2001; Terada *et al*., 2005). In rabbits, PepT1 mRNA expression was higher in the duodenum and jejunum than the ileum, while the colon had shallow expression compared to the small intestine (Freeman *et al*., 1995). In contrast, PepT1 mRNA was consistent throughout the length of the small intestine of chickens, pigs, and ruminants (Chen *et al*., 1999). Similarly, 8 week old rats and mice showed no significant differences in the PepT1 mRNA expression along the small intestine (Lu and Klaassen, 2006). Furthermore, PepT1 mRNA was detected in the duodenum, jejunum, and ileum of 28 days old Tibetan piglets, and the jejunum exhibited the highest expression (Wang, 2009).

The effect of developmental changes on the localization and the concentration of PepT1 has been studied in different species. Hussain *et al*. (2002) investigated the localization of PepT1 in rats between 18 and 21 days of gestation and in mature animals. PepT1 distribution was exclusively in the apical brush border membrane in prenatal and mature rats. Furthermore, immunolocalization of PepT1 in the subapical cytoplasm and basolateral membrane has been observed immediately after birth (Hussain *et al*., 2002). During the developmental changes in rats, the highest PepT1 mRNA levels were detected at 4 days of age and declined by 28 days (Miyamoto *et al*., 1996). Similarly, in newborn rats, the expression of PepT1 mRNA peaked 3-5 days after birth, then rapidly decreased and increased again by the time the animals were weaning (Shen *et al*., 2001). In another study in rats, intestinal PepT1 mRNA expression was stable from postnatal age from day 4 to day 21 and decreased after day 21 (Rome *et al*., 2002). In chickens, PepT1 mRNA increased linearly with development (Chen *et al*., 2005), and the greatest expression of PepT1 mRNA was reported in the duodenum (Gilbert *et al*., 2007). Another study investigated the PepT1 levels in the later stage of embryos until two weeks after the post-hatch period (Li *et al*., 2008). There was a linear increase in PepT1 mRNA levels in all parts of the chick intestine from embryonic day 18 to post-hatch day 20. Expression was greater in the proximal intestine compared to the ileum (Li *et al*., 2008). Furthermore, PepT1 mRNA was also detected at the gestational age of 29.2 weeks and postnatal age of 2.4 weeks in human infants, suggesting that PepT1 is potentially important in neonates' suckling period (Mooij *et al*., 2016). Furthermore, in Tibetan piglets, a continuous increase in PepT1 mRNA was observed from day 1 to day 14 in the duodenum and proximal jejunum and then declined from day 21 to day 35. Another study conducted in miniature pigs at the

age of 1, 2, 3 and 6 (after weaning) weeks demonstrated that PepT1-mediated dipeptide transport in the ileal segment was highest in the youngest age group (1 week), but dipeptide transport in post-weaned pigs was higher than suckling piglets (Nosworthy *et al*., 2013). These data suggest that peptide transport in the small intestine is vital in the first weeks of life and the post-weaning period. Overall, PepT1 is expressed along the small intestine in various species, and the intensity of the expression varies along the intestinal length with the developmental stages.

1.3 Regulation of PepT1

Nutritional and pathological status alter the expression/activity of the PepT1 transporter. Studies have proven that in situations of gut stress such as fasting, malnutrition, intestinal failure, or parenteral nutrition, PepT1 expression is maintained or increased, whereas other nutrient transporters typically decline in number (Spanier *et al*., 2011). In addition to gut stress, PepT1 expression can be manipulated by dietary factors such as protein content of the diet.

1.3.1 Dietary factors

Expression/activity of PepT1 is partially substrate driven; it can be manipulated by the composition of diet delivered to the small intestine. Several experimental models have demonstrated that a high protein diet upregulates the PepT1 transporter activity (Ferraris *et al*., 1988; Ericksen *et al*., 1995). Ferraris *et al*. (1988) investigated the effect of dietary

protein on the transport of carnosine, a substrate for PepT1, in a mouse model. In this study, mice were fed with either a high protein (72%) or low protein (18%) diet for two weeks and carnosine uptake was measured in the isolated small intestine. Mice fed with a high protein diet showed greater absorption of carnosine than mice fed with a low protein diet. Similarly, in another study, PepT1 mRNA expression in the middle and distal small intestine was upregulated in rats fed with a high protein diet (Erickson *et al*., 1995). Shiraga and colleagues reported that a high-protein diet enhanced the transport of glycyl-sarcosine in rats, compared to a protein-free diet. This study also showed that an elevation in transporter activity was accompanied by enhanced PepT1 mRNA and protein expression in the rat small intestine (Shiraga *et al*., 1999). Caco-2 cells grown in a culture media with glycyl-glutamine demonstrated an enhanced uptake of glycyl-sarcosine and upregulated PepT1 protein expression compared to cells grown in the control media (Walker *et al*., 1998). Furthermore, incubation of Caco-2 cells in a growth medium supplemented with single dipeptide upregulated the PepT1 promoter activity (Shigara *et al*., 1999). Boudry *et al*. (2014) demonstrated that high protein formula enhances the colonic PepT1 transporter activity in low-birth-weight neonatal piglets. On the other hand, the administration of an amino acid mixture reduced the PepT1 mRNA expression in the rat jejunum (Ogihara *et al*., 1999).

Expression of PepT1 transporters is not only influenced by protein intake; the quality of protein and glucose, and fat content of the diet also alter the PepT1 expression. Chickens fed with soybean meal showed greater PepT1 mRNA expression compared to cornmeal (Gilbert *et al*., 2008). Furthermore, the oral administration of glucose reduced

PepT1 protein expression in the apical membrane and reduced the intestinal absorption of cephalexin in rats (Arakawa *et al*., 2016). In another study, PepT1 protein expression was decreased in mice fed with a high-fat diet compared to a regular diet (Wisniewski *et al*., 2014). The above evidence suggests that not only the quantity and quality of proteins, but other nutrients also manipulate the expression/activity PepT1. In summary, high protein diet and presence of luminal peptides upregulates the PepT1 expression and activity.

1.3.2 Fasting/malnutrition

Fasting/malnutrition has a tremendous impact on the small intestinal physiology, reducing the villus density and height, decreasing cell proliferation, and increasing cell apoptosis which leads to gut atrophy (Chappell *et al*., 2003). A study conducted in brush border membrane vesicles (BBMV) derived from rats fasted for 24 h showed a significant increase in glycyl-glutamine uptake, PepT1 protein and mRNA expression compared to BBMV from non-fasted rats (Thamotharan *et al*., 1999). Similar responses were observed in broiler chickens after 24 h of feed restriction (Madsen *et al*., 2011). Moreover, *in vivo* studies reported that 16 h fasting could upregulate the expression of PepT1 protein by two-fold, resulting in a significant increase in glycyl-sarcosine transport in wild-type mice but not in Slc15a1 knockout animals (Ma *et al*., 2012). Similarly, Ogihara and colleagues reported 4 four days of food restriction significantly increased the PepT1 protein expression in the rat jejunum (Ogihara *et al*., 1998). Ihara and colleagues also demonstrated an increase in PepT1 mRNA in the proximal gut by 179% and 164% in rats that were starved for four

days and semi-starved (50% of the daily intake) for ten days respectively, compared to control (Ihara *et al*., 2000).

Human volunteers fasted for 14 days (on 50 calories per day with multivitamins) showed a significant decrease in amino acid transport, but no significant change in transport of peptides was observed (Vazquez *et al*., 1985). Furthermore, Chen *et al*. (2005) conducted a study in chickens to investigate the impact of restricted feeding of 18% and 24% of crude protein (CP) and ad-libitum feeding of 12 and 24% CP. PepT1 mRNA expression was significantly lower in chickens fed the 12% diet and ad-libitum 24% CP diet than that of chickens fed a restricted diet containing 18 or 24% CP diets (Chen *et al*., 2005). Similar responses were observed in another study; feed restricted broiler chickens had greater PepT1 mRNA expression than control (Gilbert *et al*., 2007). Starvation induced the absorption of beta-lactam antibiotics-cefadroxil (substrate for PepT1), and PepT1 mRNA expression, and the results were most prominent in the upper small intestine (Naruhashi *et al*., 2002).

It is clear from a number of animal models that fasting/malnutrition induces mRNA expression of PepT1, leading to a greater number of PepT1 transporters in the small intestine; this adaptation may occur as physiological compensation to accommodate a reduction in nutrient availability.

1.3.3 Intestinal diseases

Inflammatory bowel diseases (IBD) are characterized by chronic inflammation of the gastrointestinal tract and include ulcerative colitis (UC) and Crohn's disease (CD) (de Souza and Fiocchi, 2016). While PepT1 is poorly expressed in a healthy colon, several studies have reported an enhanced expression of PepT1 in colon and small intestine of patients with intestinal diseases. PepT1 mRNA and protein expression were aberrantly upregulated in the colonic mucosa of the IBD patients, but not in the healthy colon, suggesting that PepT1expression is induced by intestinal inflammation (Merlin *et al*., 2001, Adibi *et al*., 2003). Similar responses were reported in the inflamed colonic tissue of both UC and CD patients (Wojtal *et al*., 2009). A significant increase in PepT1 expression was identified in biopsy specimens from patients with colon cancer (Viennois *et al*., 2016). Furthermore, the expression of PepT1 was upregulated in the colon of mice with AOM/DSS induced colitis-associated cancer. In addition, co-administration of Lys-Pro-Val, an anti-inflammatory peptide transported through PepT1, significantly decreased the AOM/DSS induced tumorigenesis (Viennois *et al*., 2016), suggesting that PepT1 may be a useful target for colon cancer treatment strategies.

Studies conducted in cell cultures also have demonstrated that PepT1 is able to transport small bacterial-derived peptides such as formyl-Met-Leu-Phe (fMLP) (Merlin *et al*., 1998), L-Ala-γ-D-Glu-meso-DAP/Tri-DAP (Dalmasso *et al*., 2010) and muramyl dipeptide (Vavricka *et al*., 2004). Furthermore, various animal and cell culture studies have reported that PepT1-mediated fMLP uptake induces the inflammatory responses (Merlin *et*

al., 2001; Buyse *et al*., 2002; Wu *et al*., 2013). These findings suggest that PepT1 would be involved in the amplification of inflammation by enhanced transport of inflammatory peptides into enterocytes.

Short bowel syndrome (SBS) is a clinical condition associated with intestinal diseases that arises because of the resection of the intestinal tissue in the management of the disease. Extensive resection of the proximal small intestine leads to mucosal hyperplasia in the distal small intestine due to the loss of absorptive capacity (O'Brien *et al*., 2001). Patients with SBS experienced upregulated PepT1 expression in the colon, compared to a faint expression in the healthy subjects. However, the expression of PepT1 was not changed in the ileum of SBS patients compared to controls (Ziegler *et al*., 2002). The authors have suggested that the upregulation of colonic PepT1 in SBS patients may be due to the accrual of malabsorbed dietary peptides in the colon. PepT1 protein was detected in the colon of rats that underwent 80% SI resection, when measured at two weeks post-operation, but it was absent in the control rats. Furthermore, to determine the transporter activity, cephalexin was perfused into the colon. The arterial concentration of cephalexin was five to nine times higher in the SI resected rats than the other two groups (Shi *et al*., 2006). In addition, the bacterial peptide fMLP induced inflammation in the colon of SI resected rats, but not the bowel transection controls. Moreover, the inclusion of glycyl-glycine reduced cephalexin absorption by 70-80% and eliminated fMLP-induced inflammation. The authors concluded that bowel resection induced the colonic PepT1 activity in rats which may also serve as a potential medium for transporting bacterial product fMLP (Shi *et al*., 2006)

Adibi and colleagues compared jejunal absorption of amino acids and dipeptides in the sprue patients. The absorption rates of both free glycine and glycyl-glycine from the jejunum were markedly reduced in sprue patients compared to healthy individuals; however, the reduction was more severe for free glycine (15-fold) than for glycyl-glycine (4-fold) (Adibi *et al*., 1974). Similarly, the absorption rate of alanine and glycine from an amino acid solution was impaired in patients with coeliac disease compared to control. In contrast, absorption rate of both amino acids was maintained when delivered as glycyl-alanine in coeliac disease patients. (Silk *et al*., 1974). The upregulation of PepT1 expression in the situation of intestinal diseases may be an adaptive mechanism when the nutritional status is compromised in the gut or might be in response to macronutrients in the colon and or exacerbated by the presence of bacterial peptides in the colon. Therefore, the enteral delivery of dietary peptides may enhance intestinal nitrogen absorption, which could be used as a nutritional strategy for patients with intestinal stress-induced PepT1 expression, such as IBD.

1.3.4 Parenteral nutrition

Parenteral nutrition (PN) is an intravenous supply of nutrition, and is a well-established life-saving technique for infants with compromised gastrointestinal function. Although PN has several benefits, it is also associated with several gut-related complications. Total or partial absence of enteral nutrition induces intestinal atrophy in neonatal and adult animal models which is evident by reduced villus height and crypt depth, loss of mucosal mass and DNA content, decreased crypt cell proliferation and mucosal

protein synthesis, and increased apoptosis (Levine *et al*., 1974; Burrin *et al*., 2000; Ganessunker *et al*., 1999; Kansagra *et al*., 2003; Niinikoski *et al*., 2004). A study conducted in neonatal piglets reported that PN feeding also reduces the superior mesenteric artery blood flow due to the lack of intraluminal nutrients (Niinikoski *et al*. 2004). Furthermore, studies have also shown that PN feeding weakened the gut barrier function, which leads to intestinal infection and inflammation (Ganessunker *et al*., 1999; Burrin *et al*., 2000; Kansagra *et al*., 2003). Ihara and colleagues (2000) demonstrated that expression of PepT1 mRNA in the proximal small intestine was upregulated (161%) in the parenterally fed rats compared to control (Ihara *et al*., 2000). Another study also reported an increase in PepT1 mRNA expression in the ileum of rats that were parenterally fed for 6 days compared to an enterally-fed group (Howard *et al*., 2004). In summary, lack of enteral nutrition induces the expression of PepT1, which may be a compensatory mechanism to ensure optimal amino acid absorption when luminal nutrients become available. Therefore, providing dietary nitrogen as dipeptides may enhance nitrogen retention and improve intestinal health and barrier function.

1.3.5 Insulin

Insulin is the critical hormone serving as a mediator in metabolic regulation, and it has a wide range of cellular effects. Studies have shown that there is a link between insulin and the regulation of PepT1 activity. Thamotharan and colleagues (1999) reported that insulin treatment of Caco-2 cells stimulated glycyl-glutamine uptake by enhancing the expression of PepT1 protein on the apical membrane, but not PepT1 mRNA levels. This

study further investigated whether insulin binding to its receptor is necessary for the dipeptide uptake. This was performed by incubating the cells with insulin and genistein. Inhibition of protein tyrosine kinase activity by genistein completely blocked the insulin-mediated effect on glycyl-glutamine uptake, which confirmed that the stimulation of dipeptide uptake requires insulin binding to its receptor (Thamotharan *et al*., 1999). In another study, glycyl-sarcosine absorption was determined in normal (control), insulin-dependent diabetic, and insulin-treated rats. The dipeptide flux rate was higher in the jejunum of the insulin-treated group compared to non-treated diabetic controls. Furthermore, immunoblotting and western blot analysis confirmed the downregulation of PepT1 in diabetic rats compared to the other two groups. However, in diabetic rats treated with insulin, the expression of PepT1 was similar to the normal group (Bikhazi *et al*., 2004).

Watanabe et al. (2003) also reported that the disappearance of cephalexin from the duodenum, plasma cephalexin concentration, and BBMV expression of PepT1 was higher in hyperinsulinemic GK and Zucker rats compared to controls. Another study showed that short term basolateral stimulation by insulin led to greater glycyl-sarcosine uptake than either control or apical stimulation in Caco-2 cell monolayers, but PepT1 mRNA expression did not differ among the treatments. Furthermore, to study insulin-induced glycyl-sarcosine uptake mechanisms, cells were pretreated with brefeldin A and colchicine. Disruption of the Golgi apparatus by brefeldin A did not affect the insulin-mediated peptide uptake. Golgi apparatus is necessary for the processing of newly synthesized PepT1 protein. However, pre-treatment of cells with colchicine, which disorganizes the microtubules, thereby disrupting the translocation of proteins determined for membrane

insertion, inhibited the stimulatory effects of insulin on the uptake of glycyl-sarcosine in Caco-2 cell monolayers (Nielsen *et al*., 2003). In summary, based on the studies mentioned above, the binding of circulating insulin to the basolateral receptor facilitates the translocation of the PepT1 protein to the apical membrane, thereby increasing the uptake of substrates present in the intestinal lumen.

1.4 Substates for PepT1

In the early stages of the investigation of peptide transport, it was believed that only dipeptides were taken up by PepT1. A study by Matthew *et al*. (1975) reported that only di- and tripeptides could move across the apical membrane. Similarly, Adibi and Morse have studied the absorption of peptides with more than four glycine residues such as tetra-, penta-, and hexa-glycine in human jejunum. They have reported no uptake of peptides with four or more amino acid residues (Adibi and Morse, 1977). Studies conducted in human intestinal cell lines and cloned ovine PepT1in xenopus oocytes have reported that substrates must have 2 or 3 amino acid residues to be transported through PepT1, and single amino acids or tetra-peptides cannot be transported. Single amino acids do not have the structural features to bind to the transporter and tetra-peptides cannot fit into the PepT1 binding pockets (Terada *et al*., 2000; Pan *et al*., 2001; Vig *et al*., 2006).

Multiple studies have revealed that PepT1 has an enormous range of substrates, including more than 400 dipeptides and 8000 tripeptides derived from dietary or body protein digestion (Adibi *et al*., 2003; Daniel *et al*., 2004; Newstead *et al*., 2016). However,

transporter affinity varies based on the peptide properties (Brandsch *et al*., 2004). Transport of peptides requires an inward-directed proton gradient, which is maintained by a sodium proton exchanger. PepT1 also can transport prokaryotic peptides such as fMLP and Tri-DAP. The versatility of PepT1 has made it a target for studies of drug transport across the small intestine, including anticancer (bestatin), angiotensin-converting enzymes inhibitors (captopril and benazepril), β-lactam antibiotics (ciclacillin and flucloxacillin), antiviral drugs (valacyclovir) and L-DOPA-L-Phe (Tamai *et al*., 1997; Nakanishi *et al*., 2000; Brandsch *et al*., 2013; Zhang *et al*., 2013). Overall, PepT1 is capable of transporting numerous dietary di and tripeptides, bacterial peptides, peptide-mimic drugs, and angiotensin-converting enzymes inhibitors. However, substrate affinity and transport efficiency depend on many factors.

1.4.1 The affinity of different peptides to PepT1

Although PepT1 has a wide range of substrate specificity, substrate affinity for PepT1 varies based on the size, charge, substitutions at the N and C termini and hydrophobicity of the peptides. The substrates are categorized into three groups based on the affinity constant (Ki), as high affinity (Ki < 0.5 mM), medium affinity (Ki 0.5 – 5 mM), and low affinity (Ki > 5mM) (Brandsch *et al*., 2004). For example, a study conducted in the human intestinal cell line reported that alanyl-alanine has a high affinity for PepT1 with Ki of 0.08 mM, whereas lysyl-lysine is classified as a medium affinity substrate with a Ki of 3.4 mM, and prolyl-serine has low affinity with a Ki of 14 mM (Eddy *et al*., 1995; Brandsch *et al*., 1999,). Furthermore, studies have reported that peptides with affinities

higher than 15 mM are considered to non- substrates; an example is sarcosyl-sarcosine with a reported Ki of 15.9 mM (Daniel *et al*., 1992; Brandsch *et al*., 2004).

A study by Vig *et al*. (2006) investigated the structural requirements for transport by PepT1 using hPepT1 transfected kidney cells. This study revealed that dipeptides and tripeptides were substrates of the PepT1, where dipeptides (Gly-Gly, Val-Val) were better substates than corresponding tripeptides (Gly-Gly-Gly, Val-Val-Val) due to optimum size and properties for binding and transport. In contrast, individual amino acids and tetra peptides did not transport through PepT1 indicating that amino acids do not have the structural features to bind to PepT1 and the PepT1 binding pocket is not large enough to accommodate tetrapeptides (Vig *et al*., 2006). Another study conducted in Caco-2 cells also revealed that the affinity for the transporter was greater in neutral dipeptides (Gly-Pro and Val-Val) than di-anionic (Asp-Asp and Glu-Glu) or di-cationic (Arg-Arg and Lys-Lys) peptides (Eddy *et al*., 1995). The influence of C-terminal amino acid residues on the dipeptide absorption was studied using 12 glycyl-containing dipeptides and human volunteers. Faster absorption was observed with glycyl-containing dipeptides with a neutral amino acid in their C-terminus when compared to dipeptides containing cationic amino acids in this position (Steinhardt and Adibi, 1986).

Another study conducted in mammalian PepTl expressed in xenopus oocytes investigated the binding affinity of Phe-Tyr and peptide derivatives including as Ac-Phe-Tyr, Phe-Tyr-NH_2, and Ac-Phe-Tyr-NH_2. This study revealed that the modification of N-terminus of Phe-Tyr drastically reduced the binding affinity of the dipeptides (Phe-Tyr – 0.01 mM, Ac-Phe-Tyr – 8.41 mM, Ac-Phe-Tyr-NH2 – 9.97 mM). Modification of C-

terminus also decreased the binding affinity, but to a lesser extent than in the case of N-terminus (Phe-Tyr-NH2 – 0.94 mM), which suggests that free N-terminus is the primary binding site for peptides (Meredith *et al*., 2000).

Hydrophobicity of the amino acid residues also affect the binding affinity of a peptide. Studies have reported that larger and more hydrophobic amino acids at the N-terminus resulted in increased PepT1 activation. Gebauer and colleagues showed that binding affinity of X-Ala peptides positively correlated with the hydrophobicity of N-terminus amino acid (Gebauer *et al*., 2003). Another study conducted in a human renal cell line showed that the affinity of Phe-Gly, Tyr-Gly, and Trp-Gly were greater than Gly-Gly. At the same time, similar trends were reported at the C-terminous, for example, Gly-Tyr and Gly-Phe were better substrates compared to Gly-Gly, Gly-Asp and Gly-Glu (Vig *et al*., 2006).

Overall, most di and tripeptides are transported through PepT1. Peptide transport through PepT1 is influenced by charge, hydrophobicity, size and side chain flexibility.

1.4.2 Competitive and chemical inhibition of peptide transport

Specific peptides or molecules that mimic the peptide configuration with a high affinity to the PepT1 transporter competitively inhibit other peptide influx. For example, glycyl-sarcosine is a non-physiological dipeptide that is resistant to hydrolysis by intestinal peptidases (Daniel 2004); as such, it has been used widely in studies to characterize the transporter expression and function (Nosworthy *et al.* 2013; Stelzl *et al.* 2017; Gleeson *et*

al. 2018). Glycyl-sarcosine is advantageous in studies of competitive inhibition of the transporter because of its high affinity for PepT1 (Vig *et al*. 2006). Peptides with neutral amino acids, including glycine, have high affinity for the transporter, particularly compared to peptides with cationic amino acids such as lysine (Vig *et al.* 2006). A study by Nguyen *et al*. (2007) also demonstrated that glycyl-sarcosine acted as an inhibitor of PepT1 mediated glycyl-glutamine transport.

Experimental peptide transport inhibition may also be achieved with the use of 4-aminomethyl benzoic acid (4-AMBA). The 4-AMBA binds to the transporter but is not translocated through PepT1 and as such, competitively inhibits the peptide influx (Meredith *et al*., 1998). The 4-AMBA was used in several studies to characterize the effect of PepT1 substrates (Darcel *et al*., 2005; Diakogiannaki *et al*., 2013; Dranse *et al*., 2018,). For example, Darcel *et al*. (2005) demonstrated that peptones mediated vagal afferents through PepT1 activation using peptones with 4-AMBA (Darcel *et al*., 2005). These competitive peptide transport inhibitors are valuable experimental tools in research that focus on the role of PepT1 or peptide transport in health and diseases.

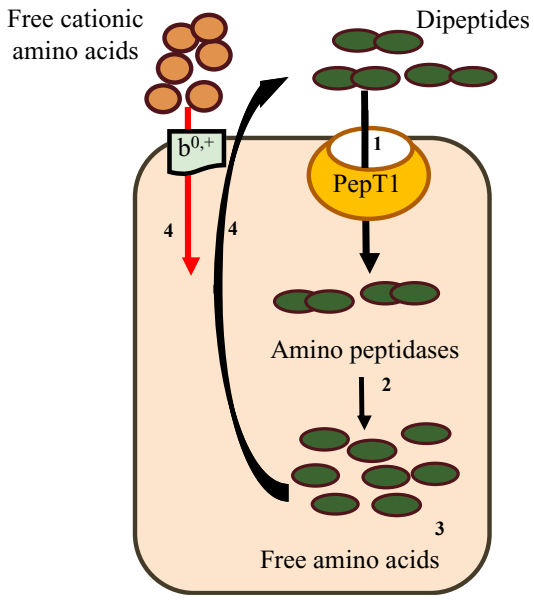
1.5 rBAT/$b^{0,+}$ transporter

Cationic amino acids are transported into enterocytes via Na^+ independent amino acid transporter system called rBAT/$b^{0,+}$ (Palacin, 1994). rBAT/$b^{0,+}$ is responsible for the transport of cationic amino acids such as lysine and arginine, and the neutral amino acid dimer cystine (Munck and Munck, 1997; Chillarón *et al*., 1996). $B^{0,+}$ is responsible for 85%

of the arginine (Wenzel *et al*., 2001) and 47% of lysine (Thwaites *et al*., 1995) uptake into enterocytes. The association of $b^{0,+}$ with rBAT protein by a disulfide bond to form heterodimeric complex is necessary to fully activate $b^{0,+}$ for the transport of cystine and dibasic amino acids (Sakamoto *et al*., 2009). rBAT/$b^{0,+}$ is an obligatory amino acid exchanger that exports an intracellular amino acid in order to take up an extracellular amino acids under specific conditions (Chillarón *et al*., 1996). In summary, rBAT/$b^{0,+}$ transports cationic amino acids and dimer cystine across the apical membrane of the small intestine, which is an obligatory exchanger.

1.5.1 PepT1 Mediated Trans-stimulation of rBAT/$b^{0,+}$AT

Studies have shown that a functional interaction exists between uptake of free amino acids and peptides at the cellular level and provide evidence for an additional role of PepT1 as a mediator of free amino acid transport. rBAT/$b^{0,+}$ is an obligatory exchanger that can exchange intracellular amino acids for extracellular amino acids under specific conditions (Chillarón *et al*., 1996). Wenzel *et al*. (2001) investigated the ability of peptides to enhance free amino acid uptake in Caco-2 cells, using combinations of amino acids and dipeptides. This study revealed that arginine uptake was enhanced when the Caco-2 cells were pre-incubated with cationic amino acid-containing dipeptides. It was speculated that the21nhanceed arginine uptake was due to a high intracellular concentration of free amino acids that resulted from the hydrolysis of dipeptides taken up into the cell through PepT1 (Figure 1). This concept was called “trans-stimulation” of the rBAT/$b^{0,+}$ transporter.



1. PepT1 mediates the uptake of dipeptides into enterocytes
2. Intracellular hydrolysis of dipeptides into free amino acids by amino peptidase enzymes
3. Results in increase in intracellular free amino acid concentration
4. Exchange of intracellular free amino acid for extracellular amino acid

Figure 1.1: PepT1 mediated trans-stimulation of rBAT/$b^{0,+}$ transporter

Enhanced arginine uptake was abolished when the cells were pre-incubated with an intracellular aminopeptidase inhibitor. In this study, enhanced arginine uptake was observed when the cells were pre-incubated with lysyl-lysine, glycyl-arginine, or glycyl-histidine, whereas trans-stimulation of arginine uptake by glycyl-arginine and glycyl-histidine was moderate compared to lysyl-lysine (Wenzel *et al*., 2001). Intracellular hydrolysis of glycyl-arginine and glycyl-histidine would yield only one substrate for rBAT/$b^{0,+}$, because rBAT/$b^{0,+}$ does not transport glycine (Thwaites *et al*., 1995; Palacin *et al*., 1998). Wenzel et al. 2001 also reported that 20 mM glycine or 10 mM glycyl-glycine did not influence the arginine transport (Wenzel *et al*., 2001). Furthermore, pre-incubation of cells with the aminopeptidase inhibitor amastatin with glycyl-arginine hindered the enhanced arginine uptake by glycyl-arginine. This suggested that intracellular hydrolysis of dipeptides is necessary in the process of trans-stimulation because an increased

concentration of intracellular amino acids could not be achieved without the hydrolysis of dipeptide (Wenzel *et al.,*2001).

Interestingly, PepT1 mediated trans-stimulation not only influenced the amino acid absorption, also influenced the transport of drugs which are similar in structure to amino acids (Nguyen *et al*., 2007). Nguyen *et al*. (2007) demonstrated that PepT1-mediated uptake of glycyl-glutamate improved the transport of gabapentin (an amino acid-like drug). However, inclusion of glycyl-sarcosine reduced the effective transport. Furthermore, co-perfusion with lysine or arginine also significantly reduced the gabapentin uptake. Authors speculated that, glycyl-sarcosine competed with glycyl-glutamine transporter, thereby depleting the intracellular amino acid pool, and compromising the trans-stimulation effect. The authors concluded that PepT1 mediated uptake of a dipeptide led to the stimulation of uptake of gabapentin through the transport system rBAT/$b^{0,+}$ (Nguyen *et al*., 2007). Therefore, understanding the interaction between PepT1 and rBAT/$b^{0,+}$ will give a new insight into amino acid transport, particularly for the cationic amino acids arginine and lysine.

1.6 Arginine

Arginine contains four nitrogen atoms per molecule, serves as the most abundant nitrogen carrier in body fluids and body proteins and plays a critical role in multiple metabolic pathways. Beyond tissue protein synthesis, arginine serves as a precursor for the synthesis of nitric oxide, ornithine, polyamines, proline, creatine, agmatine, glutamate, and

protein. Furthermore, arginine plays a versatile role in ammonia detoxification via the urea cycle. Rate-limiting enzymes such as arginosuccinate synthase, the two arginase isoenzymes, the three nitric oxide synthase isoenzymes, and arginine decarboxylase are involved in regulating the synthesis and catabolism of arginine (Morris, 2004a). In adults, *de novo* synthesis of arginine from citrulline occurs in the kidney (Featherston et al., 1973). Arginine is also synthesized from citrulline in the liver through the urea cycle (Morris, 2004b). However, the rapid hydrolysis of hepatic arginine to ornithine and urea, due to high arginase activity in the hepatocytes results in no net arginine synthesis (Flynn *et al*.,2002). In contrast to adults, *de novo* arginine synthesis occurs in the enterocytes of the small intestine from proline, glutamate, and glutamine in neonates (Tomlinson *et al*., 2011, Flynn and Wu, 1996) due to the low activity of arginase (Bertolo and Burrin, 2008). A study that quantified the synthesis of ornithine, citrulline and arginine from proline in enterocytes showed that proline oxidase activity and the intestinal synthesis of citrulline and arginine were high in the newborn piglets but were drastically decreased in 7-d-old pigs and reduced further in 14- to 21-d-old suckling pigs (Wu 1997). Furthermore, rate of citrulline and arginine synthesis from glutamine also decreased with the increasing postnatal age from 7 to 21 days (Wu *et al*., 1994 and 1995). In summary, arginine is a vital amino acid primarily involved in synthesizing many metabolites and detoxifying ammonia. The *de novo* biosynthesis of arginine occurs in the small intestine in neonates and in the kidneys in adults.

1.6.1 Importance of arginine supplementation in neonates

Arginine is one of the essential amino acids in neonates, whereas it is conditionally essential in adults. Arginine requirement is high in young mammals to maintain optimal growth and metabolic processes. Although arginine is essential in neonates, its concentration is remarkably low in many species' milk, including pigs, rats, and humans (Wu *et al*., 1994; Davis *et al*., 1994). Sow milk has been estimated to provide around 49% and 31% of 2- and 7-days old pigs' daily requirement, respectively (Wu and Knabe., 1995). However, sow milk contains considerable amounts of arginine precursors including glutamine, glutamate and proline (Wu *et al*., 1994), suggesting that neonates rely heavily on de novo synthesis of arginine from dietary precursors.

Plasma arginine and ammonia are two sensitive indicators of arginine status in neonatal piglets and human infants, and arginine deficiency in mammals is associated with elevated plasma ammonia concentration (Johnson *et al*., 1972; Batshaw *et al*., 1984; Brunton *et al*., 1999). Kim *et al*. (2004) demonstrated that plasma ammonia concentrations were significantly higher in 14- and 21-day old pigs fed sow milk replacer compared to measures at 7 days old, but values were lower with the addition of 0.2 and 0.4% arginine to the diets. Supplementing 0.2 and 0.4% arginine decreased the plasma ammonia by 20 and 35% and urea by 19 and 33%, respectively. The supplementation of 0.2 and 0.4% arginine to piglets increased the daily weight gain by 28 and 66% and body weight by 15 and 32%, respectively, compared with piglets fed sow-milk replacer between 7 and 21 days of age. Findings of this study clearly demonstrated the importance of adequate arginine

availability to maximize the growth and metabolic process (Kim *et al*., 2004). In another study, piglets that received a sow-milk replacer diet supplemented with 0.6% arginine showed an increase in daily weight gain and protein synthesis in skeletal muscle compared to a non-supplemented group (Yao *et al*., 2008). Furthermore, studies have reported that supplementation of arginine improved the growth and enhanced the intestinal development and function in low-birth-weight piglets (Wang *et al*., 2012; Zheng *et al*., 2018). The effect of arginine on growth was greater in low-birth weight piglets than their normal weight litter mates (Kim and Wu., 2009), suggesting that endogenous arginine synthesis may be limited in the low-birth-weight piglets. Thus, dietary arginine supplementation is effective in increasing rate of growth and enhancing metabolic processes in piglets.

Preterm infants commonly receive total parenteral nutrition (TPN) in early life due to the inability to tolerate enteral feeding. Several studies reported hypoargininemia and hyperammonemia syndrome in preterm infants receiving TPN (Heird *et al*., 1972; Batshaw *et al*., 1984, Wu *et al*., 1986). Similar results were observed in TPN-fed piglets where arginine was provided at the NRC requirement (Brunton *et al.*, 2003). These studies suggested that TPN solutions provide inadequate arginine for preterm infants. Pediatric TPN solutions provide varying level of arginine range from 0.7 $g.kg^{-1}d^{-1}$ to 1.8 $g.kg^{-1}d^{-1}$ (Brunton *at al*., 2000). Supplementing arginine in TPN ameliorated hyperammonemia (Heird *et al*., 1972) and reduced the incidence of necrotizing enterocolitis (Amin *et al*., 2002). Later studies suggest that PN-associated hyperammonemia may be due to lack of intestinal synthesis of citrulline and arginine due to under-expression of key enzymes, the onset of gut atrophy and lack of luminally-delivered precursors. Underdeveloped arginine

synthetic pathways and reduced intestinal mass in preterm infants reduced the de novo arginine and citrulline synthesis (Miller *et al*., 1996) Similar findings were reported in neonatal piglets (Blachier *et al*., 1993; Murphy *et al*., 1996; Stoll *et al*., 1998; Robinson *et al*, 2018). Brunton *et al*. (1999) reported that neonatal piglets receiving a PN solution that contained proline but no glutamine, ornithine, citrulline, or arginine, showed arginine deficiency which led to hyperammonemia and death. The arginine deficiency was partially alleviated when the same amino acid mixture was delivered enterally, but moderate hyperammonemia still occurred (Brunton *et al*., 1999). These results suggest that luminal arginine precursors are necessary because the parenterally delivered proline was not adequately accessible to the gut; however, in all feeding situations neonates have a dietary requirement for arginine that is dictated by the route of feeding.

Weaning from sow milk induces stress in piglets and is associated with reductions in food intake and weight gain. During weaning there is an increased risk for diarrhea, intestinal dysfunction, and atrophy. Piglets start to adopt a dry diet (maize and soybean meal) during weaning. Changes in intestinal morphology may cause loss of villus height and micro-vessel injury and contribute to impaired absorption and transport of nutrients (Hampson., 1986; McCracken *et al*., 1999; Boudry *et al*., 2004; Yang *et al*., 2016). An adequate supply of nutrients is necessary to maintain the optimal development of the gut structure. Reduced feed intake during the initiation of weaning is accompanied by a low dietary intake of arginine, which contributes to intestinal epithelial damage in early weaning (Zhan *et al*., 2000; Wu *et al*., 2004). Arginine deficiency during weaning also reduces the gene expression in the vasculature (Zhan *et al*., 2000). In a study of weaning

piglets fed with maize or soybean meal-based diet supplemented with 1% arginine, arginine supplementation induced vascular endothelial growth factor expression and intestinal development when compared to non-supplemented piglets (Yao *et al*., 2011). In addition, cellular and humoral immunity in piglets was enhanced when a milk-based formula was supplemented with 0.4-0.8% arginine and fed for two weeks. This study showed that arginine supplemented (0.4-0.8%) piglets had heavier spleens, a larger thymus, and altered production of leukocytes, cytokines, and antibodies (Tan *et al*., 2009).

To summarize, milk from sows and possibly humans is low in arginine concentration relative to whole-body needs, so the neonate relies on de novo arginine synthesis in the intestinal epithelium. In situations where intestinal metabolism is compromised, particularly in preterm birth or during weaning, dietary arginine supplementation is necessary to maximize growth and optimize metabolic processes.

1.6.2 Arginine and lysine antagonism

Lysine is an indispensable amino acid for mammals required to synthesize protein, collagen, and carnitine. Lysine is the first limiting amino acid in most cereal grain-based animal diets. Therefore, supplementing lysine is a common practice in swine production. The lysine arginine antagonism has been proven in various models, including rats, chicks, dogs, guinea pigs, and humans. In chicks, reduced feed intake and impaired arginine utilization were observed when the lysine content was 2–3.5% in the feed. Czarnecki *et al*. (1985) reported that puppies fed a diet (0.4% L-arginine and 0.91% L-lysine) with excess

L-lysine developed orotic aciduria, depressed urea formation, hyperammonemia, reduction in weight gain, and emesis (classic signs of arginine deficiency). These signs were absent in the control group. The authors stated that arginine absorption was not affected by excess lysine and the mechanism of this antagonism was not clear. In rats, the growth rate was decreased when the lysine content of the diet was 2.8% (Jones *et al*., 1966). However, supplementing arginine to the diet containing excess lysine resulted in increased growth, substantiating an antagonistic relationship of lysine on arginine metabolism in the above-mentioned studies.

Lysine and arginine share same intestinal and renal transporters. However, there were no evidence to support that the competition for the transporter was causing the observed antagonism in above mentioned studies. In chicks and dogs, competition was reported in renal reabsorption when excess lysine was fed; urinary excretion of arginine was higher in the excess lysine group compared to control diet (Czarnecki *et al*., 1985; Jones *et al*., 1967). In chicks, renal arginase activity was increased by excess lysine intake, which was apparent after 2-4 days of feeding but a significant decrease in plasma arginine concentration was observed within 6 h (Jones *et al*., 1967). Therefore, increased arginase activity was not the only cause of the antagonism.

In pigs, supplementing arginine (0.67 to 2% of diet) reduced weight gain and feed intake and lysine supplementation did ameliorate the negative effect of excess arginine (Southern and Baker, 1982). Baker (1987) reported that weight gain and feed intake were decreased in pigs when the basal diet supplemented with lysine 2.3 and 3.45%, while it was

unaffected in the 1.15% diet when compared to the basal diet. However, the feed efficiency was similar among all groups supplemented with different lysine levels. Even though supplementing excess lysine resulted in an increase in plasma lysine concentration, it did not affect the plasma concentration of arginine, ornithine, and histidine. These studies concluded that amino acid imbalance was the main cause for adverse effect of excess arginine or lysine rather than amino acid antagonism (Southern and Baker 1982; Baker *et al*., 1987). Furthermore, previous studies conducted in our lab in a gut loop perfusion model in neonatal piglets showed that the perfusion of 20 mM lysine with 10 mM arginine did not alter the arginine uptake compared to arginine alone (Tennakoon, 2013; Aljaroudi, 2017).

A recent study by Schmidt and colleagues (2020) conducted in healthy men investigated the impact of arginine on lysine metabolism by providing arginine in a range from 50-600 mg/kg/d and lysine at 30 mg/kg/d (DRI level). High arginine intakes increased the plasma arginine concentration and decreased plasma lysine concentration below normal range. This study identified that providing 300-600 mg/kg/d of L-arginine HCl with a lysine intake that was restricted to the DRI level reduced the enteral lysine uptake and systemic lysine oxidation (Schmidt *et al*., 2020). Arginine and lysine are nutritionally essential amino acids in neonates to maintain maximal growth and development; therefore, a deficiency of either could cause potentially severe consequences. Understanding the interaction and utilizing one amino acid in the form of dipeptide may reduce the competition for the transporter, which will help to optimize the absorption and utilization of these nutritionally important amino acids.

1.7 Gut hormones

Emerging studies have investigated "non-traditional" roles for the PepT1, outside of intestinal peptide absorption and drug delivery. Interestingly, recent studies have shown that PepT1 activation influences the gut hormone release and glucose homeostasis. Nutrients and their by-products trigger the secretion of various hormones from specialized cells in the gastrointestinal tract. Gut hormones include proglucagon-derived glucagon-like peptide-1 (GLP-1) and glucagon-like peptide 2 (GLP-2), as well the as non-proglucagon derived gut peptides, glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY), neurotensin (NT) and cholecystokinin (CCK). These gut hormones play a significant role in appetite regulation, gastric emptying, and insulin secretion. GLP-1 plays a significant role in maintaining glucose homeostasis; its incretin action regulates blood glucose, decreases food intake, and promotes satiety (reviewed in Field *et al*., 2010). The binding of GLP-1 and GIP to their specific G protein-coupled receptor stimulates insulin secretion from pancreatic β-cells, induces β-cells proliferation, reduces β-cells apoptosis, improves satiety, and reduces gastric motility (Dupre *et al*., 1973; Schmidt *et al*., 1985; Leech *et al*., 2011). GLP-1 has been used as a therapeutic target in the treatment of type 2 diabetes (T2DM) and obesity (Nauck *et al*., 1993; Zander *et al*., 2002). GLP-1 is secreted from the intestinal L-cells. L-type cells are in the epithelial layer of the intestine, where they have direct contact with the luminal nutrients. L-cell density is relatively low in the proximal intestine with the greatest density in the ileum and colon (Larsson *et al*., 1975; Bryant *et al*., 1983; Eissele *et al*., 1992). GLP-1 is released into the bloodstream within 10-15 min following food intake (Herrmann *et al*., 1995). The relatively low density

of L-cells in the proximal intestine in rodents and humans suggests that proximal to distal neuronal and hormonal communication might influence the release of GLP-1 during the meal (Eissele *et al*., 1992). GIP is secreted by intestinal K cells in response to nutrient stimuli and the highest number of K cells is found in proximal small intestine, primarily in the duodenum (Buchan *et al*., 1978). Furthermore, loss of insulinotropic action of GIP and reduced GIP receptor expression in pancreatic β-cells were observed in patients with type 2 diabetes (Nauck *et al*., 1993; Meier *et al*., 2001). Miyawaki *et al*. (2002) investigated the role of GIP signaling in obesity development. In this study, wild-type mice fed a high-fat diet showed higher GIP release, fat deposition, and insulin resistance, whereas mice deficient in the GIP receptor were protected from obesity and insulin resistance, suggesting that inhibition of GIP signals may be a potential therapeutic strategy for anti-obesity drug discovery (Miyawaki *et al*., 2002). However, GLP-1 has a short half-life and is rapidly cleaved by the enzyme dipeptidyl peptidase IV (DPP-IV); GLP-1 has a half-life less than 2 min. The half-life of GIP in healthy individuals was 10 minutes but was reported as 5 minutes in patients with type 2 diabetes (Kieffer *et al*., 1995, Deacon *et al*., 2000). In summary, gut hormones influence a range of physiological process including appetite regulation, gastric emptying, and insulin secretion. Notably, GLP-1 and GIP are the two primary incretin hormones secreted from the intestine (following nutrient stimuli) that induce insulin secretion from pancreatic β cells.

1.7.1 Influence of dietary carbohydrate and fat on gut hormone secretion

Luminal nutrient content triggers the secretion of a range of gut peptides from the enteroendocrine cells (EECs). These gut hormones play a crucial role in regulating appetite.

Brennan et al. investigated the effect of macronutrients on the gut hormone release using lean and obese males. This study reported that there was no significant hormone response by a high carbohydrate diet, and a significant increase in plasma CCK level was reported immediately after ingestion of high protein and high-fat diet (Brennan *et al*., 2012). *In vivo* and *in vitro* studies have shown that glucose (Gribble and Reimann 2016; Sun *et al*., 2017) and fructose (Kuhre *et al*., 2014) also trigger the gut peptides GLP-1, CCK and PYY release. However, in humans, 3-O-methylglucose, a non-metabolized SGLT-1 substrate also increased GLP-1, but the mechanism remains unknown (Wu *et al*., 2012). Furthermore, ingestion of lipids also stimulates gut hormone release including CCK, GLP-1 and GIP via the signaling of triglyceride metabolites (Ekberg *et al*., 2016; Mandoe *et al*., 2018). Another study in isolated vascularly perfused rat ileum investigated the effect of luminal administration of glucose, peptones and short chain fatty acids on the secretion of PYY, GLP-1 and neurotensin (NT) (Dumoulin *et al*., 1998). The authors reported that glucose (250 mM) induced the remarkable increase of all three gut peptides, whereas a physiological level of glucose did not stimulate the peptide release. Short chain fatty acids at 20 mM concentrations showed an early and transient increase in the release of gut peptides. Furthermore, peptone (5%) induced a constant release of PYY, GLP-1 and NT (Dumoulin *et al*., 1998). Overall, a variety of luminal nutrients stimulates the release of gut hormones. However, the threshold concentration of these nutrients required for each hormone release varies.

1.7.2 Protein intake and gut hormone release

Numerous studies have investigated the effect of dietary protein on the release of gut hormones from EECs using animal-based, milk-based, or plant-based protein sources. In this regard, several studies conducted in rodents and humans have reported that small intestinal protein or peptone infusion stimulates the gut hormones release from the EECs (Ryan *et al*., 2013; Steinert *et al*., 2014). Clinical studies have shown that dietary protein intake increases the circulating level of CCK, GLP-1, and PYY (Bowen *et al*., 2006; Lejeune *et al*., 2006; van der Klaauw *et al*., 2013; Steinert *et al*., 2014). Furthermore, in rodents, intestinal infusion of protein hydrolysate activates the CCK-dependent vagal afferent firing, and high protein intake induces increased activation of brain areas involved in the control of food intake (Darcel *et al*., 2005). Ingestion of a whey protein meal (16.7 g protein, 25 g carbohydrate) invoked a significant increase in serum insulin, GIP, and GLP-1 compared to a white wheat bread meal (3.7 g protein, and 25 g carbohydrate) in healthy humans (Salehi *et al*., 2012). Furthermore, serum collected from the individuals given a whey or white bread meal was used in subsequent *in vitro* experiments with mouse-derived pancreatic islets. Significantly higher insulin secretion was observed when the islet cells were incubated with serum obtained at 15 and 30 min after ingestion of whey protein meal compared to the white bread meal. Interestingly, the addition of GIP receptor-antagonist ((Pro(3))GIP[mPEG]) abolished the insulin stimulatory effect of whey serum (Salehi *et al*., 2012). The *in vivo* study discussed above clearly demonstrated that whey protein increases the plasma GLP-1, GIP, and insulin levels, and the *in vitro* study confirmed that binding of GIP to the receptor is necessary to stimulate the insulin secretion from isolated pancreatic

islets. Furthermore, a whey protein load before a carbohydrate meal significantly increased the secretion of insulin and incretin hormones (GLP-1 and GIP), slowed gastric emptying, and led to lower postprandial glycemia in type 2 diabetic patients (Ma *et al*., 2009). In addition, a high protein diet (30%) significantly increased the postprandial GLP-1 and satiety compared to an adequate protein (10%) diet in healthy individuals (Lejeune *et al*., 2006).

A study by Frid *et al*. (2005) also assessed the effect of whey protein supplementation on gut hormone responses. In type 2 diabetic patients, whey protein induced a greater postprandial GIP response compared to a reference group; however, there were no differences in GLP-1 between the reference and test diet group (Frid *et al*., 2005). In another study, luminal administration of albumin egg hydrolysate (AEH) to isolated rat ileum and colon significantly increased the GLP-1 release into the portal effluent, whereas infusion of an amino acid mixture (HN25) or saline had no effect on GLP-1 release. Furthermore, similar results were observed when STC-1 cells were incubated with AEH and HN25. The authors suggested that this specific effect of peptones on GLP-1 release must relate to the small peptides, which is aligned with the lack of effect of free amino acids (Cordier-Bussat *et al*., 1998). Recent study by Dranse *et al*. (2018) reported that intra-duodenal infusion of casein hydrolysate reduced the glucose production by liver compared to saline control in rats. Furthermore, addition of GLP-1 receptor antagonist exendin-9 reversed the ability of casein hydrolysate to reduce glucose production, confirming the importance of GLP-1 signaling in the gluco-regulatory role of casein hydrolysate administration (Dranse *et al*., 2018). Furthermore, studies conducted in cell or animal models and humans have shown

that peptones or dietary protein or protein hydrolysate stimulates the CCK secretion (Cordier-Bussat *et al*., 1997; Liddle *et al*., 1986; Choi *et al*., 2007; Brennan *et al*., 2012). In summary, protein intake, regardless of the protein source, increases the release of gut hormones, including GLP-1, GIP, PYY, and CCK.

1.8 Insulinotropic and glucose-lowering effects of intact protein, protein hydrolysate and amino acids

In recent years, numerous human and animal studies have investigated the effect of dietary protein on the insulin and glucose response. Type 2 diabetic patients who consumed a high protein (30%) diet for five weeks showed an improved glucose tolerance compared to patients who consumed a normal protein (15%) diet. Improved glucose tolerance was evident by a decreased area of the glucose response curve and by lowered glycated hemoglobin levels (Gannon *et al*., 2003). This study suggested that a high protein diet may improve the blood glucose in patients with type 2 diabetes. Furthermore, when the protein source was compared, the whey versus casein diet showed a more significant insulin response; however, subjects felt more satiated after the casein and soy protein diets than the whey. Similarly, supplementation of whey protein to a high glycemic breakfast and lunch induced insulin secretion and improved the blood glucose response in individuals with type 2 diabetes (Frid *et al*., 2005). Combined feeding of protein hydrolysate with glucose had a higher peak insulin concentration compared to an equal amount of glucose alone in healthy humans (Calbet and MacLean., 2002). Another study investigated the effect of a carbohydrate meal alone and the carbohydrate meal given with protein

hydrolysate on the insulin response in patients with a long-term diagnosis of type 2 diabetes versus healthy controls. Combined feeding of carbohydrate and protein showed higher de novo insulin synthesis in control and diabetic patients which was evident by increased plasma insulin, proinsulin and C-peptide levels compared to the carbohydrate treatment. This greater insulin response stimulated the glucose clearance and led to lower postprandial glycemia (Manders *et al*., 2005).

Several *in vitro* studies have shown that amino acids also significantly affect insulin release by pancreatic β-cells. For example, inclusion of L-arginine and L-ornithine in the medium induced insulin secretion from pancreatic islets that were exposed to glucose (Blachier *et al*., 1989). The authors suggested that the secretory response is due to the plasma membrane depolarization by cationic amino acids. In another study, incubation of pancreatic β-cells in the medium with leucine also stimulated insulin release (Sener and Malaisse., 1980). Furthermore, intravenous administration of individual essential amino acids stimulated the insulin release with various capacities. Thirty grams of arginine in human subjects showed an insulin response that was equal to that with 30 g of a mixture of 10 amino acids (arginine, lysine, phenylalanine, leucine, valine, threonine, methionine, histidine, isoleucine, and tryptophan) (Floyd *et al*., 1966; Floyd *et al*., 1968). Furthermore, orally administered drinks containing a mixture of amino acids (arginine, leucine, and phenylalanine) and drinks with free leucine, phenylalanine, and wheat protein hydrolysate with glucose showed a significant insulin response compared to the carbohydrate-only drink (van Loon *et al*., 2000). These studies have suggested that combination of carbohydrate meal with free amino acids particularly arginine, leucine and phenylalanine

may be a tool to elevate the plasma insulin. However, there was no clear evidence on the effect of these amino acids on postprandial glycemia in healthy and diabetic individuals. Only particular amino acids at physiological levels or higher in the plasma could stimulate insulin secretion directly from pancreatic β-cells, but that would be different from the effects of dietary peptides inducing an effect (Sener and Malaisse., 1980; Xu *et al*., 2001; Yang *et al*., 2010).

Previous studies have shown a relationship between certain amino acids and insulin release (Blachier *et al*., 1989; Van Loon *et al*., 2000; Calbet and MacLean, 2002) or have attributed the whey-induced insulin stimulatory effect to BCAAs, lysine and threonine mediated insulinemic response (Nilsson *et al*., 2007), but all these studies were performed with carbohydrate (glucose) added to the amino acid mixture or whey protein or whey hydrolysate. A study by Power et al. reported a significant increase in plasma insulin after the ingestion of 45 g of whey protein as either intact protein (WPI) or hydrolysate (WPH) and suggested that the insulinotropic response of whey protein is independent of glucose in the diet (Power *et al*., 2009). Furthermore, in the absence of carbohydrates, there was no significant relationship between the change in plasma branched chain amino acids and insulin release from either WPI or WPH diet. Later, Salehi et al. confirmed that incretin hormones play a major role in insulinotropic effect of a protein diet (Salehi *et al*., 2012). In summary, protein intake (intact or hydrolysate) stimulates insulin release and regulates the postprandial glucose response.

1.9 Influence of PepT1 on gut peptides release and glucose homeostasis

Clinical and animal studies have demonstrated that ingestion of protein in the diet increases the secretion of gut hormones and improves glucose levels (Gannan *et al*., 2003; Manders *et al*., 2005; Frid *et al*., 2005; Ma *et al*., 2009). However, researchers are still debating about the underlying mechanism. Darcel *et al*. (2005), reported that activation of vagal afferents and CCK release in the rat duodenum by peptones required the involvement of PepT1. Inclusion of a PepT1 inhibitor (4-AMBA) abolished the peptone-mediated response in vagal afferents and gastric motility (Darcel *et al*., 2005).

In later studies, intestinal peptide transporter PepT1 was identified as a nutrient sensor and mediator of protein-induced gut hormone release from EECs. An *in vivo* study by Diakogiannaki and colleagues (2013) in primary cultures from the murine colon investigated if and how peptones and small peptides induce the GLP-1 secretion. This study reported a significant increase in relative GLP-1 release when the cells were incubated with small peptides (Gly-Sar, Leu-Gly-gly, Gly-Phe) or peptones (meat hydrolysate) compared to control; peptones showed a more significant effect on GLP-1 release than di- or tripeptides (Diakogiannaki *et al*., 2013). Diakogiannaki and colleagues further demonstrated that oligopeptide-mediated GLP-1 release occurred through the PepT1-mediated membrane depolarization and the Ca^{2+} voltage channel opening. GLP-1 response to Gly-Sar was sensitive to pH and blocked when the cells were incubated with PepT1 inhibitor 4-AMBA. Furthermore, GLP-1 release was reduced in PepT1 knockout mice compared to wild-type mice. The addition of nifedipine, a calcium channel blocker, to the

medium also abolished the Gly-Sar mediated GLP-1 secretion (Diakogiannaki *et al*., 2013). Overall, dietary protein might be an important factor in mediating the metabolic processes that are regulated by GLP-1 secretion.

Significant involvement of PepT1 in the relationship between peptides and gut hormone release has been described in mechanistic studies using various cell culture models. For example, the role of PepT1 in oligopeptide-mediated growth hormone (hGH) release, membrane potential and intracellular calcium secretion was investigated using gastrointestinal hormone secreting STC-1 cells that were transfected with human PepT1 cDNA plasmid and human growth hormone cDNA plasmid (pXGH) or mock-transfected with only pXGH (Matsumura *et al*., 2005). Dipeptides stimulated the hGH secretion only from the PepT1 transfected cells, while in mock-transfected cells, dipeptides did not induce hGH secretion, suggesting that peptide transport through PepT1 is necessary to triggers the hGH secretion. Furthermore, dipeptide-induced hormone release was pH dependent and showed greater response at pH 5.5. In addition, membrane depolarization was only observed in PepT1 transfected cells and the inclusion of a Ca^{2+} channel blocker reduced the hormone secretion, which suggests that oligopeptide-mediated hormone secretion depends on Ca^{2+} influx through voltage dependent Ca^{2+} channels. This study suggested that binding to PepT1 is necessary for the oligopeptide's stimulation of gastrointestinal hormone secretion, as demonstrated in the STC-1 cell model (Matsumura *et al*., 2005). Intestinal organoid culture is a model that contains all the cell types of intestinal epithelium and has been recently used to study the effect of nutrients on the incretin hormone release. The effect of Gly-Sar on GLP-1 secretion was studied in proximal small intestinal

organoids from wild type and PepT1 knockout mice (Zietek *et al*., 2015). Gly-Sar induced GLP-1 release in the organoids from wild-type mice but the GLP-1 response to Gly-Sar was not evident in organoids prepared from PepT1 knockout mice (Zietek *et al*., 2015). These studies have clarified the role of PepT1 in dietary peptide mediated gut hormone secretion, demonstrating that transport through PepT1 is a critical step in the process.

Several studies have reported that a high protein diet improves glucose tolerance in healthy and diabetic animal models and human (Gannan *et al*., 2003; Frid *et al*., 2005). However, a limited number of studies have investigated the critical role of PepT1 in mediating glucose homeostasis. A recent study by Dranse *et al*. (2018) investigated the role of PepT1 mediated protein sensing in the regulation of glucose homeostasis in healthy rodents and in a model of early-onset insulin resistance and obese rodents. In this study, upper small intestinal infusion of casein increased the glucose tolerance compared to saline treatment in healthy and obese rodents. Inhibition of peptide transport by the addition of 4-AMBA reversed the ability of casein to increase the glucose clearance (Dranse *et al*., 2018). Overall, the transport of peptides through PepT1 induces gut hormone release through membrane depolarization and Ca^{2+} channel opening. As such, protein mediated PepT1 activation also regulates glucose homeostasis in adults.

1.10 Protein intake in relation to insulin and gut hormones in infancy

Early nutrition has the potential programming effect of the heath in later life. A high protein intake in infancy has been suggested to confer a risk for obesity and chronic disease later in life (Koletzko *et al*., 2005). A large randomized controlled trial (EU CHOP) revealed that infants who received high protein infant formula during the first year of life had higher body weight and BMI at two years of age compared to infants received lower protein infant formula (Koletzko *et al*., 2009). Standard infant formulas are higher in protein content than human milk, generally leading to a greater daily protein intake in formula-fed infants (Heinig *et al*., 1993; Dewey *et al*., 1996; Koletzko *et al*., 2005). Many studies have reported that formula-fed infants had higher body weight than infants fed with breastmilk (Dewey *et al*., 1998; Kramer *et al*., 2004; Koletzko *et al*., 2005). Breastfeeding in infancy appears to have a protective effect against the later risk of obesity compared to feeding a standard infant formula. A recent study that compared a standard infant formula to a protein-reduced formula has shown that standard formula feeding was linked with higher plasma amino acids, insulin, and insulin-like growth factor -1 and promoted weight gain; the infants fed the protein-reduced formula milk had outcomes similar to breastfed infants (Koletzko *et al*., 2019). Another study reported that formula-fed infants had higher circulating GLP-1 than breast fed infants after 4 months of feeding. The authors speculated that this may contribute to long-term diabetes risk (Diaz *et al*., 2015). Several observational studies and a systematic review reported that breastfeeding in infancy was associated with reduced risk of type 2 diabetes where breastfed infants had lower blood glucose and serum insulin in infancy and marginally

lower insulin in later life than formula fed counterparts (Arenz *et al*., 2004; Owen *et al*., 2005; Voortman *et al*., 2016; Patro-Golab *et al*., 2016). Furthermore, significant postprandial responses in gut hormones were observed in formula-fed infants, and the responses were smaller in breast-fed infants (Salmenperä *et al*., 1988). In summary, high protein formula feeding in infancy is associated with high circulating GLP-1 and insulin, which may contribute to an increased risk of obesity and type 2 diabetes in later life. In adults, PepT1 has been identified as protein sensing mechanism and regulates the glucose homeostasis. However, the role of PepT1 as a protein sensing intermediary mechanism has not been studied in neonates.

CHAPTER 2: Problem investigation

2.1 Rationale

PepT1 is responsible for transporting amino acids in the form of di/tripeptides into the enterocytes, which is an energy-efficient process. Moreover, studies conducted in cells and animal models have suggested that are interactions between dipeptides and amino acids uptake in the small intestine (Wenzel *et al*., 2001; Tennakoon, 2013). Arginine is an essential amino acid in neonates, and it is low in sow's milk relative to whole-body needs (Wu and Knabe, 1995). Therefore, neonates rely on *de novo* arginine synthesis for growth and to sustain metabolic processes. Arginine is involved in many synthetic processes, including detoxification of ammonia and nitric oxide (NO) synthesis. Lysine is an essential amino acid for protein synthesis and the first limiting amino acid in the swine diet due to the low concentration of lysine in a grain-based diet. Both arginine and lysine share $b^{0,+}$ transporter; therefore, providing one in excess may negatively influence other amino acids' absorption. The intestinal presence of PepT1 and the transport of peptides have been established in neonatal piglets and human neonates. Thus, understanding the interactions between dipeptides and amino acids uptake would be beneficial for the efficient supply of nutritionally essential amino acids or amino acid-like drugs.

In preterm infants, parenteral nutrition is a life-saving strategy to provide nutrients; however, prolonged parenteral nutrition due to gut immaturity causes intestinal atrophy. The high-risk transition period from parenteral to enteral nutrition increases the risk of intestinal infection and inflammation. Similarly, in swine production, the weaning of

piglets from sow milk severely affects intestinal morphology and leading to intestinal stress. However, PepT1 expression is maintained during intestinal stress while specific free amino acid transporters are reduced in number (Satoh *et al*., 2003), and expression of PepT1 can be induced by providing substrates. Further studies have shown that absorption of amino acids as peptides is preferred during intestinal diseases. Therefore, it is important to investigate the structural and functional benefits of enteral delivery of amino acids as dipeptide in a piglet model of compromised intestinal function.

PepT1 has been identified as a protein sensing mechanism in the upper gut and may be involved in the regulation glucose homeostasis. Breastfeeding is associated with a reduced risk of type 2 diabetes where breastfed infants had lower blood glucose and serum insulin in infancy and marginally lower insulin in later life than formula-fed counterparts (Owen *et al*., 2005). In contrast, infants who received infant formula during the first year of life had higher body weight and BMI at two years of age (Koletzko *et al*., 2009). High protein diets improve blood glucose metabolism in healthy, obese, and diabetic models. However, high protein feeding in infancy with standard formulas is associated with an increased risk of obesity and chronic diseases in later life when compared to breastfeeding, which is naturally lower in protein. To date, the association between PepT1 activation and gut hormone release and glucose metabolism has not been studied in the neonate. Therefore, I characterized whether the same hormonal effects related to PepT1 activation that have been reported in mature rodent models and models of diabetes also occur in the neonate.

2.2 Objectives and hypotheses

The primary objective of this thesis was to understand the potential role of PepT1 in maintaining health in neonates. Three specific objectives were investigated and are described in individual chapters (Chapters 3 to 5).

Objective 1 The Role of Lysyl-lysine in Enhancing Free Arginine Uptake in the Small Intestine (Chapter 3)

In an intestinal in situ perfusion model in neonatal piglets, the objectives were to:

(a) determine whether the presence of lysyl-lysine enhanced the uptake of free arginine through the trans-stimulation of $b^{0,+}$ receptor

(b) determine whether the addition of glycyl-sarcosine competitively inhibited the trans-stimulation activity by reducing lysyl-lysine uptake and intracellular lysine availability for trans-stimulation

Hypotheses for chapter 3 were:

(a) the presence of lysyl-lysine will enhance the uptake of free arginine into enterocytes through the trans-stimulation of $b^{0,+}$ receptor

(b) the addition of glycyl-sarcosine will competitively inhibit the trans-stimulation activity by reducing the intracellular lysine availability

Objective 2 Investigating Potential Advantages of Re-Feeding with a Dietary Dipeptide into an Atrophied Gut (Chapter 4)

In a gut atrophy neonatal piglet model, the objectives were to:

(a) determine whether delivering lysine in the form of lysyl-lysine into an atrophied gut would lead to advantages in growth and intestinal recovery, when compared to equimolar free lysine

(b) determine whether delivering lysyl-lysine with glycyl-sarcosine would reduce the lysyl-lysine absorption and lysyl-lysine mediated beneficial effects

Hypotheses for chapter 4 were:

(a) delivering lysine as the dipeptide lysyl-lysine (Lys-lys) versus a free amino acid into an atrophied gut will increase the lysine availability for protein synthesis

(b) delivering Lys-lys with glycyl-sarcosine (Gly-sar) will reduce the Lys-lys absorption, thereby reducing the lysine availability for protein synthesis

Objective 3 Characterizing the Influence of PepT1 on the Gut hormone release and Glucose metabolism (Chapter 5)

In an intestinal in situ perfusion model in neonatal piglets, the objectives were to:

(a) characterize the effect of PepT1 activation and dietary peptide transport on gut hormone secretion

(b) characterize the influence of PepT1 activation and dietary peptide transport on glucose response

Hypotheses of chapter 5 were:

(a) activation of PepT1 when dietary peptide transport occurs will lead to gut hormone release and alter the glucose homeostasis

(b) co-perfusion of protein hydrolysate with a PepT1 inhibitor will abolish the effect

CHAPTER 3: Lysyl-lysine enhanced the intestinal absorption of L-arginine in Yucatan miniature piglets

This chapter consists of four major experiments, and Bimal Tenakoon performed the first three experiments as part of his MSc work. I performed the fourth experiment, and my contributions to this experiment included defining the concept and interventions, completing the animal work, conducting the laboratory analyses and statistical analyses, and interpreting the data. This chapter represents work that was presented at Canadian Nutrition Society Annual Conference (2018), and the conference abstract was published in Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 43, 2018). Part of this work was also presented at Canadian Nutrition Society Annual Conference (2011), and the conference abstract was published in Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 36, 2011). The initial manuscript draft (with 3 experiments) was written by B. Tennakoon and J. Brunton and my contribution was incorporating the fourth study into each section. The manuscript is formatted to follow the guidelines of British Journal of Nutrition, the journal of choice for this work.

3.1 Abstract

Peptide transporter-1 (PepT1) and amino acid transport systems both contribute to amino acid uptake by intestinal epithelial cells, 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 21 d old Yucatan miniature piglets, we demonstrated that L-arginine uptake was significantly enhanced by 181% when perfused simultaneously with 20 mmol/l lysyl-lysine, compared to the control condition of arginine alone. In contrast, loops perfused with 20 mmol/l lysyl-glycine did not alter arginine uptake. We speculated that enhanced uptake of arginine was likely due to trans-stimulation of rBAT/$b^{0,+}$ cationic amino acid transporter. Dipeptides taken up into enterocytes via PepT1 may undergo rapid hydrolysis to release free lysine. High intracellular lysine trans-stimulates the rBAT/$b^{0,+}$ anti-transporter to enhance arginine uptake in exchange for lysine. When lysyl-lysine was perfused with an amino peptidase inhibitor (amastatin), the potentiating effect of 20 mmol/l lysyl-lysine was abolished, suggesting that trans-stimulation was affected when intracellular hydrolysis of dipeptides was impeded. Furthermore, co-perfusion of lysyl-lysine with glycyl-sarcosine, which is a hydrolysis resistant peptide with higher affinity to PepT1 than lysyl-lysine, abolished the lysyl-lysine mediated arginine uptake. To our knowledge, we are the first to demonstrate the interaction between arginine absorption and lysine-containing dipeptides at the cellular level in an *in situ* neonatal model.

3.2 Introduction

The products of dietary protein degradation are small chain peptides and free amino acids, which are absorbed by a variety of free amino acid transporter systems (Erickson and Kim, 1990), or by a single di- and tri- peptide transporter, PepT1 (Daniel, 2004). The amino acids L-arginine and L-lysine share the Na^+-independent cationic amino acid $b^{0,+}$ transport system (Wenzel *et al*., 2001). Arginine is involved in the synthesis of many biologically important molecules, such as ornithine, citrulline, creatine, polyamines and nitric oxide (Marletta, 1989; Brosnan and Brosnan, 2007; Marini *et al*., 2012). Studies conducted in piglets have shown that dietary arginine is essential (Southern and Baker, 1983; Brunton *et al*., 1999). Furthermore, arginine supplementation above sow-milk concentrations enhanced the growth of young milk-fed piglets (Kim and Wu, 2004). Lysine is also essential for pigs; it is considered the first limiting amino acid in the pig diet (Baker, 2007). As arginine and lysine share same transporter for intestinal uptake, arginine-lysine antagonism has been reported in chickens and dogs (Kadirvel and Kratzer, 1974; Czarnecki *et al*., 1985). However, the importance of the antagonism between these two cationic amino acids is controversial in pigs (Edmonds and Baker, 1987).

The proton-coupled oligopeptide transporter PepT1 is responsible for dietary di- and tripeptide transport in the intestine (Daniel, 2004). A previous study conducted in our laboratory demonstrated the presence of PepT1 along the entire length of the small intestine in pre-weaned Yucatan miniature piglets (Nosworthy *et al*., 2013). Furthermore, higher ileal dipeptide transport was observed in piglets at post-weaning age (6 weeks), after

transitioning from sow milk to a cereal-based diet (Nosworthy *et al*., 2013). A synergistic relationship appears to exist between arginine uptake and PepT1 activity, as an *in vitro* study conducted in a Caco-2 cell line demonstrated enhanced arginine uptake when cells were pre-incubated with specific dipeptides (Wenzel *et al*., 2001). This is likely due to the fact that the $b^{0,+}$ system has been recognized as an obligatory amino acid exchanger, in that it trades intracellular amino acids for extracellular amino acids under specific conditions, a concept that has been named trans-stimulation (Chillaron *et al*., 1996). As such, the intracellular concentration of substrates for the $b^{0,+}$ system such as lysine may affect luminal arginine uptake. In this series of experiments, we tested the hypothesis that efficient uptake of lysine as a dipeptide via PepT1 would enhance arginine uptake because subsequent hydrolysis of the di-lysine would raise the intracellular lysine concentration, which could then be exchanged for arginine at the brush border via the $b^{0,+}$ system. In the present study, we have determined the effect of lysyl-lysine, lysyl-glycine and their constituent free amino acids on arginine uptake using an in situ intestinal perfusion model in suckling piglets. To determine if the effect relies on intracellular dipeptide hydrolysis to raise free amino acid concentration, we pre-perfused small intestinal segments with the aminopeptidase inhibitor amastatin. Furthermore, to determine if the enhanced arginine uptake depends on the PepT1 mediated uptake of lysyl-lysine, we perfused the intestinal segments with lysyl-lysine with graded amount glycyl-sarcosine which is a hydrolysis resistant dipeptide with high affinity to PepT1 transporter. We hypothesised that the addition of glycyl-sarcosine will competitively inhibit the trans-stimulation activity by reducing the intracellular lysine availability.

3.3 Experimental methods

3.3.1 In situ perfusion (gut loop model)

Three weeks old sow-fed Yucatan miniature piglets were obtained from Animal Care Services, Memorial University of Newfoundland and were randomized to one of three experimental protocols (n = 5 or 6 per experiment). 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 to minimize any effect of fasting on PepT1 function or expression. The gut loop model was adapted from Nosworthy et al, who successfully validated a continuously perfused gut loop model for dipeptide transport studies in pigs (Nosworthy *et al*., 2013). Pre-anesthetics were given by IM injection and included ketamine (20 mg/kg) and acepromazine (0.5 mg/kg). Piglets were subsequently intubated and maintained under general anesthesia with 0.6–1.0% isoflurane (Abbott Laboratories, Montreal, QC) delivered with oxygen (1.5 l/min). Five or six gut loops, 10 cm each, were isolated along the proximal intestine, and were separated by 50 cm. The first jejunal loop was placed 15 cm distal to the ligament of Treitz. Inlet and outlet cannulas (inner diameter, 0.2 cm, outer diameter, 0.3 cm; Watson Marlow) were secured at the ends of each loop. The loops were flushed with warmed (37°C) PBS (125 mmol/l NaCl, 15.9 mmol/l Na_2HPO_4, 1.2 mmol/l NaH_2PO_4, pH 7.4, 37°C) to remove any chyme that was present. In the first animal undergoing the perfusion study for each experimental protocol, the isolated gut loops were randomly assigned to the experimental perfusate solutions; subsequently, the allocation of the perfusates was systematically changed to avoid an effect

of location. All of the perfusate solutions were made up of PBS buffer and contained 10 mmol/l arginine (Ajinomoto North America). A series experiments were conducted to study our objectives. ***Experiment 1:*** The first experiment was conducted to determine the effect of lysyl-lysine on arginine uptake in the jejunum. To this end, five jejunal loops were perfused with buffers that contained 10 mmol/l L-arginine in combination with either 0, 10, 20 or 50 mmol/l lysyl-lysine (Bachem Americas) or with 20 mmol/l free L-lysine (Sigma). The concentrations were chosen based on a previous study (Wenzel *et al*., 2001). ***Experiment 2:*** The second experiment was conducted to determine the effect of lysyl-glycine on L-arginine uptake. Six gut loops were perfused with 10 mmol/l L-arginine in combination with either 0, 10, 20 or 50 mmol/l lysyl-glycine (Bachem Americas), 20 mmol/l L-lysine or 20 mmol/l glycine. ***Experiment 3:*** The third experiment utilized four gut loops to assess the effect of the aminopeptidase inhibitor amastatin on L-arginine uptake. Experimental buffers contained 10 mmol/l L-arginine alone or with 20 mmol/l lysyl-lysine with or without 10 µmol/l amastatin (Sigma). The two gut loops which were designated with amastatin treatments were pre-incubated with a 10 µmol/l amastatin solution for 10 min prior to starting the perfusion. ***Experiment 4:*** The final experiment was conducted to confirm the trans-stimulation activity of the $b^{0,+}$ transporter and the role of PepT1 in the trans-stimulation activity using six gut loops. In this experiment gut loops were perfused with buffers that contained 10 mmol/l L-arginine, or the same arginine concentration as control with either, 20 mmol/l lysyl-lysine, 20 mmol/l glycyl-sarcosine, 20 mmol/l lysyl-lysine with 5 mmol/l gly-sar, 20 mmol/l lysyl-lysine with 10 mmol/L glycyl-sarcosine or 20 mmol/l lysyl-lysine with 20 mmol/l glycyl-sarcosine. The first three treatments were used to investigate the trans-stimulation activity of the $b^{0,+}$ transporter and

the last three treatments were used to study the competitive inhibition of lysyl-lysine uptake. In all of the experiments, a loop perfused with L-arginine alone was used as the control condition. The amino acid and dipeptide-containing PBS buffers were adjusted to pH 6. Radio-labeled arginine was added in the form of ^{3}H-arginine (2220 kBq per 60 ml) (American Radiolabeled Chemicals) to the perfused buffers in all experimental conditions. The solutions were perfused for 120 min and buffers were sampled (1 ml) every 30 min. At the end of each experiment, loops were excised by cautery, flushed with cold 0.9% saline, cut longitudinally, placed on a glass plate in an ice bath and scraped with a microscope slide to remove the mucosa. The mucosa was weighed, frozen in liquid nitrogen and stored at -80°C for future analyses.

3.3.2 Arginine uptake

Arginine uptake was determined by ^{3}H-arginine disappearance from the perfused buffers. Sampled perfusate (100 µl) was mixed with ScintiVerse (Fisher Scientific) and disintegrations per minute (DPM) was measured over 15 min in a TriCarb 2810 TR liquid scintillation counter (Perkin Elmer Life and Analytical Sciences). Specific radioactivity was calculated at baseline (prior to perfusion) and in each of the perfusate samples taken over the course of the 2-h study. The specific radioactivity was then used to determine the total arginine uptake.

3.3.3 Tissue free amino acid concentration of intestinal mucosal samples

The method for the measurement of tissue free amino acids was adapted from Nichols *et al.* (2008) using 100 mg of isolated mucosa. Amino acid concentrations were determined following pre-column derivatization with phenyl isothiocyanate (PITC) and separation on a C18 reverse-phase column (Waters) using high performance liquid chromatography (HPLC).

3.3.4 Statistical analysis

Data were analyzed using repeated measures ANOVA, followed by a Dunnett's post-hoc test (when the treatment groups were compared with control group) or Bonferroni's post-hoc test (when the treatment groups were compared with each other). All treatments were tested in the same animal. Means ± SD were considered significantly different at $P < 0.05$ (GraphPad Prism 7.0).

3.4 Results

3.4.1 Arginine uptake

3.4.1.1 Effect of differing concentrations of lysyl-lysine and lysyl-glycine on arginine uptake

The uptake of arginine from the proximal jejunum was significantly higher (181%) when 20 mmol/l lysyl-lysine was added to the perfusate, compared to arginine alone ($P < 0.05$) (Figure 3.1A). There were no significant differences in arginine uptake with 10 (139%) and 50 mmol/l lysyl-lysine (131%) compared to the control (100%) condition. In

this experiment, lysyl-glycine did not enhance arginine uptake at any concentration in perfused loops of proximal jejunum compared to arginine alone (Figure 3.1B). However, when 20 mmol/l free glycine was included in the perfusate, arginine uptake was 47% higher than in the control condition ($P < 0.05$) (Figure 3.1B).

3.4.1.2 Effect of the aminopeptidase inhibitor amastatin on arginine uptake

Arginine uptake measured with and without amastatin did not change, indicating there was no effect on arginine transport by amastatin (Figure 3.1C). Similar to the previous experiment (Figure 3.1A), significantly higher arginine uptake was measured when perfused with 20 mmol/l lysyl-lysine compared to arginine alone ($P < 0.05$), and the effect was abolished when 20 mmol/l lysyl-lysine was perfused with amastatin simultaneously (Figure 3.1C).

3.4.1.3 Effect of inclusion of glycyl-sarcosine with lysyl-lysine on arginine uptake

As we reported in previous experiments (Figure 3.1A), arginine uptake was significantly higher (187%) when arginine co-perfused with 20 mmol/l lysyl-lysine compared to arginine alone or arginine with glycyl-sarcosine ($P<0.01$). A significant reduction ($p<0.05$) in arginine uptake was observed when 20 mmol/l glycyl-sarcosine was co-perfused with lysyl-lysine. Furthermore, co-perfusion of arginine with 5 mmol/l and 10 mmol/l glycyl-sarcosine with lysyl-lysine showed 163% and 123% arginine uptake respectively compared to control. Furthermore, arginine co-perfused with 20 mmol/l glycyl-sarcosine had no influence on arginine uptake (101%) compared to control (Figure 3.1D).

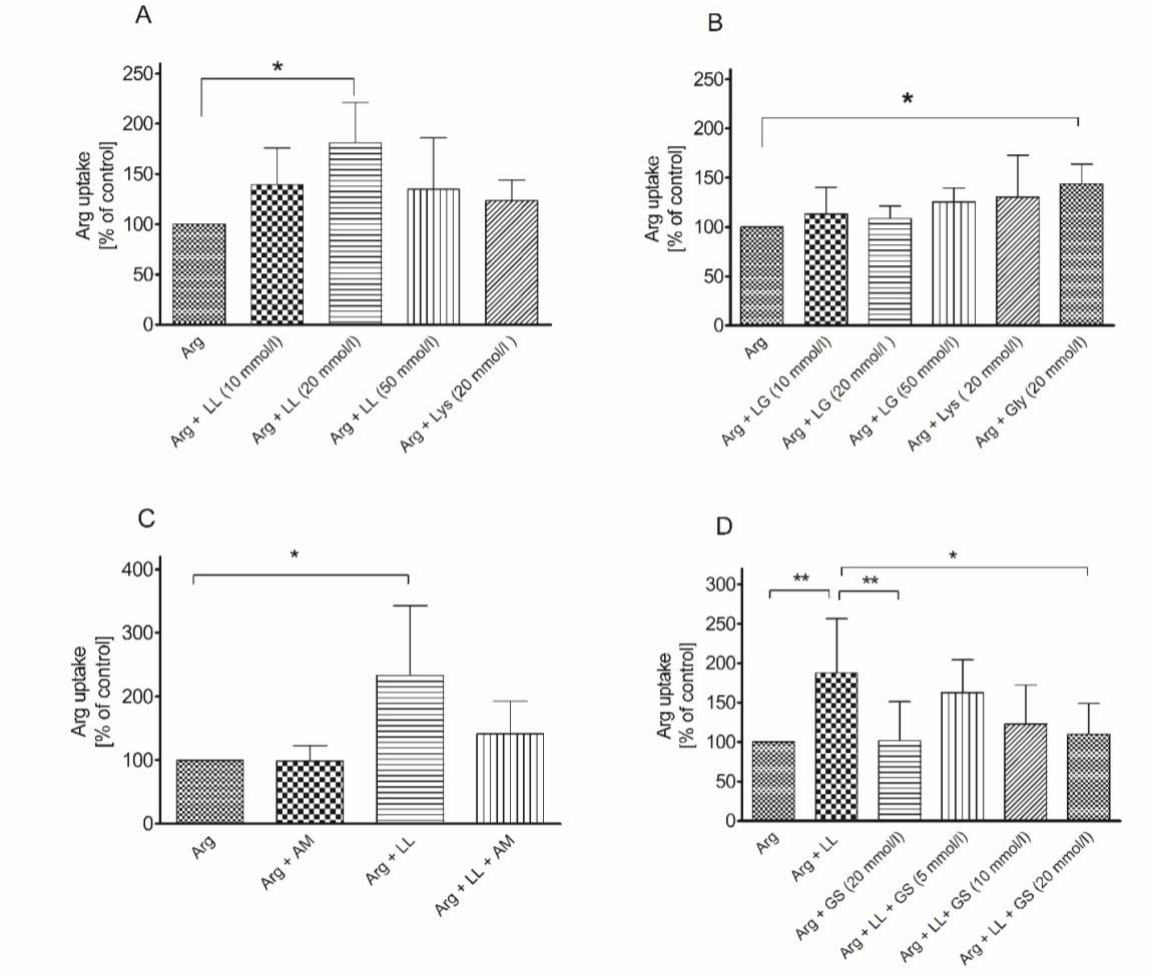


Figure 3.1: Arginine uptake from closed loops of the proximal intestine. Data are expressed as a percentage of control (Arg). The arginine concentration was maintained at 10 mmol/l in all experimental conditions, and arginine uptake was determined based on the ^{3}H-Arginine disappearance. A) The effect of varying concentrations of lysyl-lysine (LL). B) The effect of lysyl-glycine (LG). C) The effect of inhibition of hydrolysis of LL (20 mmol/l) by amastatin (AM). D) The effect of lysyl-lysine (20 mmol/l) with varying concentrations of glycyl-sarcosine (GS). Values are mean ± SD (n=5). Data were analyzed by repeated measures ANOVA, with Dunnett's (A-C) or Bonferroni's (D) post-hoc test; *p <0.05 and **p <0.01.

3.4.2 Tissue free amino acid concentrations in jejunal mucosa

Mucosal tissue free arginine and ornithine concentrations did not differ amongst mucosa samples taken from loops in the lysyl-lysine experiment, regardless of the concentration or form of lysine that was delivered in the perfusate (Table 3.1). Similarly, in the lysyl-glycine experiment, mucosal tissue free arginine and ornithine concentrations were similar, regardless of the concentrations or form of lysine and glycine delivered into the loop (Table 3.2). However, compared to the control loop with no lysine in the perfusate, tissue free lysine concentration was significantly higher in intestinal loops exposed to 20 or 50 mmol/l lysyl-lysine (Table 3.1). Similarly, 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 mmol/l lysyl-glycine compared to the control loop ($P < 0.05$) (Table 3.2). Of note, during the HPLC analysis of standards, the retention time for the lysyl-glycine peak and the L-lysine peak were found to be very close; as such, when the lysyl-glycine concentrations are high it is likely that these two peaks merged, and lysine concentrations may be overestimated in those samples. Tissue free glycine concentration was significantly higher than the control condition in loops perfused with 20 mmol/l glycine. Tissue free glycine concentrations in loops perfused with 10, 20 or 50 mmol/l lysyl-glycine were not significantly different (Table 3.2). The addition of amastatin led to similar tissue free arginine and ornithine concentrations, in that no differences were observed in response to changes in the luminal buffers (Table 3.3). In the other two experimental conditions (table 3.1 and 3.4) without amastatin, there was significantly higher tissue free lysine concentration with 20 mmol/l lysyl-lysine; however,

in this experiment, tissue free lysine concentrations were similar in all experimental groups (Table 3.3). In the fourth experiment, the mucosal free arginine and ornithine concentrations did not vary among the treatments. The mucosal free lysine concentration was significantly higher in the loop perfused with 20 mmol/l lysyl-lysine compared to control, 20 mmol/l glycyl-sarcosine. Furthermore, loop perfused with 20 mmol/l lysyl-lysine with 20 mmol/l glycyl-sarcosine had significantly lower mucosal free lysine compared to 20 mmol/l lysyl-lysine treatment (Table 3.4).

Table 3.1: Tissue free arginine, ornithine and lysine concentrations in intestinal mucosa treated with 10 mmol/l arginine (Arg) with differing concentration lysyl-lysine (LL) or with 20 mmol/l lysine (Lys)

	Treatment				
Mucosal amino acid (μmol/g)	**Arg**	**Arg + 10 mmol/l LL**	**Arg + 20 mmol/l LL**	**Arg + 50 mmol/l LL**	**Arg + 20 mmol/l Lys**
Arginine	0.18±0.07	0.20±0.16	0.28±0.12	0.19±0.06	0.22±0.15
Ornithine	0.22 ±0.07	0.22±0.03	0.21±0.04	0.24±0.15	0.28±0.07
Lysine	0.50 ±0.21	1.02±0.41	2.09‡±0.82	2.90‡±1.07	1.19±0.73

Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test. Within a row, data with superscripts are different from control (Arg), $P < 0.05$.

Table 3.2: Tissue free arginine, ornithine and lysine concentrations in intestinal mucosa treated with 10 mmol/l arginine (Arg) with differing concentration lysyl-glycine (LG) or with 20 mmol/l lysine (Lys) or 20 mmol/l glycine (Gly).

	Treatment					
Mucosal amino acid (µmol/g)	**Arg**	**Arg + 10 mmol/l LG**	**Arg + 20 mmol/l LG**	**Arg + 50 mmol/l LG**	**Arg + 20 mmol/l Lys**	**Arg + 20 mmol/l Gly**
Arginine	0.30 ± 0.13	0.37 ± 0.10	0.38 ± 0.12	0.49 ± 0.33	0.24 ± 0.13	0.16 ± 0.11
Ornithine	0.23 ± 0.07	0.38 ± 0.21	0.36 ± 0.10	0.34 ± 0.11	0.22 ± 0.01	0.22 ± 0.15
Lysine	1.60 ± 2.25	3.46 ± 2.76	5.22 ± 3.18	7.32 ± 2.70‡	2.32 ± 2.01	1.39 ± 1.73
Glycine	3.35 ± 1.47	4.55 ± 1.15	4.77 ± 0.63	6.34 ± 2.48	2.89 ± 0.43	11.65 ± 6.89‡

Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test. Within a row, data with superscripts are different from control (Arg), $P < 0.05$.

Table 3.3: Tissue free arginine, ornithine and lysine concentrations in intestinal mucosa treated with 10 mmol/l arginine (Arg) or 10 mmol/l arginine and 20 mmol/l lysyl-lysine (LL) with or without 10 µmol/l amastatin

	Treatment			
Mucosal amino acid(µmol/g)	**Arg**	**Arg + Amastatin**	**Arg + 20 mmol/l LL**	**Arg + 20 mmol/l LL + Amastatin**
Arginine	0.40±0.07	0.37±0.18	0.82±0.61	0.32±0.06
Ornithine	0.46±0.14	0.51±0.33	0.55±0.41	0.45±0.19
Lysine	0.87±0.19	0.98±0.34	5.65±3.53	2.77±0.59

Values are mean ± SD (n=4). Data were analyzed by repeated measures ANOVA followed by Dunnett's Multiple Comparison Test.

Table 3.4: Tissue free arginine, ornithine and lysine concentrations in intestinal mucosa treated with 10 mmol/l arginine (Arg), 10 mmol/l arginine with 20 mmol/L lysyl lysine (LL), 10 mmol/l arginine with 20 mmol/l glycyl-sarcosine (GS) or 10 mmol/L arginine with 20 lysyl-lysine with differing concentration of glycyl-sarcosine

	Treatment					
Mucosal amino acid (µmol/g)	**Arg**	**Arg + 20 mmol/L LL**	**Arg + 20 mmol/l GS**	**Arg + 20 mmol/l LL + 5 mmol/l GS**	**Arg + 20 mmol/l LL + 10 mmol/l GS**	**Arg + 20 mmol/l LL + 20 mmol/l GS**
Arginine	0.11±0.04	0.22±0.13	0.15±0.06	0.20±0.21	0.19±0.14	0.11±0.01
Ornithine	0.13±0.05	0.16±0.03	0.15±0.11	0.16±0.06	0.12±0.06	0.13±0.06
Lysine	0.65±0.26^{b}	1.51±0.71^{a}	0.62±0.24^{b}	0.77±0.37^{ab}	0.76±0.32^{ab}	0.57±0.25^{b}

Values are mean ± SD (n=6). Data were analyzed by repeated measures ANOVA followed by with Bonferroni post-hoc test. Different letters indicate the significant differences ($p< 0.05$).

3.5 Discussion

To our knowledge, we are the first to demonstrate a positive effect of dipeptides on arginine uptake in an *in vivo* gut loop model. In the presence of 20 mmol/l lysyl-lysine, arginine uptake was greater compared to when arginine was perfused alone (Figure 4.1A). The enhanced arginine uptake was likely mediated via the rBAT/$b^{0,+}$ system, via a phenomenon that is termed trans-stimulation. Trans-stimulated arginine uptake was initially described in human rBAT injected xenopus oocytes that had elevated intracellular cationic amino acid concentrations (Chillaron *et al*., 1996). The potential for lysine dipeptides to enhance arginine uptake was later described in a study conducted in Caco-2 cells, which found that 10 mmol/l lysyl-lysine significantly increased arginine uptake (Wenzel *et al*., 2001). We found no effect of 10 mmol/l lysyl-lysine on arginine uptake in our *in vivo* model; intracellular free amino acid concentration generated by 10 mmol/l 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 (Chillaron *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 (Table 4.1). Thus, intracellular cationic free amino acid concentration may induce trans-stimulation, as arginine uptake was significantly higher when perfused with 20 mmol/l lysyl-lysine. However, we also found that the addition of 50 mmol/l lysyl-lysine did not produce any benefit to arginine uptake. To follow-up on this result, we measured the amino acid and dipeptide

concentrations of the buffers that were sampled during the perfusion studies. When lysyl-lysine was mixed to a concentration of 50 mmol/l, we determined that extensive hydrolysis occurred both prior to and 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 mmol/l (data not shown) and luminal free lysine concentration had increased to 32 mmol/l. 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 lysyl-lysine hydrolysis in the 10 mmol/l and 20 mmol/l lysyl-lysine treatments was 8.5 and between 12-16.4 mmol/l, respectively, at study end; thus, the lower free lysine concentrations may not have interfered with rBAT/$b^{0,+}$ -mediated arginine uptake.

Previous work demonstrated that intracellular free amino acid concentration is 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. We hypothesized that the uptake of 20 mmol/l lysyl-glycine would not enhance arginine uptake to the same extent as lysyl-lysine, because glycine is not substrate for the rBAT/$b^{0,+}$ system (Thwaites et al., 1995); thus, glycine produced by intracellular hydrolysis of lysyl-glycine should not contribute to enhanced arginine uptake. Indeed, arginine uptake was not significantly higher with 10 (113% of control), 20 (108%) or 50 (125%) mmol/l lysyl-glycine, compared to the control loops (Figure. 1B). It was surprising that the 50 mmol/l lysyl-glycine treatment did not produce a significant effect

on arginine uptake, as intracellular hydrolysis should result in a similar free lysine concentration as 20 mmol/l lysyl-lysine. Furthermore, the transport affinity for dipeptides containing neutral amino acids is higher than those containing acidic amino acids (Vig *et al*., 2006), so lysyl-glycine should be taken up by PepT1 into enterocytes more efficiently than lysyl-lysine. We measured the tissue free lysine and glycine concentrations in perfused gut loop mucosa in the second experiment and found that glycine concentrations did not increase significantly with increasing exposure to glycine (as lysyl-glycine), but lysine was higher with 50 mmol/l lysyl-glycine, but this value may be artificially high due to inadequate peak separation with lysyl-glycine in the HPLC method. There was large variability in both lysine and glycine values, driven by one piglet with very high free glycine and lysine concentrations (Table 4.2).

To initiate trans-stimulation, the intracellular free amino acid concentration has to reach the Km value of 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 mmol/l lysyl-lysine on arginine uptake. 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 (Wenzel *et al.*, 2001). Importantly, these authors also demonstrated that trans-stimulation

produced by incubation of cells with free amino acids was not affected by the presence of aminopeptidase inhibitor.

Our fourth experiment also demonstrated that co-perfusion of 20 mmol/l lysyl-lysine with arginine enhanced the arginine uptake, likely via the trans-stimulation activity of rBAT/$b^{0,+}$. Furthermore, the addition of glycyl-sarcosine to the lysyl-lysine significantly reduced the lysyl-lysine mediated enhanced arginine uptake. Glycyl-sarcosine is a non-physiological hydrolysis resistant dipeptide (Winckler *et al*., 1999) with higher affinity to PepT1 than lysyl-lysine (Eddy *et al*., 1995; Brandsch *et al*., 1998; Brandsch *et al*., 2004). Therefore, we speculated that glycyl-sarcosine would have competitively inhibited the lysyl-lysine uptake, thereby reducing the intracellular lysine availability to reach the Km value of the intracellular binding site of the rBAT/$b^{0,+}$ to facilitate the trans-stimulation activity. Furthermore, significantly lower arginine uptake and lower mucosal free lysine in the gut loop perfused with arginine alone, 20 mmol/l glycyl-sarcosine, and lysyl-lysine with 20 mmol/l glycyl-sarcosine compared to lysyl-lysine further confirms that higher intracellular free lysine is necessary to stimulate the trans-stimulation activity of the rBAT/$b^{0,+}$ transporter. Similarly, a previous study by Nguyen *et al.* (2007) investigated the effect of PepT1 mediated transport of glycyl-glutamate on gabapentin uptake. Gabapentin is an amino acid-like drug which transported into the enterocytes through rBAT/$b^{0,+}$. The authors reported a direct relationship between the PepT1 mediated transport of dipeptide and the trans-stimulated uptake of gabapentin. Furthermore, the inclusion of and methionine sulfoximine (glutamine synthase inhibitor) or 20 mmol/l glycyl sarcosine reduced the glycyl-glutamate mediated uptake of gabapentin in the rat ileum (Nguyen *et*

al., 2007). Therefore, based on the results from the fourth experiment, co-perfusion of lysyl-lysine enhanced the uptake of arginine, and the transport of lysyl-lysine through PepT1 is necessary to increase the intracellular lysine concentration to induce the trans-stimulative uptake of amino acid arginine.

The cationic amino acids arginine and lysine both use the rBAT/$b^{0,+}$ system for transport into the intestinal epithelium (Van Winkle *et al*., 1988). Studies conducted in chicks (Austic and Scott, 1975) and dogs (Czarnecki *et al*., 1985) have reported arginine and lysine antagonism for intestinal uptake. However, the importance of the antagonism between these two cationic amino acids is controversial in pigs, as Edmonds and Baker (1987) did not demonstrate an antagonistic effect on arginine with excess dietary lysine (Edmonds and Baker, 1987). In the current study, we also did not observe lysine-arginine antagonism, as 20 mmol/l of free lysine 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 equimolar free lysine. However, high free lysine concentration generated by spontaneous and brush border hydrolysis during the perfusion of 50 mmol/l lysyl-lysine seemed to negatively influence trans-stimulated arginine uptake. Lack of arginine-lysine antagonism in piglet intestine could be due to higher transporter capacity, or uptake of the 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.

It was surprising to measure high concentrations of ^{3}H-labeled ornithine and citrulline in the buffers following the perfusion study. Arginine hydrolysis by arginase results in the production of urea and ornithine, and ornithine can be converted into citrulline in the urea cycle (Wu *et al*., 1997). Interestingly, a study conducted in developing pig enterocytes reported that arginase activity in intestinal enterocytes was minimal during the first 21 days of life, and subsequently increased (Wu *et al*., 1995). Our piglets were 21 days old, thus on the cusp of potential developmental changes. As a post-hoc analysis, we measured arginase activity in mucosal samples from four of our pigs but could not detect any significant activity (results not shown). Thus, the underlying mechanism for the appearance of millimolar quantities of ornithine and citrulline remains to be determined.

In summary, lysyl-lysine enhanced arginine uptake, perhaps because PepT1 enabled trans-stimulation to occur through efficient uptake of lysyl-lysine into intestinal epithelial cells. PepT1 activity has been shown to absorb dietary amino acids as peptides in a manner that is faster and more efficient than free amino acid absorption (Adibi, 1971). Understanding the interactions between peptide and free amino acid absorption is an important step towards the development of cost-effective lower nitrogen diets for domestic swine, or therapeutic formulas for nutritionally vulnerable neonates.

Financial Support: The study was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (grant number 261557–2006 RGPIN).

Conflict of Interest: None

Authorship: The authors are solely responsible for the work described in the present article. J. A. B. was responsible for formulating the research question, J. A. B. and R. F. B. were responsible for designing the study. B.C.T. and D. K. conducted the experiments, the analytical work and generated the results. All authors were involved in the statistical analyses, data interpretation and writing of the manuscript.

CHAPTER 4: Lysine dipeptide enhances gut structure and whole-body protein synthesis in neonatal piglets with intestinal atrophy.

This study was presented at Canadian Nutrition Society Annual Conference (2019), and the conference abstract was published in Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 44, 2019). Part of this work was also presented at Canadian Nutrition Society Annual Conference (2020), and the conference abstract was published in Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 45, 2020). This chapter was published in the Journal of Nutrition (April, 2022). The co-authors of this work are Dalshini Kirupananthan, Robert F Bertolo, and Janet A. Brunton. DK, JAB and RFB were responsible for designing the study, and DK carried out the animal work in addition to the laboratory and statistical analyses.

4.1 Abstract

Parenteral nutrition (PN) is often a necessity for preterm infants; however, prolonged PN leads to gut atrophy, weakened gut barrier function, and a higher risk of intestinal infections. Peptide transporter-1 (PepT1) is a di/tripeptide transporter in the gut, and unlike other nutrient transporters, its activity is preserved with the onset of intestinal atrophy from PN. As such, enteral amino acids in the form of dipeptides may be more bioavailable than free amino acids when atrophy is present. In Yucatan miniature piglets with PN-induced intestinal atrophy, we sought to determine the structural and functional effects of entersl refeeding with lysine as a dipeptide, compared to free L-lysine. Piglets aged 7-8 d were PN-fed for 4 d to induce intestinal atrophy. Subsequently, piglets were refed with enteral diets with equimolar lysine supplied as lysyl-lysine (n=7), free lysine (n=7), or lysyl-lysine with glycyl-sarcosine (n=6) (to determine whether competitive inhibition of lysyl-lysine uptake would abolish PepT1-mediated effects). The diets provided lysine at 75% of requirement and were gastrically delivered for 18 h. Whole-body and tissue specific protein synthesis as well as indices gut structure and barrier function were measured. Villus height, mucosal weight and free lysine concentration were higher in the lysyl-lysine group compared to the other two groups ($P<0.05$). Lysyl-lysine led to greater whole-body protein synthesis compared to free lysine ($P<0.05$). Mucosal myeloperoxidase activity was lower in the lysyl-lysine group ($P<0.05$), suggesting less inflammation. The inclusion of glycyl-sarcosine with lysyl-lysine abolished the dipeptide effects on whole-body and tissue-specific protein synthesis ($P<0.05$), suggesting that improved lysine availability was mediated by PepT1. Improved intestinal structure and

whole-body protein synthesis suggests that feeding strategies designed to exploit PepT1 may help to avoid adverse effects when enteral nutrition is re-introduced into the compromised gut of neonatal piglets.

4.2 Introduction

Intestinal peptide transporter-1 (PepT1) is primarily expressed in the apical membrane of small intestinal epithelial cells and is responsible for the transport of dietary di- or tripeptides into enterocytes from the intestinal lumen (Daniel, 2004). PepT1 is expressed along the small intestine of many species, including humans, rodents, and pigs (Terada *et al*., 1996; Groneberg *et al*., 2001; Nosworthy *et al*., 2013). Nutritional and pathological status has been shown to alter the expression (Ihara *et al*., 2000; Satoh *et al*., 2003) and activity (Vazquez *et al*., 1985) of the PepT1 transporter in the intestine. In situations of intestinal stress, PepT1 expression is maintained or increased, whereas other nutrient transporters are typically downregulated (Ihara *et al*., 2000; Satoh *et al*., 2003). In addition to intestinal stress, PepT1 expression also responds to substate availability in the intestinal lumen (Erickson *et al*., 1995; Walker *et al*., 1998; Shiraga *et al*., 1999). Therefore, providing dietary protein in the form of peptides under conditions of intestinal injury may be beneficial to sustain the amino acid supply for growth and maintenance of metabolic processes.

The therapeutic use of dipeptides versus free amino acids in neonates has application in a variety of circumstances. For example, premature infants commonly

experience compromised enteral intake in their first weeks of life due to developmental immaturity and/or morbidity related to prematurity; as a result, parenteral nutrition (PN) is often a medical necessity. Impaired intestinal barrier function is a consequence of PN feeding, leading to an increased risk for necrotizing enterocolitis (Burrin *et al*., 2000; Niinikoski *et al*., 2004). Furthermore, the introduction or re-introduction of enteral feeding into the compromised gut of parenterally-fed infants conveys even greater risk for infection and inflammation (Stoll, 1994; Bjornvad *et al*., 2008; Fallon *et al*., 2012). In another example, in swine production, piglets experience intestinal trauma at weaning, with the abrupt transition from suckling to a cereal-based diet (Pluske *et al*., 1996). Villus damage and morphological changes in the gut occur, accompanied by reduced feed intake, disturbed immune function and altered intestinal microflora, and an interruption in growth (Miller *et al*.,1986; Hampson *et al*., 1986; Barnett *et al*., 1989; Montagne *et al*., 2004). For both human and swine neonates, transitional feeding represents a period of undernutrition. Lysine is the first limiting amino acid in cereal-based diets and is primarily used for protein synthesis (Tomé and Bos, 2007); indeed, restricted lysine in the diet has been shown to acutely reduce whole-body protein deposition in weaned piglets (Totafurno *et al*., 2019). Therefore, providing a highly bioavailable form of lysine may be beneficial for rapid recovery of the gut and to sustain growth during transition feeding.

The objective of this study was to determine if the enteral delivery of lysine as the dipeptide lysyl-lysine, compared to L-lysine, resulted in structural and functional benefits in the gut and whole-body in a model of gut recovery. Our primary hypothesis was that

delivering enteral lysine as a dipeptide in a PN-induced atrophied gut model would increase the lysine availability for protein synthesis.

4.3 Methods

4.3.1 Surgery, animal care, and study procedures

Twenty (20) male Yucatan miniature piglets at 7-8 days old were obtained from a breeding herd at Memorial University of Newfoundland (St. Johns, Canada). Animal protocols were approved by the University's Institutional Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. Upon arrival, animals were placed under general anesthesia and underwent surgical procedures to implant two venous catheters (jugular and femoral) and a gastric catheter, as previously described (Dodge et al., 2012). The jugular catheter was implanted into the jugular vein and advanced to the superior vena cava for the delivery of PN diet and isotope infusion. The femoral catheter was implanted into the femoral vein and advanced to the inferior vena cava and was used for blood sampling and drug delivery. The gastric catheter was implanted for enteral delivery of the test diets. Following surgery, piglets received antibiotics intravenously (trimethoprim and sulfadoxine; Borgal, Intervet Canada Ltd., Canada) until day 4 and analgesic (buprenorphine hydrochloride; Temgesic, Canada) every 8 hours for two days. Piglets were housed individually in circular metabolic cages with a dual-port swivel and tether system (Lomier Biomedical, Canada) that allowed for free movement while facilitating diet infusion. The room was lit from 0700 to 1900 h, and the temperature was maintained at 28°C with additional heat lamps. Piglets were weighed daily, and diet

infusion rates were adjusted accordingly. Small intestinal atrophy was induced by parenteral feeding for 4 full days post-surgery (Dodge *et al*., 2012) and refeeding of test enteral diets was initiated on the evening of day 4. On the morning of following day, whole-body protein synthesis and tissue-specific protein synthesis were measured using amino acid tracers (described below).

4.3.2 Parenteral and enteral diets

The complete elemental parenteral diet (Table 4.1) was administered through the jugular catheter starting immediately following surgery (day 0) and was provided at 50% of the total requirement. The next morning the rate was increased to 75% of the requirement, then to 100% in the afternoon of day 1 and continued until the afternoon of day 4 when enteral diets were introduced. The piglets were randomly assigned to one of three experimental enteral diets that was initiated after the PN feeding period. The enteral diets were initiated at 50% of target rate, with the balance of diet provided by PN, and progressively advanced to 100% enteral feeding over the next 5 h; enteral feeding then continued for a further 12 h (Figure 4.1). The enteral test diets were completely elemental and were identical to each other except for the form of lysine (Table 4.1). In the test diets, lysine was provided as either free L-lysine (n=7) as the control (Lys), or as an equimolar amount of lysine as the dipeptide lysyl-lysine (n=7) (Lys-Lys) to demonstrate the effect of the dipeptide on efficiency of lysine uptake from the enteral diet. A third test diet provided the same amount of lysine as lysyl-lysine in addition to glycyl-sarcosine (n=6) (Lys-Lys+Gly-Sar), to determine if benefits of providing lysine as a dipeptide could be abolished by glycyl-

sarcosine by competitively inhibiting lysyl-lysine uptake by PepT1. The total lysine in the enteral diet was maintained at 75% (0.50 $g \cdot kg^{-1} \cdot d^{-1}$) of the daily lysine requirement recommended by the National Research Council for piglets: the marginally deficient lysine intake was designed to ensure that any benefit of the dipeptide would be detectable due to the greater availability of lysine. The glycyl-sarcosine in the diet was provided at twice the molar concentration of lysyl-lysine to ensure competitive inhibition. All of the diets (parenteral and enteral) were made in the investigators' laboratory under aseptic conditions (Brunton et al., 2012). The complete diet was delivered at 288 $mL \cdot kg^{-1} \cdot d^{-1}$ to provide 13.15 g of amino $acids \cdot kg^{-1} \cdot d^{-1}$ (Ajinomoto, Canada, Evonik Industries AG, Germany, Sigma Aldrich, Canada or Bachem, USA) and non-protein energy delivery of 1.1 $MJ \cdot kg^{-1} \cdot d^{-1}$ from dextrose (Sigma Aldrich, Canada) and lipids (SMOFlipid; Baxter, Canada). Lipids, vitamins, and minerals were added to the diet bags immediately prior to infusion, as described previously (Brunton et al., 2012), and the diets were continuously infused via pressure-sensitive peristaltic pumps.

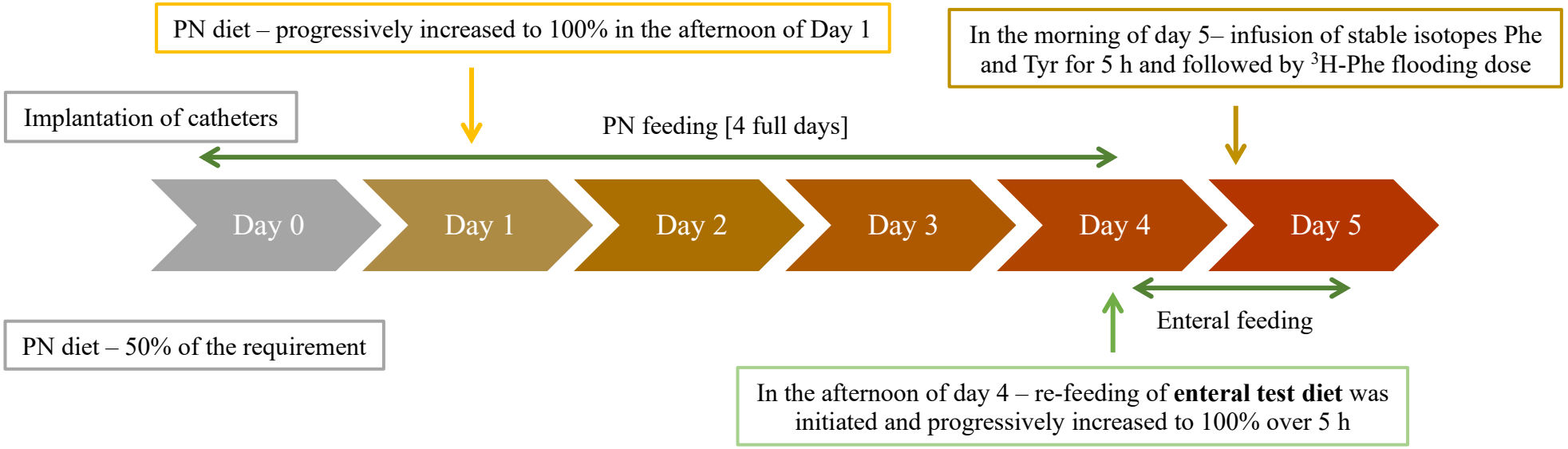


Figure 4.1: Schematic diagram of the feeding regimen

Table 4.1: Amino acid profile of the parenteral and enteral diets

	Parenteral diet	Enteral diets		
		Lys	Lys-Lys	Lys-Lys + Gly-Sar
Amino acids		$g \cdot kg^{-1} \cdot day^{-1}$		
Alanine	1.41	1.41	1.41	1.41
Arginine	0.87	0.87	0.87	0.87
Aspartate	0.80	0.80	0.80	0.80
Cysteine	0.18	0.18	0.18	0.18
Glutamate	1.37	1.37	1.37	1.37
Glycine	0.35	0.35	0.35	0.35
Histidine	0.40	0.40	0.40	0.40
Isoleucine	0.60	0.60	0.60	0.60
Leucine	1.36	1.36	1.36	1.36
Lysine[1]	1.07	0.50	0.00	0.00
Lysyl-lysine[2]	-	-	0.50	0.50
Methionine	0.25	0.25	0.25	0.25
Phenylalanine	0.72	0.72	0.72	0.72
Proline	1.08	1.08	1.08	1.08
Serine	0.74	0.74	0.74	0.74
Taurine	0.06	0.06	0.06	0.06
Tryptophan	0.27	0.27	0.27	0.27
Tyrosine	0.11	0.11	0.11	0.11
Valine	0.69	0.69	0.69	0.69
Threonine	0.53	0.53	0.53	0.53
Glycyl-sarcosine[3]	-	-	-	0.50

[1]lysine in the enteral diet was provided at 75% of the NRC requirement for young swine
[2]lysine was provided as lysyl-lysine-HCL at 0.53 g/kg/day for the dipeptide treatment groups
[3] glycyl-sarcosine was provided at twice the molar concentration of lysyl-lysine in the diet

4.3.3 Tracer infusion protocols to measure whole-body and tissue-specific protein synthesis

Whole-body protein synthesis was determined with the use of stable isotopes of phenylalanine and tyrosine, as previously described (Dinesh *et al*., 2021). Briefly, on day 5, a primed, constant delivery of L-[D_5]-phenylalanine (prime 6.39 μmol·kg^{-1}; constant 20 μmol·kg^{-1}·h^{-1}), L-[3,5-D_2]-tyrosine (prime 2.74 μmol·kg^{-1}; constant 9 μmol·kg^{-1}·h^{-1}) and L-[D_4]-tyrosine (prime 2.74 μmol·kg^{-1}) (Cambridge Isotope Laboratories, Inc., USA) were given via gastric catheter to measure whole-body protein dynamics. Constant isotope delivery during the 5 h experiment was carried out as repeated half-hourly doses. Blood samples were collected every 30 minutes to measure tracer: tracee ratios.

Thirty minutes prior to the necropsy, a bolus flooding dose of 37 MBq·kg^{-1} (1 mCi·kg^{-1}) of ^{3}H phenylalanine (Moravek Biochemicals and Radiochemical Inc, USA) with 1.5 mmol·kg^{-1} unlabeled phenylalanine (Sigma-Aldrich, Canada) was administrated intravenously over 5 min to measure the tissue-specific protein synthesis (Garlick *et al*., 1980).

4.3.4 Necropsy and tissue collection

Thirty minutes after initiation of phenylalanine bolus, piglets were anesthetized, and liver and muscle (gastrocnemius and longissimus dorsi) samples were collected, and frozen in liquid nitrogen. The small intestine (SI) was removed, total length was measured, and a 3 cm section of the proximal jejunum was flushed with cold saline and immersed in neutral buffered 10% formalin (Fisher Scientific, Canada) for histologic analyses. The next 50 cm

of proximal jejunum was isolated and flushed with cold saline. The mucosa was harvested from the intestinal section which was slit longitudinally and positioned on a glass plate on ice. The mucosa was removed using a glass slide with even pressure, was weighed and was flash-frozen in liquid nitrogen.

4.3.5 Histology

Formalin-fixed jejunum sections were processed, and villus height and crypts depths were measured in a blinded manner by a single investigator (DK) as described in Nosworthy *et al*. (2016).

4.3.6 Protein synthesis and amino acid determination

Amino acid analyses in the plasma and tissue samples were performed using phenylisothiocyanate (PITC) derivatization and HPLC, as previously described (Robinson *et al*., 2016). The specific radioactivity (SRA) of tissue free and protein-bound phenylalanine was measured in liver, mucosa and muscles and the fractional rate of protein synthesis (K_s) was calculated as follows:

$$K_s\ (\%\cdot day^{-1}) = (SRA_{bound}/SRA_{free}) \times (1440/t) \times 100$$

where SRA_{bound} and SRA_{free} are tissue-bound phenylalanine and free phenylalanine expressed in $DPM\cdot mmol^{-1}$, respectively, and t is the duration of tracer incorporation in minutes (Brunton *et al*., 2012).

4.3.7 Plasma isotope analyses

Plasma samples were prepared for GC/MS analysis as previously described (Lamarre *et al*., 2014), with minor modifications. Briefly, 50 µL plasma was transferred to screw-capped 2 mL GC vials (screw cap liner was silicon/PTFE) and 20 µL of 0.5 mol·L^{-1} phosphate buffer (pH 8.0) and 130 µL of 100 mmol·L^{-1} pentafluorobenzyl bromide (PFBBr; Sigma Aldrich, Canada) solution in acetone (Sigma Aldrich, Canada) were added. The vial was tightly capped immediately, thoroughly mixed for 1 min and incubated at 60°C for 1 h using a block heater (Fisher Scientific, USA) to allow alkalization by PFBBr. Samples were allowed to cool at room temperature for 5 min, 335 µL of n-hexane was added and mixed well for 1 min to terminate the reaction. The organic phase (top layer) was transferred into a vial fitted with an insert for the mass spectrometry analysis. GC (6890N Network GC System) oven was preheated to 50°C, and 2 µL of the sample was injected onto a DB-5MS column (0.25 mm x 30 m x 0.22 µm) (Agilent Technologies: Mississauga, ON). Helium was used as a carrier gas at a 1.1 mL·min^{-1} constant flow rate. The GC conditions were as follows: initial oven temperature was maintained at 50°C for 3 min, then the temperature was increased to 280°C at a rate of 30°C·min^{-1}, and then the temperature was held at 280°C for 4 min (programme run time was 14.67 min). Electron impact collision energy 70eV was used to ionize the samples upon entering a quadrupole MS. The mass selective detector was operated in selected ion monitoring (SIM) with mass-to-charge ratio (m/z) 300 and m/z 305 for Phe and D_5-Phe and m/z 316, 318 and 320 for Tyr, D_2-Tyr and D_4-Tyr.

Isotope enrichment was calculated as enrichment at steady-state minus the background measurement at baseline. Whole-body flux of each isotope was calculated using the equation (Tomlinson *et al*., 2011):

$$Q=i[(E_i/E_p)-1]$$

where Q is the flux of the amino acid in the pool, i is the rate of infusion of the tracer ($\mu mol \cdot kg^{-1} \cdot h^{-1}$), E_i is the enrichment of the tracer in the infusion, and Ep is the enrichment of the tracer in the plasma at steady state.

Whole-body protein synthesis was calculated based on the following equation (de Betue *et al*., 2017):

$$Q_{Phe} = PS + Ox = I + PB$$

Where PS is the rate of protein synthesis, Ox is the rate of phenylalanine oxidation to tyrosine, PB is the rate of phenylalanine release from protein breakdown, and I is the rate of phenylalanine intake from the diet.

Phenylalanine to tyrosine conversion was calculated by the formula

$$Ox = Q_{Tyr(M+2)} [E_{Tyr(M+4)} / E_{Phe(M+5)}]$$

where $Q_{Tyr(M+2)}$ is the tyrosine flux in the plasma, $E_{Tyr(M+4)}$ and $E_{Phe(M+5)}$ are the respective enrichments of the product D_4-tyrosine and D_5-phenylalanine.

Protein synthesis ($\mu mol \cdot kg^{-1} \cdot h^{-1}$) was derived from PS = Q_{phe} - OX and expressed as g of protein·kg of $BW^{-1} \cdot d^{-1}$ as described previously (Thompson et al., 1989).

4.3.8 PepT1 and occludin protein analysis

Isolation of brush border membrane vesicles (BBMV) and western blotting to determine PepT1 protein expression in the BBMV were performed as previously described (Nosworthy et al., 2016) using mouse anti-PepT1 (sc-373742) as the primary antibody. Similarly, occludin protein in the mucosal tissue was determined using the primary antibody mouse anti-occludin (sc-133255). The reference protein β-actin expression was measured using mouse anti-β-actin as the primary antibody (sc-47778). The goat anti-mouse IgG- horseradish peroxidase (HRP) (sc-2031) was used as a secondary antibody for all three proteins, and the antibodies were purchased from Santa Cruz Biotechnology, USA.

4.3.9 Myeloperoxidase (MPO) activity

Myeloperoxidase activity in the jejunal mucosa was measured using mucosal scrapings as described previously (Kim *et al*., 2012). Myeloperoxidase enzyme activity was reported as IU·mg $protein^{-1}$, where IU was the enzyme needed to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature.

4.3.10 Statistical analysis

All data are presented as means with standard deviations. All the analyses were performed using one-way ANOVA with Dunnett's post hoc test, in which the Lys-Lys

group was compared to the Lys and the Lys-Lys+Gly-Sar. Correlation analysis was performed using linear regression analysis. $P < 0.05$ was considered statistically significant. GraphPad Prism (Version 7; San Diego, California) was used to perform statistical analysis and to generate the graphs.

4.4 Results

4.4.1 Piglet growth

Animals in the Lys-Lys treatment group did not differ in age (7±1 d), initial body weight (1.69 ± 0.16 kg), growth rates (64 ± 13 $g \cdot kg^{-1} \cdot d^{-1}$), or final weight (2.27 ± 0.17 kg) compared to other two treatment groups at the end of day 5.

4.4.2 Intestinal morphology

Lys-Lys piglets had greater SI length compared to the piglets fed Lys-Lys+Gly-Sar **(Table 4.2).** Jejunal mucosa weight and villus height were also significantly greater in piglets re-fed with Lys-Lys compared to other two treatments (**Table 4.2**)**.** Crypt depth was significantly lower in Lys-Lys+Gly-Sar piglets compared to Lys-Lys group (**Table 4.2**).

4.4.3 Plasma isotopic enrichment and whole-body protein synthesis

A steady-state of isotopic enrichment during the L-[D_5]-phenylalanine and L-[3,5-D_2]-tyrosine infusions was achieved in plasma by 3.5 h for all piglets (data not shown). Whole-body protein synthesis was significantly higher in the Lys-Lys piglets compared to

groups fed Lys or Lys-Lys+Gly-Sar (**Figure 4.2**). Plasma phenylalanine and tyrosine concentrations did not differ in the Lys-Lys group compared to other two groups at the end of the 5 h of isotope infusion (Supplemental Table 2).

4.4.4 Tissue-specific protein synthesis

The liver, mucosa and muscle protein synthesis rates were significantly lower in the Lys-Lys+Gly-Sar group compared to Lys-Lys group; however, no differences in tissue specific rates of protein synthesis were apparent between the Lys-Lys and Lys groups (**Figure 4.3**).

4.4.5 Plasma and tissue free lysine concentrations

Lys-Lys+Gly-Sar piglets had significantly lower plasma lysine concentration compared to the Lys-Lys piglets ($P < 0.001$). Plasma lysine did not differ between the Lys and Lys-Lys groups when sampled 17 h after enteral feeding was initiated (Supplemental Table 4.1) (**Figure 4.4a**). However mucosal tissue free lysine concentration was significantly higher in the Lys-Lys piglets fed compared to the Lys or Lys-Lys+Gly-Sar groups ($P = 0.01$) (Supplemental Table 4.2) (**Figure 4.4b**).

Table 4.2: Intestinal morphology in piglets enterally fed with free L-lysine, lysyl-lysine, or lysyl-lysine with glycyl-sarcosine[1]

Variable	Lys	Lys-Lys	Lys-Lys + Gly-Sar	*P* value[2]
Intestinal length, cm·kg body weight^{-1}	247 ± 15	251 ± 22	228 ± 12*	0.02
Jejunal mucosa weight, mg·cm^{-1}	55 ± 11*	71 ± 11	49 ± 6*	0.007
Villus height (μm)	266 ± 29*	279 ± 20	204 ± 19*	<0.0001
Crypt depth (μm)	88 ± 8	88 ± 5	68 ± 10*	<0.0001

[1]Values are mean ± SD, free L-lysine (Lys, n=7), lysyl-lysine (Lys-Lys, n=7), or lysyl-lysine with glycyl-sarcosine (Lys-Lys+Gly-Sar, n=6).

[2] Analyses were performed using one way ANOVA with Dunnett's post hoc test. Asterisk (*) indicates a significant difference compared to Lys-lys treatment. ($p < 0.05$).

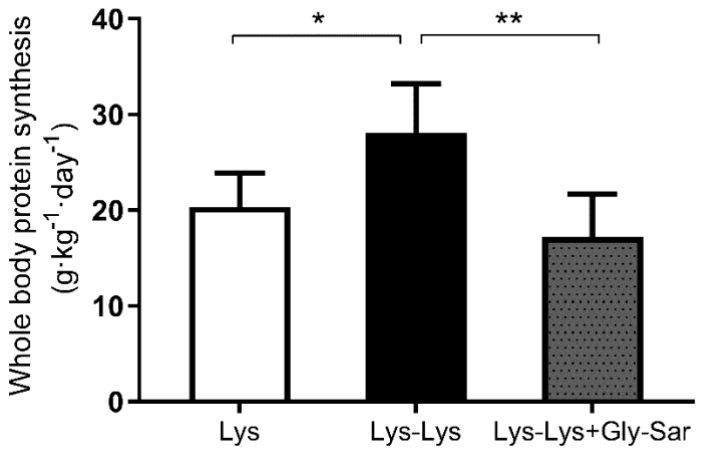


Figure 4.2: Whole-body protein synthesis in piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean ± SD, Lys (n=7), Lys-Lys (n=7), and Lys-Lys+Gly-Sar, (n=6). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. *P < 0.05 and **P <0.01. Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine.

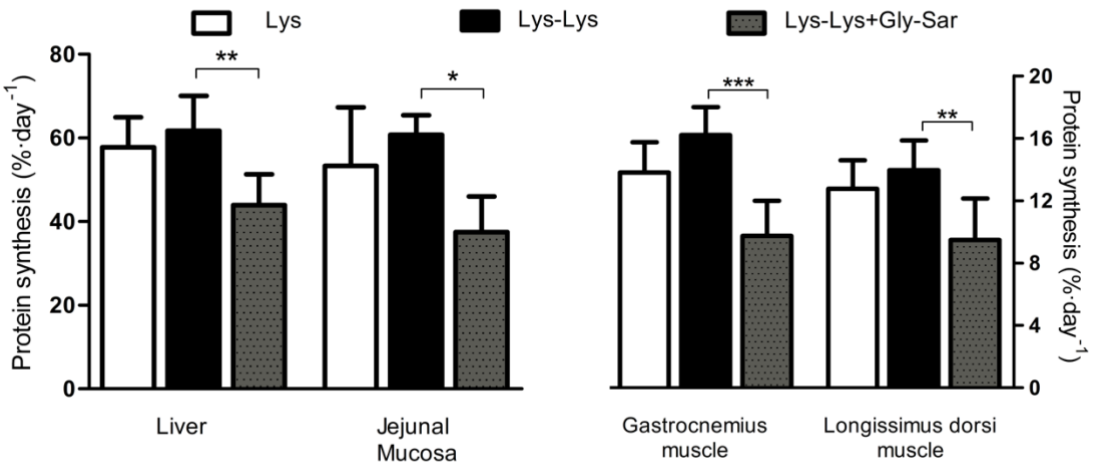


Figure 4.3: Fractional rates of protein synthesis in the liver, jejunal mucosa, gastrocnemius and longissimus dorsi muscles in piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean ± SD, Lys (n=7), Lys-Lys (n=7 except for liver and gastrocnemius muscle n=6), and Lys-Lys+Gly-Sar (n=6). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. *P < 0.05, **P <0.01 and ***P<0.001. Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine.

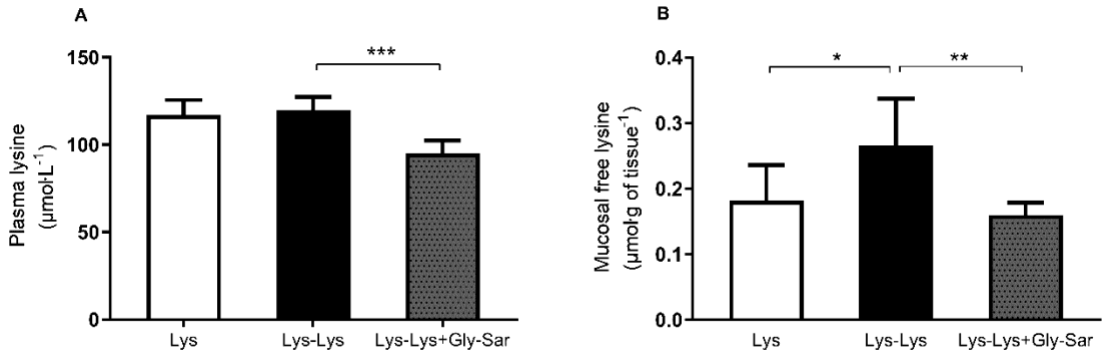


Figure 4.4: Plasma lysine concentration (A) and jejunal mucosal free lysine concentration (B) in piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean± SD, Lys (n=7), Lys-Lys (n=7), and Lys-Lys+Gly-Sar, (n=6). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. ***P<0.001, **P<0.01, *P<0.05. Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine**.**

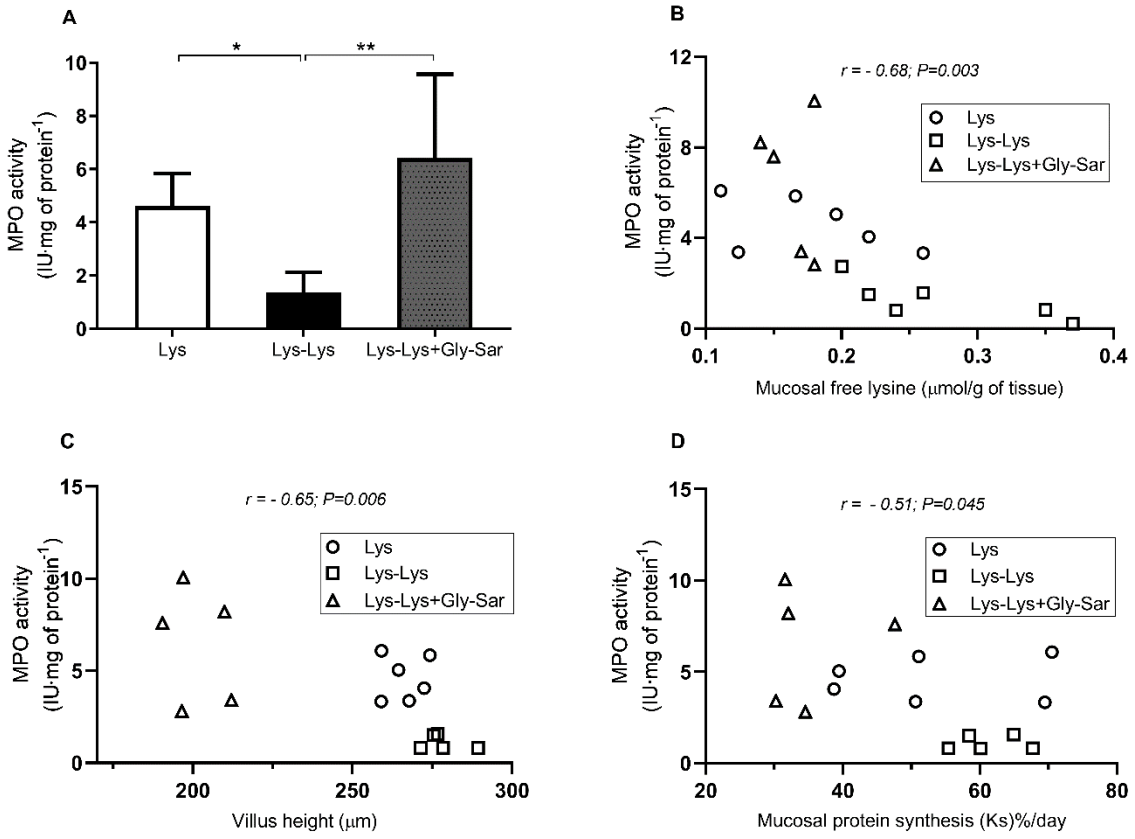


Figure 4.5: Myeloperoxidase (MPO) activity in the jejunal mucosa (A), and correlations between MPO activity and mucosal free lysine (B), villus height (C), and mucosal protein synthesis (D) in the piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean ± SD, Lys (n=7), Lys-Lys (n=6) and Lys-Lys+Gly-Sar (n=5). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. *P < 0.05 and **P <0.01 (A). Each symbol represents an individual piglet (B-D). Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine.

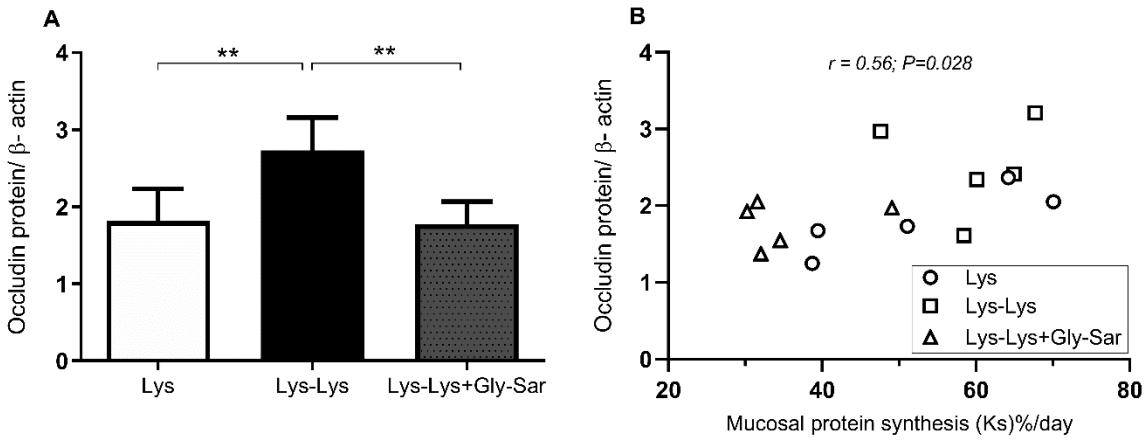


Figure 4.6: Relative expression of occludin protein in the jejunum (A), and the correlation between relative expression of occludin protein and rate of mucosal protein synthesis (B) in piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean ± SD, Lys (n=5), Lys-Lys (n=5) and Lys-Lys+Gly-Sar (n=5). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. **P <0.01 (A). Each symbol represents an individual piglet (B). Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine.

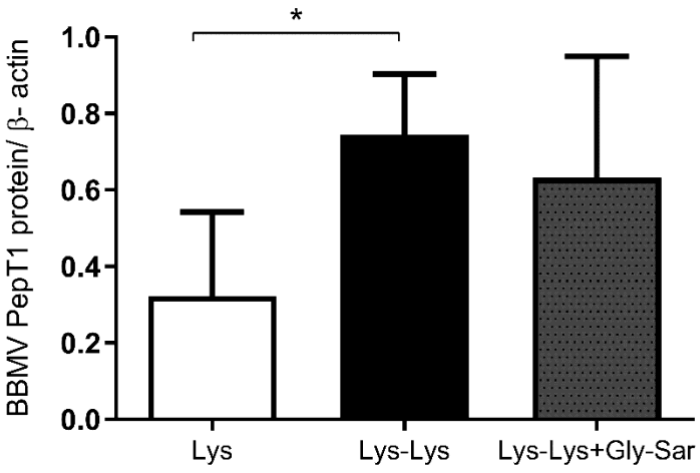


Figure 4.7: Relative expression of PepT1 protein in the jejunum brush border membrane from piglets with PN-induced intestinal atrophy following 18 h of enteral refeeding with diets containing Lys, Lys-Lys or Lys-Lys+Gly-Sar. Values are mean ± SD, Lys (n=5), Lys-Lys (n=5) and Lys-Lys+Gly-Sar (n=5). Data were analyzed using one-way ANOVA with Dunnett's post hoc test. *$P < 0.05$. Lys, free L-lysine; Lys-Lys, lysyl-lysine; Lys-Lys+Gly-Sar, lysyl-lysine with glycyl-sarcosine.

4.4.6 MPO activity

Myeloperoxidase enzyme activity was measured in the jejunal mucosa as an indicator of neutrophil infiltration. Piglets in the Lys or Lys-Lys+Gly-Sar groups generated higher MPO activity compared to the Lys-Lys piglets ($P<0.01$) (**Figure 4.5a**). MPO activity was negatively correlated with mucosal free lysine concentration, villus height, and rate of mucosal protein synthesis (**Figure 4.5b-d)**.

4.4.7 Occludin and PepT1 protein

The expression of occludin protein was measured as a representative intestinal tight-junction protein in the jejunal mucosa. Occludin protein expression was highest in the Lys-lys treatment group compared to other two treatments (**Figure 4.6a**). Furthermore, occludin protein expression correlated positively with the rate of jejunal mucosal protein synthesis (**Figure 4.6b**). Lys-lys treatment group also had significantly greater PepT1 protein expression in the brush border compared to the Free lysine group, but was similar to the Lys-lys+Gly-sar treatment groups (**Figure 4.7)**

4.5 Discussion

Previous studies have shown that when intestinal stress is induced by prolonged lack of enteral stimulation, such as during starvation, fasting or parenteral nutrition, the activity and/or expression of PepT1 transporter is sustained or up-regulated even though specific amino acid transporters are down-regulated (Vazquez *et al*., 1985; Ogihara *et al*., 1999; Ihara *et al*., 2000; Ma e*t al*., 2012). Therefore, we hypothesized that delivering

dietary lysine as a dipeptide into an atrophied gut would increase lysine bioavailability for protein synthesis. We used parenteral nutrition to induce intestinal atrophy in this study, and then re-introduced an enteral diet that provided lysine at 75% of the NRC requirement for piglets; the lower lysine protocol ensured that any advantage of the dipeptide by increasing the availability of lysine would be noticeable.

One of the most exciting outcomes of this study was that simply changing the form of lysine to a dipeptide led to greater whole-body protein synthesis. This strongly suggests that the efficiency of absorption is improved with the use of the dipeptide in an injured gut. The advantage may be related to a reduced abundance of free amino acid transporters, or an enhanced abundance of peptide transporter, or both. We did not measure free amino acid transporters but did find greater PepT1 protein in the brush border of piglets fed dipeptides, supporting the concept that transporter expression is induced by dietary substrates (Erickson et al., 1995; Walker *et al*., 1998; Shiraga *et al*., 1999).

The fate of the lysine dipeptide once transported into the intestinal epithelial cell could include transport into the portal blood as an intact dipeptide, or intracellular hydrolysis to free lysine. Higher mucosal lysine concentration in the Lys-lys group suggests efficient hydrolysis of the dipeptide occurred intracellularly. Enhanced lysine availability when fed as a dipeptide was also demonstrated by the outcomes of intestinal morphology. Similar to mucosal lysine concentration, feeding the dietary dipeptide resulted in greater mucosal weight and villus height. In addition to whole-body protein synthesis, we also measured hepatic, jejunum mucosal, and muscle protein synthesis as a functional indicator

of lysine availability. Despite improvements in gut morphology and whole-body protein synthesis, it was surprising that the rate of protein synthesis in the mucosa was not significantly higher with the lysyl-lysine treatment. We measured mucosal protein synthesis 18 hours after the introduction of enteral feeding. It is possible that higher rates of protein synthesis occurred earlier in the re-feeding period, which could lead to the improvements in gut morphology. So, it is possible that we missed the window of greater protein synthesis, but this cannot be confirmed. Even though we did not see differences in the rate of mucosal protein synthesis between the free lysine and lysyl-lysine treatments, the relative amount of occludin protein in the jejunal mucosa was positively correlated with the rate of mucosal protein synthesis, suggesting specific proteins could have been induced.

Another important finding was that glycyl-sarcosine completely abolished the beneficial effects measured with lysyl-lysine alone. The addition of glycyl-sarcosine to the diet with the lysine dipeptide provided important insight into the fate of lysyl-lysine in the intestinal lumen. One of the difficulties of conducting *in vivo* studies in conscious, ambulatory animals is being confident that the dipeptide remains intact in the gut lumen, to be available for transport by PepT1. Glycyl-sarcosine is a hydrolysis-resistant dipeptide often used to assess PepT1 transporter activity. Because glycyl-sarcosine has a higher affinity to PepT1 than lysyl-lysine (Eddy *et al*., 1995; Brandsch *et al*., 1998; Brandsch *et al*., 2004), we hypothesized that delivering glycyl-sarcosine with lysyl-lysine would interfere with lysyl-lysine absorption, reducing the lysine available for protein synthesis. As we predicted, the inclusion of glycyl-sarcosine resulted in outcomes that were suggestive of lysine deficiency, as evidenced by lower whole-body protein synthesis,

mucosal free lysine concentration, and mucosal weight. Furthermore, poorer outcomes of villus height, crypt depth, SI length, plasma lysine, and tissue protein synthesis in the lysyl-lysine with glycyl-sarcosine group clearly suggest that the dipeptide lysyl-lysine remained intact in the intestinal lumen and the high-affinity glycyl-sarcosine competitively inhibited lysyl-lysine uptake. If significant hydrolysis of lysyl-lysine had occurred in the intestinal lumen or if the transport of lysyl-lysine was entirely dependent on paracellular movement, the inclusion of glycyl-sarcosine would not have so clearly abolished the beneficial effects of the lysine dipeptide. We used glycyl-sarcosine because it is hydrolysis-resistant, and also because sarcosine is not a proteinogenic amino acid and glycine is dispensable and not known to stimulate protein synthesis. If total hydrolysis of glycyl-sarcosine occurred following uptake from the intestinal lumen, then the treatment increased total dietary amino acids by less than 2%, and likely did not affect protein synthesis.

A previous study from our group showed that PepT1 mRNA expression and the transport of dipeptide in the small intestine of piglets were consistent over the suckling period (1-3 weeks). However, higher ileal dipeptide transport was observed in piglets at post-weaning age (6 weeks), after transitioning from sow milk to a cereal-based diet. It is likely that the transporter expression increased in the lower gut, secondary to exposure to dietary peptides when the less digestible cereal-based diet reached that section of the gut (Nosworthy *et al*., 2016). Therefore, we expected that the diet with dipeptides would induce PepT1 protein expression in BBMV, and it did.

Parenteral feeding adversely affects the mucosal integrity and gut barrier function, which can lead to subsequent bacterial translocation and inflammation (Niinikoski *et al*., 2004). Occludin is one of the tight junction proteins that plays a vital role in the maintenance of gut barrier integrity, as decreased occludin expression is associated with increased intestinal permeability and reduced gut barrier function (Fries *et al*., 1999; Kucharzik *et al*., 2001; Al-Sadi *et al*., 2011). In our study, changing the form of dietary lysine to a dipeptide increased the relative occludin protein expression. This outcome suggests that occludin synthesis may have been affected by lysine availability. Furthermore, dietary lysyl-lysine also appeared to lower mucosal inflammation, as indicated by the lower mucosal MPO activity. In addition, MPO activity was negatively correlated to mucosal free lysine, as well as to mucosal protein synthesis, suggesting lower inflammation was a result of higher protein synthesis due to more available lysine. Moreover, a significant inverse correlation between relative occludin protein expression and mucosal MPO activity (data not shown), also suggested that barrier integrity is key to limiting inflammation. Better lysine availability mediated by dipeptide transport likely facilitated more rapid mucosal recovery, restoring barrier function and leading to reduced neutrophil infiltration. This further supports the importance of exploiting PepT1 to facilitate efficient uptake of nutritionally important lysine by an injured gut.

Several studies have shown that PepT1 expression and activity is preserved under various pathological conditions. However, none of these studies have investigated specific structural and functional benefits of providing dipeptides during the situation of intestinal injury. A study conducted in healthy adults and patients with coeliac disease reported that

the absorption rate of alanine and glycine from glycyl-L-alanine dipeptide solution was similar in coeliac disease patients and controls. However, the absorption rate of both amino acids from the free amino acid solution was impaired in patients with coeliac disease compared to healthy adults (Silk *et al*., 1974). Furthermore, the absorption rate of glycine as both free glycine and glycyl-glycine was markedly reduced in the jejunum of sprue patients compared to healthy individuals, with a more severe reduction for free glycine (15-fold) than for glycyl-glycine (4-fold) (Adibi *et al*., 1974). Ihara and colleagues demonstrated that expression of PepT1 mRNA in the proximal small intestine was up-regulated by over 160% in the parenterally fed rats compared to controls (Ihara *et al*., 2000). In humans, starvation significantly decreased the transport of amino acids, but no significant change was observed in the transport of peptides (Vazquez *et al*., 1985). Most the above-mentioned studies were performed in adults or mature animal models and investigated the PepT1 expression and peptide transport during situations of intestinal stress. However, they did not report the functional importance of enhanced peptide transport during intestinal injury. According to our knowledge, this is the first study to provide evidence for important structural, functional and immune benefits of delivering just one of the essential amino acids in the form of the dipeptide in a neonatal model of intestinal recovery. The application of these findings has relevance to the swine industry as a strategy to offset adverse gut effects and growth impairment related to weaning. Furthermore, because the piglet is a highly relevant model for the extrapolation of data to human infants (Shulman *et al*., 1993; Odle *et al*., 2014), our results may also advance the clinical care of preterm newborns who are at high risk for feeding intolerance and necrotising colitis.

In summary, our results showed that when intestinal injury is present, lysine was more bioavailable as dipeptide. Remarkably, when dietary lysine was limited to 75% of requirement, delivering it as dipeptide resulted in a 38% improvement in the rate of whole-body protein synthesis and improved structural indices of gut health, compared to equimolar free lysine. The inclusion of glycyl-sarcosine as a competitive inhibitor of PepT1 clearly demonstrated that the efficient uptake of lysine was mediated by PepT1. The findings from this study provide a foundation for the development of nutrition strategies to sustain growth and enhance gut recovery during the high-risk transition from gut stress, such as from parenteral to enteral nutrition, or recovery from other intestinal stress such as weaning.

Acknowledgements

We thank Martin F Hurley for surgical assistance. DK, JAB and RFB were responsible for designing the study and DK conducted the research and performed the statistical analyses. DK and JAB wrote the manuscript and had primary responsibility for the final content. All authors contributed to and approved the final manuscript.

Supplemental Table 4.1: Plasma amino acid concentrations (μmol·L^{-1}) in piglet re-fed with free L-lysine, lysyl-lysine or lysyl-lysine with glycyl-sarcosine after four days of PN diet [1]

Amino acids	Lys	Lys-lys	Lys-lys + Gly-sar	*P* value[2]
Amino acids related to arginine metabolism				
Arginine	123 ± 27	132 ± 38	108 ± 27	0.59
Aspartate	36 ± 15	42 ± 17	29 ± 4*	**<0.01**
Citrulline	490 ± 138	476 ± 107	320 ± 125	**0.05**
Glutamine	316 ± 48	387 ± 118	367 ± 94	0.35
Glutamate	167 ± 52	230 ± 80	150 ± 24	**0.05**
Ornithine	125 ± 59	161 ± 13	100 ± 28*	**0.04**
Proline	612 ± 131	809 ± 161	638 ± 146	**<0.05**
Indispensable amino acids				
Histidine	83 ± 33	78 ± 40	86 ± 8	0.90
Isoleucine	158 ± 23	179 ± 42	163 ± 40	0.50
Leucine	385 ± 48	424 ± 66	361 ± 87	0.26
Lysine	**117 ± 9**	**120 ± 8**	**95 ± 7***	**< 0.01**
Methionine	67 ± 16	61 ± 11	87 ± 20	**<0.05**
Phenylalanine	178 ± 20	170 ± 17	148 ± 25	0.25
Threonine	255 ± 46	241 ± 108	420 ± 162*	**< 0.01**
Tryptophan	77 ± 35*	106 ± 17	69 ± 20*	**<0.01**
Valine	308 ± 61	351 ± 68	357 ± 95	0.44
Dispensable amino acids				
Alanine	487 ± 58	604 ± 56	362 ± 181*	**<0.01**
Glycine	961 ± 301	1126 ± 98	1300 ± 178	**0.01**
Hydroxy proline	103 ± 16	123 ± 12	106 ± 18	0.06
Serine	575 ± 122	682 ± 135	631 ± 124	0.31
Taurine	152 ± 20	193 ± 25	221 ± 48	**<0.01**
Tyrosine	95 ± 35	63 ± 14	81 ± 61	0.36
Dipeptides				
Glycyl-sarcosine	-	-	118 ± 21	-

[1]Values are mean ± SD plasma amino acid concentration from day 5 after 17 h of enteral feeding of free L-lysine (Lys, n=7), lysyl-lysine (Lys-Lys, n=7), or lysyl-lysine with glycyl-sarcosine (Lys-Lys + Gly-Sar, n=6).
[2]Analyses were performed using one way ANOVA with Dunnett's post hoc test. Asterisk (*) indicates the significant differences compaired to Lys-lys treatment ($p< 0.05$).

Supplemental Table 4.2: Mucosal tissue free amino acid concentrations (μmol·g of tissue^{-1}) in piglet re-fed with free L-lysine, lysyl-lysine or lysyl-lysine with glycyl-sarcosine after four days of PN diet[1]

Amino acids	Lys	Lys-Lys	Lys-Lys + Gly-Sar	*P* value[2]
Amino acids related to arginine metabolism				
Arginine	0.23 ± 0.04	0.32 ± 0.14	0.22 ± 0.06	0.10
Aspartate	0.73 ± 0.12	0.66 ± 0.12	0.79 ± 0.13	0.19
Glutamine	0.18 ± 0.04	0.16 ± 0.03	0.17 ± 0.04	0.59
Glutamate	1.51 ± 0.29	1.44 ± 0.18	1.46 ± 0.27	0.85
Proline	1.25 ± 0.20	1.37 ± 0.37	1.57 ± 0.25	0.15
Indispensable amino acids				
Histidine	0.11 ± 0.06	0.14 ± 0.07	0.17 ± 0.04	0.28
Isoleucine	0.37 ± 0.07	0.41 ± 0.14	0.27 ± 0.06	0.06
Leucine	0.74 ± 0.14	0.80 ± 0.28	0.57 ± 0.29	0.23
Lysine	**0.18 ± 0.05***	**0.26 ± 0.07**	**0.16 ± 0.02***	**<0.01**
Methionine	0.16 ± 0.03	0.16 ± 0.05	0.15 ± 0.12	0.96
Phenylalanine	1.04 ± 0.09	1.18 ± 0.14	0.74 ± 0.11*	<0.01
Valine	0.54 ± 0.06	0.56 ± 0.18	0.40 ± 0.11	0.08
Dispensable amino acids				
Alanine	1.77 ± 0.32	2.02 ± 0.44	1.44 ± 0.18*	0.02
Glycine	1.69 ± 0.14	1.82 ± 0.18	2.34 ± 0.40*	<0.01
Hydroxy proline	0.85 ± 0.20	0.68 ± 0.14	0.82 ± 0.24	0.25
Serine	0.87 ± 0.09	0.87 ± 0.13	0.73 ± 0.12	0.05
Taurine	1.06 ± 0.18	0.96 ± 0.10	0.89 ± 0.18	0.18
Tyrosine	0.12 ± 0.03	0.11 ± 0.01	0.17 ± 0.07	0.06
Dipeptides				
Glycyl-sarcosine	-	-	0.08 ± 0.04	-

[1]Values are mean ± SD jejunal mucosal free amino acid concentration in piglets re-fed with free L-lysine (Lys, n=7), lysyl-lysine (Lys-Lys, n=7), or lysyl-lysine with glycyl-sarcosine (Lys-Lys + Gly-Sar, n=6).
[2]Analyses were performed using one way ANOVA with Dunnett's post hoc test. Asterisk (*) indicates the significant differences compaired to Lys-lys treatment ($p < 0.05$).

CHAPTER 5: Characterizing the influence of peptide transporter 1 (PepT1) activation on protein-mediated gut hormone release and glucose response in neonatal piglets

This study was presented at Canadian Nutrition Society Annual Conference (2022), and the conference abstract was published in Applied Physiology Nutrition and Metabolism (Appl. Physiol. Nutr. Metab. Vol. 47, 2022). This chapter will be submitted for the publication in the BMC Nutrition & Metabolism Journal. The co-authors of this work are Dalshini Kirupananthan, Robert F Bertolo, and Janet A Brunton. DK and JAB were primarily responsible for designing the study with input from RFB. DK carried out the animal work, the laboratory and statistical analyses with supervision by JAB and RFB. The manuscript was drafted by DK with contributions and editing by JAB and RFB. The progress of this study was impeded by COVID-related university shout-down as well as due to animal breeding failures.

5.1 Abstract

PepT1, found in the apical membrane of the small intestine, has been identified as a protein sensing mechanism in the upper gut. PepT1 influences the secretion of peptide hormones from the enteroendocrine cells, which may beneficially affect appetite regulation and glucose homeostasis. Studies in adult humans and animal models have shown that high protein diets improve the blood glucose. In contrast, high protein feeding in infancy with standard formulas is associated with an increased risk of obesity and chronic diseases in later life when compared to breastfeeding, which is naturally lower in protein. However, the association between PepT1 activation and gut hormone release and glucose metabolism has not been studied in the neonate. The objectives of this study were to characterize the effect of PepT1 activation and dietary peptide transport on gut hormone release and glucose response in neonatal piglets. Using an in-situ perfusion model, saline was infused into the duodenum for 30 min, followed by a 4-h continuous duodenal infusion of either saline (control), casein hydrolysate, or casein hydrolysate with PepT1 inhibitor (4-aminomethyl benzoic acid). Casein hydrolysate was infused at the sow milk protein concentration. Subsequently, ^{13}C glucose was infused for 2 h to measure glucose production, and then IV glucose was given to measure glucose clearance. GLP-1 and GIP concentration were significantly higher in the casein hydrolysate treatment compared to the other two groups. Peak insulin and plasma insulin area under the curve were also higher in the casein hydrolysate treatment which resulted in faster glucose clearance compared to other two treatments. Thus, in neonates, PepT1 is also involved in inducing gut hormone release and

altering glucose homeostasis. The findings of this study will help to elucidate the risk factors associated with formula feeding in infancy and later risk of obesity.

5.2 Introduction

Obesity is one of the critical public health issues worldwide; it is the 5th leading cause of global deaths and is responsible for the development of associated non-communicable diseases (WHO, 2016). Early infant nutrition has a potential programming effect towards a later risk of obesity (Monasta *et al*., 2010; Koletzko *et al*., 2014). Sufficient protein intake and essential amino acid supply in infancy are fundamental to avoid suboptimal growth and impaired development (WHO, 2002); however, high protein intakes beyond requirement may actually be detrimental during early development.

Formula-fed infants exhibit higher body weight gain than breastfed infants, and formula-fed infants have a greater risk of becoming overweight or obese in later life (Dewey *et al*., 1998; Kramer *et al*., 2004; Koletzko *et al*., 2005; Weber *et al*., 2014). A large randomized controlled trial (EU CHOP) revealed that infants who received high protein infant formula during the first year of life had higher body weight and BMI at two years of age (Koletzko *et al*., 2009a). Formula-fed infants consume higher protein per kg of body weight than breast-fed infants (Heinig *et al*., 1993; Dewey *et al*., 1996; Koletzko *et al*., 2005). Several studies have shown that this high protein formula feeding is associated with rapid weight gain, elevated levels of GLP-1 and insulin growth factor-1, higher circulating insulin, and reduced insulin sensitivity in later life (Dewey *et al*., 1992; Heinig *et al*., 1993; Kramer *et al*., 2004; Manco *et al*., 2011; Inostroza *et al*., 2014; Diaz *et al*.,

2015; Rzehak *et al*., 2017; Azad *et al*., 2018). Higher circulating hormones such as GLP-1 and insulin in infancy may confer a risk for obesity and chronic disease later in life. A recent study by Koletzko et al. 2019 tested this "early protein hypothesis" and confirmed that high protein feeding in infancy with standard formulas is associated with an increased risk of obesity (Koletzko *et al*., 2019). However, breastfeeding in infancy has consistently been reported to provide protective effects against a later risk of obesity and adiposity (Koletzko *et al*., 2005; Koletzko *et al*., 2009a; Koletzko *et al*., 2009b; Koletzko *et al*., 2019), which may be related in part to the fact that breast milk is naturally lower in protein.

Dietary protein intake alters glucose homeostasis; multiple studies have shown that protein intake led to higher plasma insulin levels and reduced postprandial glycemia in healthy and diabetic subjects (Gannon *et al*., 1988; Gannon *et al*., 2003; Manders *et al*., 2005; Frid *et al*., 2005; Claessens. *et al*., 2008; Power *et al*., 2009; Evangelista *et al*., 2021). Thus, high dietary protein intake appears to be beneficial for individuals with insulin resistance. However, chronically elevated insulin stimulated by high dietary protein could be problematic in healthy subjects over the long term, increasing the risk for insulin resistance (Gavin *et al*., 1974; Kobayashi *et al*., 1978; Bertacca *et al*., 2005). Furthermore, high protein intake stimulates gut peptide release including GLP-1 and PYY, influencing appetite control (Brennan *et al*., 2012; van der Klaauw *et al*., 2013), inducing satiety, and reducing food intake in rodents and humans (Lejeune *et al*., 2006). Overall, high protein intake has beneficial effects in adults, particularly those with diabetes. Interestingly, peptide transporter 1 (PepT1) has been identified as the protein sensing mechanism in the upper small intestine and is involved in regulating glucose homeostasis in adults (Dranse

et al., 2018). PepT1 influences the secretion of peptide hormones from the enteroendocrine cells (Darcel *et al*., 2005; Diakogiannaki *et al*., 2013), which may beneficially affect appetite regulation and glucose homeostasis. However, the associations between PepT1 activation and gut hormone release and glucose metabolism have not been studied in the neonate. Therefore, this study characterized the influence of PepT1 activation and dietary peptide transport on gut hormone release and glucose kinetics in a neonatal piglet model.

5.3 Materials and Methods

5.3.1 Animals and surgical procedure

All the surgical and experimental procedures were approved by our Institutional Animal Care Committee according to the guidelines of the Canadian Council on Animal Care. Fifteen Yucatan miniature piglets at 9-14 days old were obtained from a breeding herd at Memorial University of Newfoundland (St. Johns, Canada). Animals were fasted for 3 hrs prior to the surgical procedure to eliminate the influence of gut from suckling. The piglets were randomly assigned to one of three treatments (as described below) and were balanced for sex and weight across treatments. Upon arrival, animals were sedated, intubated, and anesthetized as described previously (Dodge *et al*., 2012). Under general anesthesia, a jugular vein catheter was implanted to infuse the glucose isotope and subsequently a bolus glucose during the intravenous glucose tolerance test; saline was infused as well to maintain hydration. The carotid artery was isolated and catheterized for arterial blood sampling. A laparotomy was performed to expose the small intestine and to

catheterize the portal vein for blood sampling, and finally, a duodenal catheter was inserted to administer the treatments directly into the small intestine.

5.3.2 Experimental procedure

PepT1-mediated gut hormone release. Immediately following catheter implantation, and while still under general anesthesia, all the animals received duodenal saline infusion for 30 min, followed by continuous treatment infusions for 4 h. Treatments were administered via a syringe pump at the rate of 10 ml/h. The treatments include saline as a control, or casein hydrolysate (5.5 g/ 100 ml) to determine the effect of standard protein diet on gut hormone release and glucose homeostasis, or casein hydrolysate (5.5 g/ 100 ml) with 4-aminomethyl benzoic acid (4-AMBA, 20 mM, Sigma-Aldrich, Canada) to study the protein-stimulated gut hormone release that occurs through PepT1-medicated transport of peptides. The 4-AMBA has been identified as a PepT1 inhibitor which binds to the transporter but is not translocated through PepT1 and competitively inhibits the peptide influx (Meredith *et al*., 1998); 20 mM was chosen based on the previous studies (Diakogiannaki *et al*., 2013; Dranse *et al*., 2018). The casein hydrolysate concentration was chosen to mimic the total protein concentration in sow milk.

Gut hormone measurement. During the 4 h and 30 min infusion period, portal blood samples were collected every 15 min from baseline to the end of the first hour of the treatment infusions, to determine the effect of treatments on the gut hormones release (-30, -15, 0, 15, 30, 45, 60 min). Portal blood samples were immediately treated with DPP-IV inhibitor (Millipore, USA) to prevent the inactivation of the hormones of interest.

Endogenous glucose production (EGP) test. At 1 h and 30 min into the experiment, piglets received bolus (300 μmol/kg and constant (300 $\mu mol.kg^{-1}h^{-1}$) IV infusions of U-$^{13}C_6$ glucose stable isotope (Cambridge Isotope Laboratories, Inc., USA) for 2 h. Carotid blood samples were drawn at -10, -5, 0, 30, 60, 90, 105, 120 min to measure the EGP based on the tracer dilution technique as described below. EGP was calculated once the ^{13}C glucose reached the steady-state (t=90-120 min).

Intravenous glucose tolerance test (IVGTT). IVGTT was performed during the last hour of the experimental procedure. Blood samples were taken -10, -5, and 0 min before a bolus infusion of glucose. Piglets received an intravenous bolus of glucose (50% solution in saline; 0.5 g/kg BW) into the jugular vein immediately after the baseline blood samples were obtained. Blood samples were collected at 5, 10, 15, 20, 20, 25, 30, 35, 40, 50, and 60 min or until blood glucose concentration returned to baseline concentration. Blood glucose concentration was measured using a glucometer (Ascensia Diabetes Care, Mississauga, Ontario, Canada), and then separated plasma was stored at -80ºC for later analysis.

5.3.3 Plasma hormones and amino acid analysis

Gut hormone concentrations (GLP-1 and GIP) were measured in the portal plasma collected during the initial period (-30 to 60 minutes). Plasma GLP-1 was quantified using fluorescence-based GLP-1 active (7-36) ELISA Kits (Millipore, Watford, UK) according to the manufacturer's instructions. Plasma GIP was determined using GIP ELISA Kits (MyBioSource, San Diego, USA) based on the manufacturer's instructions. Gut hormone concentrations were expressed as a percentage of change from the baseline. Plasma insulin

concentration during the IVGTT test was assessed using human insulin ELISA Kits (Abcam, Toronto, ON). The assay was performed according to the manufacturer's guidelines. Plasma amino acid levels were measured using HPLC with the use of phenylisothiocyanate derivatives and norleucine was used as the internal standard as previously described (Dinesh *et al*., 2021).

5.3.4 Endogenous glucose production

Glucose production was measured as previously described with minor modifications (Wahjudi *et al*., 2010). Briefly, 300 µl of 0.3 M barium hydroxide and 300 µl of 0.3 M zinc sulfate were added to 100 µl plasma samples to precipitate proteins and the samples were centrifuged at 1000 x *g* for 5 min. The supernatant was transferred to glass vials and vacuum dried. In the derivatization process, 100 ml of 0.18 M methoxylamine hydrochloride (dissolved in pyridine) was added into each vial and incubated at 70°C for 60 min. Then, 100 µl of acetic anhydride (Sigma Aldrich) was added and allowed to react at 45°C for another 60 min. The sample was dried and redissolved in 100 µl ethyl acetate (Fisher Chemicals) prior to GC/MS analysis. The GC/MS conditions were injector temperature 180°C with helium as carrier gas with a flow rate of 1 ml/minute. The column temperature was held initially at 180°C for 2 min, ramped up by 5°C/min up to 250°C and then increased by 50°C/min to reach a final temperature of 300°C. The tracer to tracee ratio was calculated by monitoring m/z = 131 for D-glucose (C_1-C_2) and m/z = 133 for the stable isotope of glucose ([1,2-$^{13}C_2$] D-glucose). The endogenous glucose production was calculated as described in Stoll *et al*. (2012).

Ra = Endogenous glucose production + exogenous glucose infusion

The rate of appearance (Ra) of total glucose (endogenous + exogenous) was determined from the ^{13}C enrichment of glucose during the steady state using the isotope dilution equation. In this study, the steady state was achieved during the last 30-45 minutes.

$$Ra = [(Ei/Ep)-1] * I$$

where Ei is the enrichment of ^{13}C in the infusate, Ep is the enrichment of ^{13}C in plasma, and I is the infusion rate of D-[$^{13}C_6$] glucose ($\mu mol.kg^{-1}h^{-1}$). The glucose rate of disappearance (Rd) from plasma is assumed to be equal to Ra under the steady state. Therefore,

Endogenous glucose production = Rd – exogenous glucose infusion where exogenous glucose infusion during the study period was limited to the D-[$^{13}C_6$] glucose.

5.3.5 Plasma Glucose Assay

Plasma glucose concentration was determined using an in-house developed assay which utilized glucose oxidase/peroxidase reagent (1 capsule contains 500 units of glucose oxidase (Aspergillus niger), 100 purpurogallin units of peroxidase (horseradish), and buffer salts) (Sigma-Aldrich, Saint Louis, Missouri, USA). A stock solution of glucose (5.6 mM) was used to prepare serial dilutions to develop a standard curve (0, 0.0125, 0.025, 0.05, 0.1 and 0.2 mM). Briefly, one capsule of glucose oxidase/peroxidase reagent was dissolved in 39.2 ml of deionized water in an amber vial. 5 mg of o-dianisidine dihydrochloride powder was resuspended in 1 ml deionized water in a separate amber vial. 0.8 ml of resuspended

o-Dianisidine dihydrochloride was added to the amber vial containing the oxidase/peroxidase reagent. The reagent solution was then stored in a cooler until needed. The plasma samples were diluted as 10 µl plasma samples in 990 µl deionized water, and 60 µl of diluted plasma sample was transferred to a 96 well plate. Then, 120 µl of reagent was added to each well, incubated at 37ºC for 30 minutes with agitation, and the plate was read at 450 nm. The glucose concentration of the samples was calculated, expressed as percentage change from the baseline, and the area under the response curve was also calculated.

5.3.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 8.0., GraphPad, La Jolla, CA, USA). All data are shown as mean ± SEM. One way ANOVA was used to determine the statistical significance for the outcome with single measurement, two-way ANOVA was used to analyze measurements performed over time. Group means were compared using Tukey's multiple comparisons test and a significant difference was considered as $p < 0.05$.

5.4 Results

5.4.1 Gut hormones

Plasma GLP-1 and GIP concentrations are expressed both as a percentage of baseline (Figure 5.1A and 5.1B) and as absolute concentrations at 30 min into the treatment period (Figure 5.1C and 5.1D). Plasma GLP-1 changed significantly over time ($p=0.01$),

and was affected by treatment ($p<0.01$), and there was a significant time x treatment effect ($p<0.01$). The change from baseline for GLP-1 was greater in the casein treatment compared to casein+4-AMBA at 30- and 60-min into the treatment period; it was also greater than the saline treatment throughout the one-hour treatment period (Figure 5.1A). The casein+4-AMBA change from baseline was higher than the saline group by 45 min into the treatment period. Plasma GIP did not change significantly over time ($p=0.40$), but was affected by treatment ($p<0.01$), and there was a significant time x treatment effect ($p=0.03$). Furthermore, the change in plasma GIP as a percentage of baseline was significantly higher in the casein treatment at 30 min compared to the other two groups (Figure 5.1B). In addition, the casein hydrolysate group had significantly higher GLP-1 when measured at 30 min compared to the saline control. The inclusion of the PepT1 inhibitor 4-AMBA with the casein hydrolysate resulted in a GLP-1 concentration that was 38% lower than without the inhibitor (Figure 5.1C). GIP concentration was also significantly higher in the casein treatment compared to saline treatment; however, it did not significantly differ between casein and casein+4AMBA ($p = 0.08$) (Figure 5.1D).

5.4.2 Plasma glucose and insulin concentrations during IVGTT

Plasma glucose concentration measured during the IVGTT was expressed as a percentage of change from baseline. The peak in plasma glucose was significantly lower in the casein treatment compared to saline treatment and casein with the inhibitor treatment. However, the casein hydrolysate infusion did have a greater glucose clearance rate compared to the other two treatments and was the only treatment to almost return to

baseline at 60 min (Figure 5.2). P-values from the two-way ANOVA for treatment, time and time x treatment interaction were <0.01. In addition, the integrated area under the curve (AUC) of plasma glucose was significantly lower in the casein treatment compared to the other two treatments (Figure 5.3). To determine whether the glucose response was due to the change in plasma insulin, plasma insulin concentration was measured in plasma sampled during the IVGTT (Figure 5.4). Plasma insulin was significantly higher in the casein treatment compared to saline treatment throughout the IVGTT test period. The casein treated piglets also had significantly higher plasma insulin compared to the casein with 4-AMBA group at the initial period (0-10) and 25 and 50 min after the bolus dose of glucose (Figure 5.4). P-values determined from the two-way ANOVA for treatment, time and time x treatment interaction were <0.01. The AUC of plasma insulin was significantly higher in the casein treatment than the other two treatments, and casin+4-AMBA AUC for plasma insulin was higher than for the saline treatment (Figure 5.5).

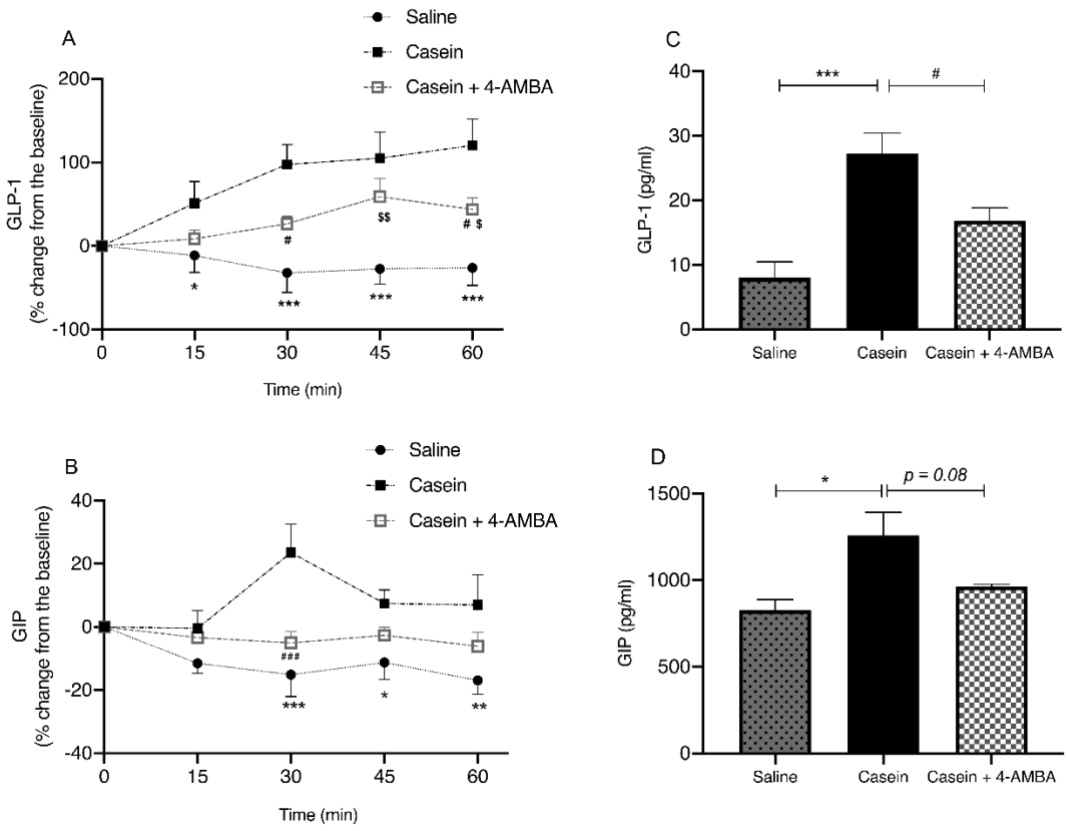


Figure 5.1: Change in plasma GLP-1 (A) and GIP (B) as a percent of baseline and plasma GLP-1 (C) and GIP (D) concentration 30 min after treatment initiation in piglets that received duodenal infusions of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical significance was determined using two-way ANOVA for A and B, and one-way ANOVA for C and D with Tukey's post hoc test, *casein compared to saline (*p<0.05, **p<0.01, ***p<0.001); $^{\#}$casein compared to casein with inhibitor 4-AMBA ($^{\#}$p<0.05, $^{\#\#\#}$p<0.001); $^{\$}$saline compared to casein with inhibitor 4-AMBA ($^{\$}$p<0.05, $^{\$\$}$p<0.01).

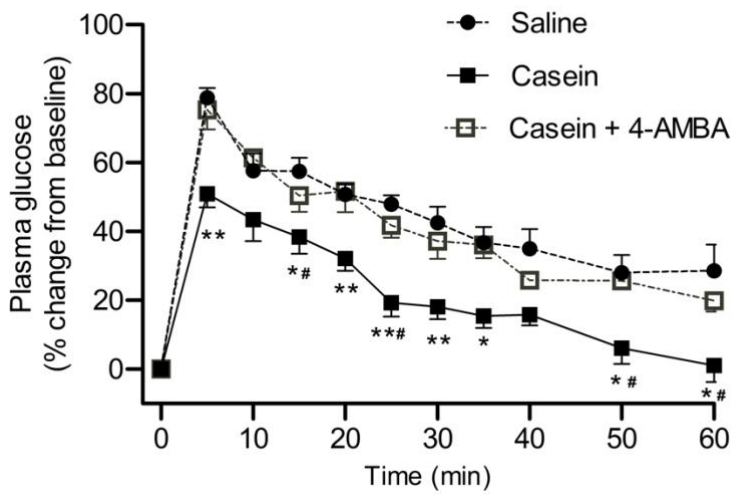


Figure 5.2: Change (as a percent of baseline) in plasma glucose over time during the IVGTT in piglets that received duodenal infusion of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical significance was determined using two-way ANOVA with Tukey's post hoc test, *casein compared to saline ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$); $^{\#}$casein compared to casein with inhibitor 4-AMBA ($^{\#}p<0.05$, $^{\#\#}p<0.01$); $^{\$}$saline compared to casein with inhibitor 4-AMBA ($^{\$}p<0.05$).

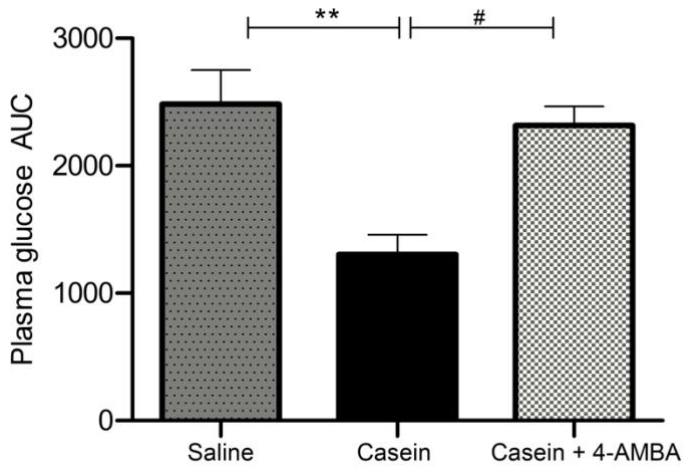


Figure 5.3: Plasma glucose integrated area under the curve over time during the IVGTT in piglets that received duodenal infusion of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, *casein compared to saline (*$p<0.05$, **$p<0.01$, ***$p<0.001$); $^{\#}$casein compared to casein with inhibitor 4-AMBA ($^{\#}p<0.05$, $^{\#\#}p<0.01$); $^{\$}$saline compared to casein with inhibitor 4-AMBA ($^{\$}p<0.05$).

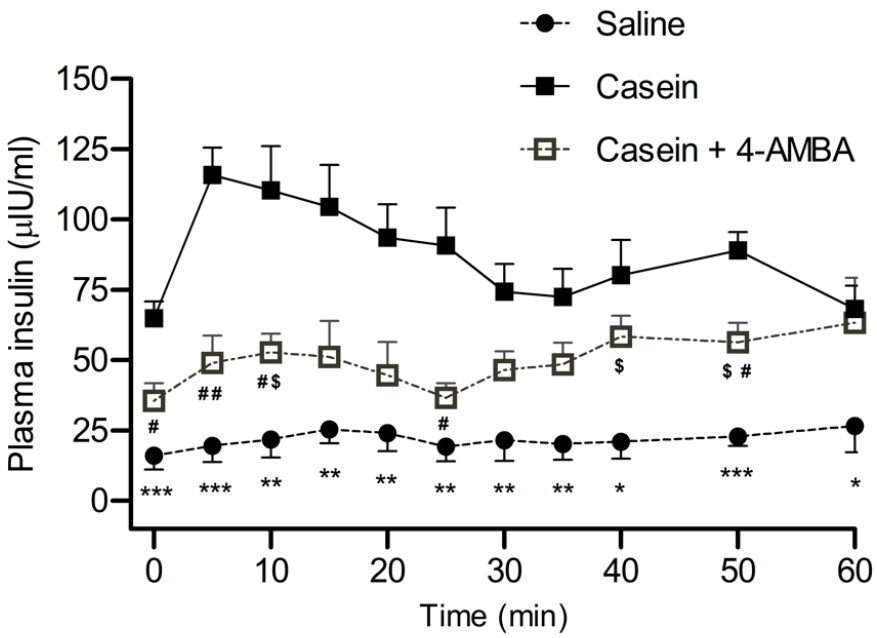


Figure 5.4: Absolute plasma insulin concentrations over time during the IVGTT in piglets that received duodenal infusion of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment, Statistical significance was determined using two-way ANOVA with Tukey's post hoc test, *casein compared to saline (*p<0.05, **p<0.01, ***p<0.001); $^{\#}$casein compared to casein with inhibitor 4-AMBA ($^{\#}$p<0.05, $^{\#\#}$p<0.01); $^{\$}$saline compared to casein with inhibitor 4-AMBA ($^{\$}$p<0.05).

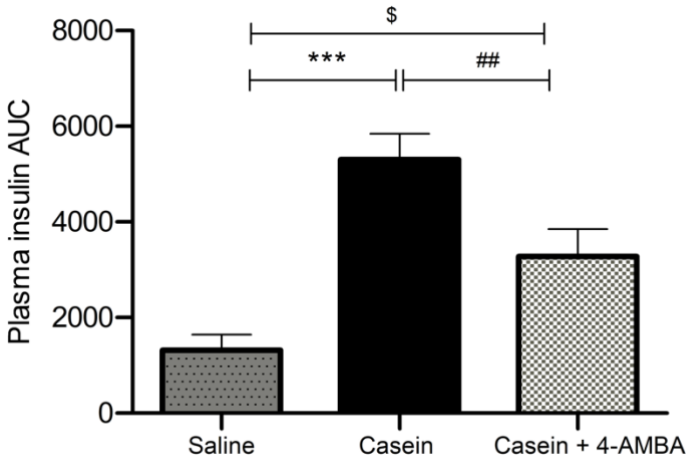


Figure 5.5: Insulin integrated area under the curve over time during the IVGTT in piglets that received duodenal infusion of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, *casein compared to saline (*p<0.05, **p<0.01, ***p<0.001); $^{\#}$casein compared to casein with inhibitor 4-AMBA ($^{\#}$p<0.05, $^{\#\#}$p<0.01); $^{\$}$saline compared to casein with inhibitor 4-AMBA ($^{\$}$p<0.05).

5.4.3 Endogenous glucose production (EGP)

EGP was determined using the ^{13}C glucose isotope dilution method during the steady isotopic state. There were no significant differences in EGP among the treatments, with the overall treatment effect of $p = 0.07$ (Figure 5.6).

5.4.4 Plasma amino acid analysis

Plasma amino acid concentrations (carotid and portal) were determined following the 60 min of duodenal treatments infusion. No significant differences in carotid and portal total plasma amino acid concentrations were observed among the treatment groups (Figure 5.7). However, portal plasma concentrations of valine, leucine, isoleucine, methionine, and proline were significantly higher in the casein and the casein+4-AMBA treatments compared to saline control (Table 5.1). Furthermore, carotid plasma concentrations of leucine, isoleucine, and proline were significantly higher in the casein and casein+4-AMBA treatments compared to saline (Table 5.2).

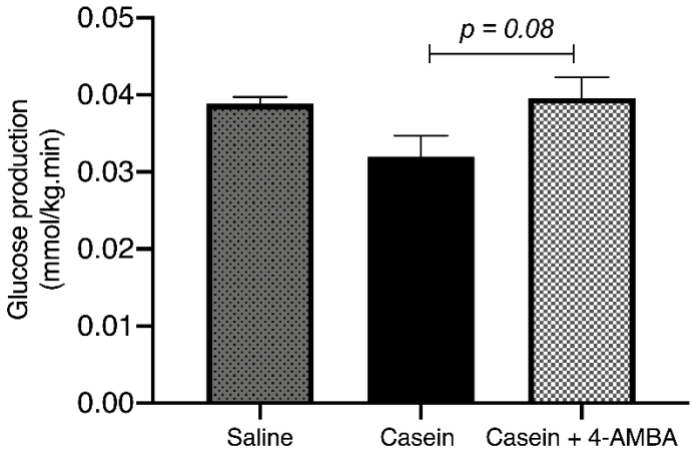


Figure 5.6: Endogenous glucose production in piglets that received duodenal infusions of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test and the overall treatment effect p = 0.07

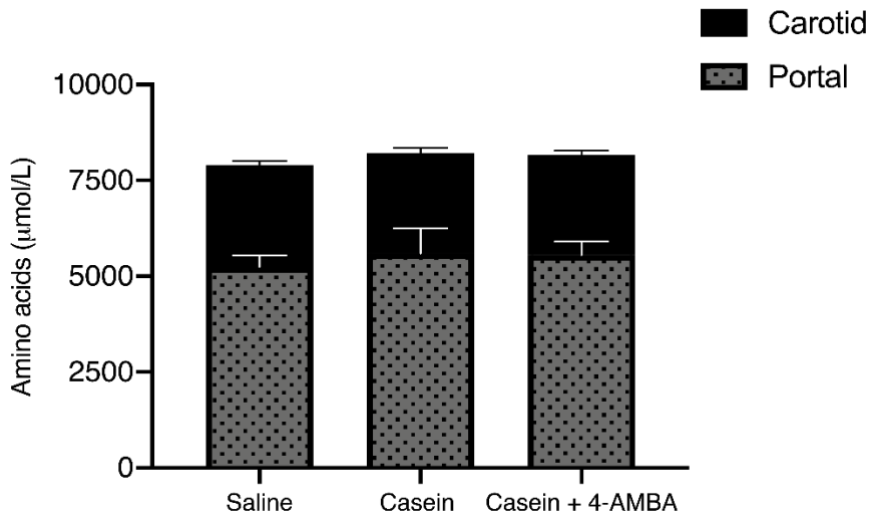


Figure 5.7: Portal and carotid total amino acid concentration in plasma obtained from piglets following 60 min of duodenal infusion of either saline, casein hydrolysate (Casein), or casein hydrolysate with PepT1 antagonist 4-AMBA (Casein+4-AMBA). Values are presented as mean ± SEM, n=5 per treatment. Statistical analysis was performed using one way ANOVA with Tukey's post hoc test.

Table 5.1: Concentration of amino acids in the portal plasma obtained from piglets following 60 min of duodenal treatment infusion[1]

Amino acids	Saline	Casein	Casein + 4-AMBA	*P* value[2]
Arginine	145 ± 20	167 ± 37	173 ± 19	0.23
Citrulline	265 ± 54	303 ± 93	300 ± 62	0.70
Glutamine	207 ± 20	241 ± 55	224 ± 48	0.24
Ornithine + Tryptophan	120 ± 37	120 ± 38	95 ± 25	0.48
Proline	326 ± 60^{b}	513± 123^{a}	418 ± 71^{ab}	0.03
Histidine	56± 18	107 ± 66	75 ± 8	0.16
Isoleucine	279 ± 38^{b}	494± 41^{a}	451± 27^{a}	<0.01
Leucine	108 ± 7^{b}	198 ± 22^{a}	228 ± 9^{a}	<0.01
Lysine	62± 21.2	83 ± 18	70± 13	0.20
Methionine	32 ± 3^{b}	70± 8^{a}	71 ± 5^{a}	<0.01
Phenylalanine	274± 64	240 ± 57	247 ± 13	0.54
Threonine	526 ± 127	460 ± 333	548 ± 77	0.83
Valine	231 ± 26^{b}	344 ± 29^{a}	400 ± 22^{a}	<0.01
Alanine	201 ± 107	147 ± 91	70 ± 38	0.09
Glycine	1295 ± 183	1058 ± 402	1048 ± 414	0.48
Hydroxy proline	162 ± 33	139 ± 59	111 ± 21	0.17
Serine	294 ± 51	325 ± 115	344 ± 59	0.57
Taurine	462 ± 72	377 ± 78	419 ± 76	0.23
Tyrosine	75 ± 24	113 ± 42	119 ± 11	0.08

[1]Values are mean ± SEM, n=5.

[2]*P* values for treatment effect by one way ANOVA; group mean differences by Tukey's post hoc test. Significant differences denoted by differing letters ($p< 0.05$)

Table 5.2: Concentration of amino acids in the carotid plasma obtained from piglets following 60 min of duodenal treatment infusion[1]

Amino acids	Saline	Casein	Casein + 4-AMBA	*P* value[2]
Arginine	77 ± 14	77 ± 18	69 ± 9	0.62
Citrulline	162 ± 26	141 ± 24	121 ± 43	0.22
Glutamine	149 ± 16	138 ± 21	117 ± 24	0.24
Ornithine + Tryptophan	46 ± 5	55 ± 10	52 ± 8	0.75
Proline	212 ± 16^{b}	237 ± 16^{a}	242 ± 15^{a}	0.03
Histidine	33 ± 10	40 ± 27	48 ± 29	0.22
Isoleucine	98 ± 12^{b}	157 ± 12^{a}	134 ± 17^{a}	<0.01
Leucine	64 ± 8^{b}	91 ± 5^{a}	79 ± 8^{ab}	<0.01
Lysine	46 ± 8	75 ± 52	50 ± 11	0.40
Methionine	23 ± 4	32 ± 9	22 ± 4	0.21
Phenylalanine	181 ± 20^{a}	132 ± 5^{ab}	128 ± 14^{b}	0.04
Threonine	349 ± 26^{a}	240 ± 95^{b}	212 ± 22^{b}	0.02
Valine	117 ± 16	166 ± 13	132 ± 24	0.06
Alanine	132 ± 40^{a}	55 ± 38^{b}	76 ± 93^{b}	<0.01
Glycine	753 ± 65^{a}	470 ± 106^{b}	579 ± 140^{ab}	0.01
Hydroxy proline	113 ± 28^{a}	66 ± 21^{b}	67 ± 12^{b}	0.01
Serine	173 ± 39	157 ± 45	178 ± 55	0.75
Taurine	269 ± 37^{a}	168 ± 45^{b}	225 ± 47^{ab}	0.02
Tyrosine	51 ± 7	57 ± 6	73 ± 15	0.37

[1]Values are mean ± SEM, n=5.

[2]*P* values for treatment effect by one way ANOVA; group mean differences by Tukey's post hoc test. Significant differences denoted by differing letters ($p< 0.05$)

5.5 Discussion

Understanding the role of PepT1 in protein-stimulated gut hormone release and glucose metabolism in neonates is clinically necessary to develop ideal feeding regimens for infants and to avoid the later risk of chronic diseases. Previous studies have shown that high protein intake in adults can stimulate insulin secretion, improve glucose tolerance, slow down gastric emptying and reduce food intake (Gannon *et al*., 1988; Gannon *et al*., 2003; Manders *et al*., 2005; Frid *et al*., 2005; Lejeune *et al*., 2006; Claessens. *et al*., 2008; Power *et al*., 2009; Brennan *et al*., 2012; van der Klaauw *et al*., 2013; Evangelista *et al*., 2021). In contrast, feeding high protein formula in infancy is associated with a later risk of obesity and other chronic diseases (Koletzko *et al*., 2005). Although PepT1-mediated upper small intestinal protein sensing improved glucose homeostasis in healthy, obese, and diabetic rodents (Dranse *et al*., 2018), there has been little investigation into the associations between peptide transport, gut hormone release, and glucose metabolism in neonates. This study investigated the influence of PepT1 activation and transport of peptides on the gut hormone secretion and glucose clearance in neonates using neonatal piglets as a human infant model. We hypothesized that activation of PepT1 with dietary peptide transport would lead to gut hormone release and altered glucose homeostasis; we further hypothesized that co-perfusion of protein hydrolysate with 4-AMBA would abolish the effect. In this study, we used 4-AMBA as the inhibitor to confirm that the protein-mediated effects were due to the transport of peptides through PepT1, as 4-AMBA will bind to PepT1 but is not transported.

Our study findings demonstrated that upper small intestinal casein hydrolysate infusion stimulated the release of incretin hormones GLP-1 and GIP from the neonatal small intestine. Furthermore, the inclusion of the PepT1 inhibitor hindered this protein-mediated effect on gut hormone release, confirming that the transport of peptides through PepT1 significantly influences GLP-1 and GIP secretion (Figure 5.1). Our data are consistent with previous findings in a cell culture model, which reported that activation of PepT1 by a dipeptide induced GLP-1 secretion from L-cells, and the addition of PepT1 inhibitor 4-AMBA reduced the PepT1-mediated effect. The proposed mechanism was that PepT1 activation triggers membrane depolarization, leading to the opening of voltage-gated Ca^{2+} flux and subsequent GLP-1 release from the intestinal L-cells (Diakogiannaki *et al*., 2013). Furthermore, luminal administration of egg protein hydrolysate also increased the GLP-1 release in the rat ileum, whereas infusion of an amino acid mixture did not induce the same effect (Cordier-Bussat *et al*., 1998). A recent study also reported that luminal administration of valine induced the GLP-1 release from the isolated proximal rat small intestine (Modvig *et al*., 2021). However, the effect of amino acids on the GLP-1 release is controversial. In our study, we administrated an enzymatically hydrolyzed casein, which is a source of small peptides and free amino acids. Therefore, higher concentrations of some amino acids along with higher GLP-1 in the casein+4-AMBA treatment compared to saline suggest that free amino acids may have an effect on GLP-1 release. However, higher GLP-1 and GIP release in the casein treatment compared to casein+4-AMBA occurred in conjunction with similar carotid and portal blood amino acid concentrations (Table 5.1 and Figure 5.7), which confirms that the transport of peptides through PepT1

influenced the release of GLP-1 and GIP. Therefore, PepT1-mediated transport of peptides is involved in the protein-mediated increase in GLP-1 and GIP concentrations.

Another important finding was that the transport of peptides through PepT1 induced insulin secretion, which was evident by higher baseline plasma insulin and higher insulin response (peak insulin level and AUC of plasma insulin) during the glucose challenge in the casein treatment compared to inhibitor treatment (Figure 5.4 and 5.5). It may be that greater circulating GLP-1 and GIP concentrations were responsible for the insulin response. Previous studies have shown that ingestion of intact protein or protein hydrolysates enhanced plasma insulin levels in healthy and diabetic individuals and rodents (Gannon *et al*., 1992; Gannon *et al*., 2003; Power *et al*., 2009; Salehi *et al*., 2012). Further studies demonstrated that whey protein intake stimulated the release of GLP-1 (Salehi *et al*., 2012) and GIP (Nilsson *et al*., 2004; Salehi *et al*., 2012), and binding of these incretin hormones to their respective receptors in the pancreatic β-cells significantly increased insulin secretion from isolated pancreatic β-cells (Salehi *et al*., 2012). Therefore, we speculate that higher circulating incretin hormones in the casein treatment induced greater insulin secretion. In addition, studies have reported that milk protein ingestion increases plasma amino acids, particularly BCAAs, which are known to stimulate insulin release (Nilsson *et al*., 2004; Salehi *et al*., 2012). Consistent with previous studies, we observed significantly higher branched-chain amino acids (BCAAs), methionine, and proline in casein and casein+4-AMBA treatments compared to saline control (Table 5.1). Therefore, higher specific plasma amino acids in both casein hydrolysate treatments likely contributed to the

higher insulin response (peak insulin level and AUC of plasma insulin) when compared to saline treatment during the glucose challenge.

In response to the higher insulin concentrations in the animals receiving the casein treatment, faster glucose disposal occurred. Plasma glucose returned to baseline levels by 60 min in the casein treated piglets; the saline group and those treated with casein plus inhibitor were still 29% and 20% above baseline at the 60 min time point (Figure 5.2). Slower glucose disposal in the casein with PepT1 inhibitor compared to casein treatment suggests that protein mediated glucose response in the casein treatment influenced by the transport of peptides through PepT1. Previous studies in healthy adults and patients with type 2 diabetes have demonstrated that ingestion of protein increases insulin secretion and facilitates glucose disposal (Manders *et al*., 2005; Frid *et al*., 2005). Our results have clearly demonstrated the same protein-mediated effect is present in neonates.

We also investigated the influence of the transport of peptides on endogenous glucose production using the isotope dilution method. In general, high circulating insulin will reduce hepatic glucose production via the stimulation of hepatic glycolytic flux (Terrettaz *et al*., 1986; Halimi *et al*., 1987) and a reduction in hepatic glycogenolysis (Assimacopoulos-Jeannet *et al*., 1990; Liu *et al*., 1993). We hypothesized that higher circulating GLP-1 and insulin would occur with the casein treatment, and that this would lead to a reduction in glucose production; however, the glucose production was not significantly different among the treatment groups (Figure 5.6). Dranse et al. 2018 demonstrated that intestinal infusion of protein hydrolysate and activation of PepT1 led to

lower endogenous glucose production under pancreatic euglycemic clamp conditions in healthy rodents. The mechanism by which PepT1 activation mediated lower glucose production was investigated by using a model that included GLP-1 receptor antagonist. They found that the administration of the GLP-1 receptor antagonist reversed the ability of the protein hydrolysate to reduce glucose production, which suggested that GLP-1 mediated signaling plays a role in the protein-mediated reduction in glucose production (Dranse *et al*., 2018). In our study, the duodenal administration of casein hydrolysate induced higher circulating GLP-1 and insulin, but it did not significantly alter the endogenous glucose production. The p value suggest that the study might have been underpowered to detect a difference, with only 5 animals per group.

Increasing evidence suggests that nutrition in postnatal life has an essential role in the programming of later life health and high dietary protein intake in infancy has been implicated as a risk factor for obesity and chronic disease (Koletzko *et al*., 2005). Salmenperä *et al*. (1988) reported higher postprandial insulin and gut hormone responses measured in formula-fed versus breastfed infants at 9 mo of age (Salmenperä *et al*., 1988). A study in 4-month-old infants found that formula-fed infants had higher circulating GLP-1 than breast fed infants (Diaz *et al*., 2015); the authors speculated that higher circulating GLP-1 may lead to long-term diabetes risk. A systematic review of several observational studies reported that breastfeeding in infancy was associated with reduced risk of type 2 diabetes and breastfed infants had lower blood glucose and serum insulin in infancy and marginally lower insulin in later life than formula fed counterparts (Owen *et al*., 2006).

PepT1 has been identified as a protein sensing mechanism, and activation of PepT1 improves glucose homeostasis in an adult rodent model (Dranse *et al*., 2018). Importantly, to our knowledge, our data are the first to identify the involvement of PepT1 in protein-mediated insulin and glucose metabolism in a neonatal model. PepT1 activation enhances gut hormone release to indirectly influence insulin release, in turn, altering glucose homeostasis. Diet-induced chronically high concentrations of these hormones could certainly change the growth trajectory of a neonate. Activation of PepT1 appears to be part of the mechanism responsible for the "protein hypothesis" related to obesity in children.

CHAPTER 6: Summary

6.1 General overview

The series of research projects presented in this thesis focused on the intestinal peptide transporter 1 (PepT1) in terms of amino acid absorption, restoring protein nutrition during the high-risk transition period from parenteral to enteral feeding, and regulating gut hormone release and glucose response in neonatal piglets. The *in vivo* ligated gut loop model was used to study the PepT1-mediated trans-stimulation of amino acid uptake. In the second study, piglets received parenteral feeding to induce intestinal stress to mimic PN-induced atrophy in neonates and weaning stress in swine production. The third study utilized a duodenal infusion model to study the effects of PepT1 activation and transport of peptides on gut hormone release and glucose response in the neonatal model. These studies utilized piglets as a model species for the human infant and to represent swine production.

6.2 Investigation of the effect of lysyl-lysine on the arginine uptake

6.2.1 Overview of results

Amino acids and dipeptide interactions may enhance or interfere with amino acid absorption. Therefore, understanding the interaction may help develop new therapeutic formulas and feeding strategies to supply nutritionally essential amino acids and amino acid-like drugs. The objectives for my first study were to investigate whether the co-perfusion of lysyl-lysine enhances the arginine uptake via the trans-stimulation of $b^{0,+}$ transporter, and whether inclusion of glycyl-sarcosine with lysyl-lysine abolishes the lysyl-

lysine mediated enhanced arginine uptake. The intestinal gut loop perfusion model was used to study these objectives. We quantified the disappearance of radiolabelled arginine in the perfusate after perfusing through the isolated intestinal segments. Mucosal-free concentrations of arginine family amino acids and lysine were also determined. This study proved that co-perfusion of arginine with lysyl-lysine enhanced the arginine uptake (~80% increase) compared to arginine alone. Furthermore, the inclusion of glycyl-sarcosine with lysyl-lysine hindered the enhanced arginine uptake in a dose-dependent manner. Glycyl-sarcosine is a hydrolysis-resistant dipeptide with higher affinity to PepT1 than lysyl-lysine. Therefore, the addition of glycyl-sarcosine might have inhibited the lysyl-lysine uptake, thereby reducing the lysyl-lysine hydrolysis and the trans-stimulation activity of a $b^{0,+}$ transporter.

6.2.2 Implications and modifications

The ligated loop model was previously developed in our lab to investigate the relationship between early development and dipeptide uptake at various locations in the small intestine of piglets (Nosworthy et al., 2013). Nosworthy et al. characterized the capacity of peptide transport along the small intestine. We utilized the proximal small intestinal segments because this is the primary location of arginine uptake and PepT1 is greatly concentrated in the proximal region from suckling to post-weaning. Using a graded amount of glycyl-sarcosine led us to confirm that PepT1-mediated lysyl-lysine uptake was necessary to increase the intracellular lysine concentration and for the trans-stimulation activity of $b^{0,+}$ transporter. Furthermore, we utilized radiolabelled arginine to determine the

arginine uptake; alternatively, we could have utilized labeled dipeptides to prove the concept with greater certainty. However, labeled dipeptides are challenging to produce and expensive to purchase. Using the ligated loop model in this study allowed us to test multiple treatments in one animal and reduce animal variability. Furthermore, we initially randomized the treatments to loop sites, and subsequently allocated the treatments to specific locations to offset the potential location effect.

In this study, we investigated the trans-stimulation activity of $b^{0,+}$ transporter using healthy three week old animals. We did measure an enhanced arginine uptake through the PepT1 mediated trans-stimulation activity of $b^{0,+}$ transporter. However, the expression of PepT1 varies with developmental stages and disease status (Nosworthy et al., 2013, Ihara et al., 2000, Adibi et al., 2003), which leads to a question of whether the changes in PepT1 expression with the developmental changes influence this trans-stimulation. Future studies should focus on investigating similar trans-stimulation activity in the models that represent the developmental stages and intestinal stress, for example, post weaned (6-week-old) animals or in a PN-induced atrophied model. Weaning in piglets and PN feeding in neonates induce intestinal atrophy which limits the intestinal absorptive capacity (Miller et al., 1986, Fallon et al., 2012). Therefore, similar studies will help to improve the nutritionally important amino acid uptake during the intestinal stress period. Utilizing one treatment per animal rather using multiple treatments in one animal would allow for the determination of a functional effect of enhanced arginine uptake, and this could be done with the use of isotope tracers. In addition, future studies can also determine the effect of

lysyl-lysine on the uptake of other synthetic substrates such as arginine like drugs, via the $b^{0,+}$ transporter.

Overall, the trans-stimulation activity of the $b^{0,+}$ transporter will give new insight into the supply of the nutritionally important amino acid arginine. Furthermore, understanding the interactions between peptide and free amino acid absorption is an essential step towards developing lower nitrogen and cost-effective diets for swine production and reducing wastage in the environment. These findings will also help to develop therapeutic formulas for nutritionally vulnerable neonates to optimize amino acid uptake and utilization during intestinal stress.

This study was conducted in healthy animals, but interestingly, PepT1 expression and activity have been reported to be elevated in the situation of intestinal stress (Ihara et al., 2000, Adibi et al., 2003). I was curious to study the effect of delivering amino acids in the form of dipeptides in the situation of intestinal stress. Parenteral feeding is one of the clinical strategies used to provide nutritional support to infants who cannot tolerate enteral nutrition, which can also induce intestinal stress (Niinikoski et al., 2004). When I completed my first study, the parenteral nutrition-induced intestinal atrophy model was well developed in our lab. Therefore, I was interested in moving my research into a pre-clinical intestinal stress model.

6.3 Investigation of structural and functional benefits of delivering amino acid as dipeptide in an atrophied gut

6.3.1 Overview of results

The objective of my second project was to study the potential structural and functional benefits of enteral delivery of lysine as dipeptide in a parenteral nutrition-induced atrophied gut during the high-risk transition period. In this study, we found that switching the form of lysine from free amino acid to dipeptide increased the whole-body protein synthesis, mucosal lysine concentration, villus height, mucosal weight and improved the inflammatory status of the gut. Furthermore, inclusion of glycyl-sarcosine with lysyl-lysine produced lower rates of protein synthesis, lower mucosal weight and villus height, and increased indices of inflammation compared to lysyl-lysine treatment. If glycyl-sarcosine could abolish the effects of lys-lys alone, it suggests competitive inhibition occurred, but it also tells us that the lysyl-lysine effect was not due to intraluminal hydrolysis of the dipeptide and uptake of free lysine. These findings suggest that providing dipeptides in the situation of intestinal stress would be beneficial in terms of improving intestinal health and whole-body protein kinetics.

6.3.2 *Implications and modifications*

The piglets underwent surgical implantation of catheters at the age of 7-8 days old; we chose this age to represent neonatal age. The piglets first received parenteral nutrition (PN) for four days to induce intestinal atrophy. It has been previously reported that even two days of PN is enough to induce intestinal morphological and physiological effects in piglets (Niinikoski et al., 2004). Enteral feeding was initiated after 4 days of PN feeding

and continued for 12 hrs to study the acute effect of providing dipeptides during the high-risk transition period from PN to enteral feeding. Lysine was chosen as a test amino acid because it is an essential amino acid, the requirements for lysine are well established in the piglet, and it's not involved in many other metabolic activities other protein synthesis. Furthermore, lysine was provided as 75% of the recommended level to ensure that a small advantage of the dipeptide would not be masked by an excess availability of lysine. In addition, the inclusion of third treatment of lysyl-lysine with glycyl-sarcosine confirmed that lysyl-lysine remained intact in the intestinal lumen, and the transport occurred through PepT1.

This study utilized a stable isotope of phenylalanine to determine whole-body protein synthesis and flooding dose of radiolabelled phenylalanine to measure tissue-specific protein synthesis, which allowed us to do both tests on the same day. In this study, the stable isotope was given the intragastric route, which might have led to some loss of phenylalanine tracer to intestinal metabolism. However, the inclusion of intestinal metabolism may be a more representative measurement of whole-body kinetics (Cvitkovic *et al*., 2004). Studies have also used constant stable isotope infusion to measure tissue protein synthesis (Watt et al., 1992; Baumann *et al*., 1994; Harding *et al*., 2008). The constant infusion method may be suitable for measuring protein with slow turnover rates, such as muscle. However, the accuracy of measuring protein synthesis rates in tissues with rapid turnover rates, such as in the liver and intestinal mucosa, might be influenced by the recycling of the tracer. Furthermore, a prolonged labelling period (6 hr in this study) may also lead to an underestimation of protein synthesis rates in tissues such as the liver and

mucosa, in which a significant amount of synthesized protein is exported and escapes the measurement (Davis et al., 2001). Flooding dose allows the determination of protein synthesis over a short period of time (Garlick et al., 1980). Therefore, this study utilized the stable isotope infusion method to measure whole-body protein synthesis and the flooding dose method to determine the tissue protein synthesis for more accurate measurements in all tissues of interest.

Ideally, adding a fourth treatment with 100% lysine treatment would have helped identify the extent of the benefits of providing lysine as a dipeptide. However, due to breeding failures and complications with litter sizes, this unfortunately was not possible.

Overall, the protocol was well designed to study our objectives. Our results demonstrate that when an intestinal injury is present, lysine is more bioavailable as a dipeptide. Furthermore, delivering lysine as dipeptide resulted in a 38% improvement in the whole-body protein synthesis and improved structural indices of gut health. In a situation of intestinal stress such as parenteral feeding in preterm infants, feeding peptides would provide faster recovery over individual amino acid residues. Furthermore, in the swine industry, feeding dipeptide during the weaning period would increase the growth rate and have an exponential effect on getting to market sooner, which will reduce the meat production cost. Future studies could also use this model to investigate the structural and functional benefits of delivering other dipeptides over the amino acids in an atrophied gut.

Besides maintaining protein nutrition, PepT1 has been identified as a protein sensing mechanism in the upper gut. Furthermore, PepT1 activation improved blood glucose metabolism in healthy, obese, and diabetic adult models. In contrast, high protein formula feeding in infancy is associated with an increased risk of obesity and chronic diseases in later life when compared to breastfeeding, which is naturally lower in protein. Therefore, in our third study, we were interested in characterizing whether the effects of PepT1 activation reported in mature rodent models and models of diabetes exist in the neonatal model.

6.4 Investigating the influence of PepT1 on gut hormone release and glucose metabolism in neonates

6.4.1 Overview of results

The objectives of my third study were to characterize the influence of PepT1 activation and dietary peptide transport on gut hormone secretion, liver glucose production, and glucose clearance in neonatal piglets. In this study, piglets were fasted for three hours to minimize the impact of any prior digestive residues. Under general anesthesia, jugular, carotid, portal, and duodenal catheters were implanted, and saline was infused into the duodenum for 30 min to measure portal baseline hormone levels. In this study, saline was used as control treatment. Casein hydrolysate at sow milk protein concentration was used to study the standard protein diet on the gut hormone release and glucose metabolism. The casein hydrolysate at sow milk protein concentration with PepT1 inhibitor 4-AMBA was used to study the protein-mediated gut hormone release occurs through the transport of

peptides. Duodenal infusion of casein hydrolysate, which is rich in substrates for PepT1, stimulated the release of GLP-1 and GIP. However, the inclusion of the PepT1 inhibitor hindered this peptide-mediated effect on gut hormone release. Our results confirm that PepT1 is involved in the protein-mediated response in gut hormone release and glucose response. Protein-mediated increase in incretins led to higher circulating insulin and facilitated faster glucose disposal in the casein treatment during the IVGTT test. This study's results confirm that PepT1 is also involved in inducing gut hormone release and altering glucose homeostasis in the developing neonates. Therefore, the effect of PepT1 activation observed in the mature model does exist in neonates. However, higher circulating insulin in infancy may not be advantageous for their health later in life.

6.4.2 Implications and modifications

This study allowed us to characterize the influence of PepT1 on gut hormone release and glucose metabolism in neonatal piglets using a terminal experiment. We originally planned this study to be designed as an eight-day survival study, with individual tests for each outcome measures spread out within the study period. One of the objectives of the original study was to determine the dose-response effect of protein intake with the inclusion of a higher protein diet, which would have provided more information regarding the association between protein intake, PepT1 activation, gut hormones, and glucose kinetics in neonates. However, we shifted to the terminal study with limited treatments and a smaller sample size due to the unexpected pandemic closures and an unexplained breeding failure

rate of approximately 70% within the Yucatan herd managed by Animal Care Services at Memorial University.

In this study, we were able to distinguish the effect of protein-mediated PepT1 activation on gut hormone release and glucose response by adding a non-translocated PepT1 inhibitor 4-AMBA to casein hydrolysate. Duodenal saline infusion and portal blood sampling during the first 30 minutes allowed us to measure baseline hormone levels and calculate the treatment effect as a percentage change. DPP-IV inhibitor was added to the portal blood samples soon after the collection to prevent the degradation of incretin hormones. The rapid proteolytic degradation of GLP-1 and GIP is catalyzed by DPP-IV which diminishes the insulinotropic effects of these incretins. Then, we wanted to investigate the effect of protein mediated PepT1 activation on the glucose regulation. We were able to determine the effect of PepT1 activation on endogenous glucose production with the infusion of ^{13}C glucose. This protocol was previously established by Stoll et al to measure endogenous glucose production in neonatal piglets (Stoll et al., 2012). Isotope reached to the steady state within 60 mins of the infusion and isotope dilution method was used to calculate the EGP. We assume that the measured values represent only the hepatic glucose production because EGP was measured within the 5 h of fasting initiation, therefore less likely to produce glucose through gluconeogenesis. Also, we measured the EGP before we perform IVGTT test to avoid exogenous glucose interference. We expected to see a treatment effect in the EGP, but we did not observe the significant difference due to high variations in the values within the treatment groups. The IVGTT test was performed at last which provided information about the effect of treatments on the glucose response.

Overall, this study was well designed to achieve our specific objectives with the limited recourses during this pandemic. The limitation of this study was that we were only able to include only three treatments and utilize five animals per treatment due to continuous breeding failures and covid restrictions. Also, we could not study the dose dependent effect of protein using survival model. Therefore, future long-term survival studies should be conducted to investigate the effect of dose-dependent protein-mediated PepT1 activation on gut hormone release and glucose homeostasis. In the current study, we only utilized casein hydrolysate to characterize the effect of PepT1 on gut hormone release and glucose response. Most importantly, neonates consume mixed proteins of whey and casein, and the ratio of whey to casein changes throughout their lactation period. Therefore, future studies focusing on the effect of varying casein and whey protein ratios would help understand the actual scenario. Also, we do not know whether human milk induces a similar effect as casein hydrolysate. Furthermore, it is unknown whether all other substates for PepT1 also induce the same effect as peptides. Therefore, future studies should focus on these areas to provide a more detailed understanding of PepT1 mediated gut hormone release and glucose response in neonates.

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