2009

IDENTIFICATION AND CHARACTERIZATION OF THE GLUCAGON-LIKE PEPTIDE-2 HORMONAL SYSTEM IN RUMINANTS

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ABSTRACT OF DISSERTATION

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The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment
of the requirements for the degree of Doctor of Philosophy
in the College of Agriculture
at the University of Kentucky

By

Christina C. Taylor Edwards

Lexington, Kentucky

Director: Dr. David Harmon, Professor of Animal Science

Lexington, Kentucky

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The hormone glucagon-like peptide-2 (GLP-2) is important in the regulation of intestinal growth and blood flow in nonruminant animals. However, no research reports the existence of GLP-2 in ruminants. Therefore, this dissertation examined the existence of GLP-2 and its receptor, their response to physiological stimuli, and its ability to induce gastrointestinal growth and intestinal blood flow in ruminants.

Experiments 1 and 2 established the gastrointestinal distribution of mRNA for proglucagon (the GLP-2 precursor) and the GLP-2 receptor. Furthermore, these experiments determined the effects of changing dietary energy intake on plasma GLP-2 concentrations and proglucagon and GLP-2 receptor mRNA expression. Experiment 3 examined the effect of exogenous bovine GLP-2 on splanchnic blood flow, splanchnic nutrient flux, and gastrointestinal growth.

This research shows that ruminants possess a functional GLP-2 signaling system that responds to nutrient ingestion. Based on observed receptor distribution and growth changes with GLP-2 treatment, GLP-2 targets the small intestine and does not affect forestomach or large intestinal growth. Increases in ileal proglucagon mRNA expression and plasma GLP-2 with increasing energy intake demonstrate that GLP-2 responds to physiologic changes in nutrient intake and can be relevant to feeding practices. Furthermore, observed increases in small intestinal growth and blood flow with GLP-2 suggest that it could substantially affect the capacity of the gastrointestinal tract for nutrient absorption. Modification of GLP-2 through diet could allow for improvements in nutrient utilization and animal productivity. This research also has important implications for use of GLP-2 for human disease therapy as the observed down-regulation in the blood flow response to 10-d GLP-2 administration has never been reported in any species prior to this dissertation.

This research systematically characterized and evaluated the potential role of GLP-2 in the control of gastrointestinal growth and splanchnic blood flow in ruminants. While it extends the knowledge of hormonal control of the gastrointestinal tract in ruminants, it

also adds crucial information to the larger body of work investigating the actions of GLP-2. This dissertation research has contributed to the groundwork necessary to enable the use of GLP-2 in improving the health and productivity of a diverse group of mammalian species.

KEYWORDS: Glucagon-like peptide-2, ruminant, gastrointestinal growth, splanchnic blood flow, mRNA expression

Christina C. Taylor Edwards

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Student's Signature

April 29, 2009

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Date

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This dissertation is dedicated to those that encouraged and motivated me through the years. My parents, who taught me the love of learning. My sister, who can always make me laugh. My friends, especially Julie, Melissa, Sally, and Sandi, who encouraged me to continue but also helped me forget my frustrations. And above all, my husband Dave, who not only endured it all but helped with every single aspect of the research and writing of this dissertation. I am deeply grateful for your love and support.

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CHAPTER 1. INTRODUCTION

The gastrointestinal tract has several critical roles in the body, including digestion and absorption of nutrients and providing a protective barrier. To maintain the functional integrity of the mucosa, the epithelium of the gastrointestinal tract is constantly and rapidly renewed. All of these functions make the gastrointestinal tract a highly metabolic tissue that consumes a substantial portion of an animal's daily energy needs (1). Therefore, although the gastrointestinal tract is essential for life, greater gastrointestinal growth is a substantial energy cost and precise control of its growth is needed. Additionally, the gastrointestinal tract acts as the first barrier for nutrient entry to the body for metabolism, and as such the ability of the gastrointestinal tract to digest and absorb nutrients can have extensive implications for whole-body nutrient availability. Because the amount and profile of nutrients available to the animal is first modified by the gastrointestinal tract, understanding gastrointestinal tract growth and nutrient utilization is one route to improving animal efficiency.

One hormone that has been identified as a key player in regulation of intestinal growth is glucagon-like peptide-2 (GLP-2), a 33 amino acid peptide secreted from the L-cell, an enteroendocrine cell of the gastrointestinal tract. Since 1996, it has been well established that GLP-2 increases small intestinal epithelial growth by increasing proliferation and inhibiting apoptosis (2-12). The increased epithelial mass and crypt-villus length observed as a result of GLP-2 administration increases small intestinal surface area and thus the potential area available for absorption. In addition, GLP-2 dose-dependently increases blood flow to "intestinal" vessels such as the superior mesenteric artery and portal vein but not to other vessels such as the celiac, renal, and gastric arteries (13-16).

Given the importance of this peptide hormone to intestinal maintenance in other species studied (human, pig, rat, mouse), it can be speculated that this hormone would likely play an important role in ruminants as well. Furthermore, the GLP-2 protein sequence is highly conserved among mammals; there is 89 to 97% homology between human, mouse, rat, bovine, and pig sequences, suggesting that this sequence is critical to

mammalian metabolism (17). This evidence suggests that GLP-2 could be a key participant in growth and adaptation of intestinal tissues in cattle. However, the role of GLP-2 in the growth and function of the ruminant gastrointestinal tract is completely unknown. Ruminants have a uniquely complex gastrointestinal tract that undergoes substantial changes during the animal's lifetime. Drastic changes in the relative size and function of the components of the ruminant gastrointestinal tract occur with transition from a milk-based diet at a young age to a fiber-based diet at weaning (18). A second period of growth of the ruminant gastrointestinal tract occurs upon initiation of lactation, as one necessary adaptation of maternal tissues to meet metabolic demands of the periparturient animal is hypertrophy of the gastrointestinal tract (19). Furthermore, gastrointestinal size in ruminants can be altered by 2 common feeding practices: changing total energy intake and changing the dietary proportions of structural: non-structural carbohydrate (20-22). Because gastrointestinal growth has implications for the potential energy and nutrient profile available for productive use by the animal, understanding how gastrointestinal size is controlled in ruminants is important to maximize animal productivity.

The purpose of this dissertation is to systematically characterize and evaluate the potential role of GLP-2 in the physiological control of gastrointestinal growth and splanchnic blood flow in ruminants. Chapter 2 reviews the literature describing the physiological role of GLP-2 in non-ruminants and the available ancillary evidence suggesting a potential role for GLP-2 in ruminants. Chapter 3 reports the results of the initial study conducted to evaluate if changing energy intake could cause detectable changes in plasma GLP-2 concentrations and gastrointestinal proglucagon and GLP-2 receptor mRNA expression in ruminants. Chapter 4 examines the distribution of proglucagon and GLP-2 receptor mRNA across each organ in the ruminant gastrointestinal tract. Chapter 5 evaluates the effect of exogenously-administered GLP-2 on splanchnic blood flow, splanchnic nutrient flux, and gastrointestinal growth in the ruminant. Furthermore, effects of GLP-2 on splanchnic blood flow and nutrient flux were conducted before and after chronic GLP-2 administration to determine changes in responsiveness after extended treatment. This information is especially critical because there are no reports in the literature documenting the effect of extended GLP-2

administration on blood flow in any species. Therefore, this dissertation research furthers the understanding of the role GLP-2 plays in gastrointestinal growth and blood flow in all mammalian species, including ruminants.

CHAPTER 2. A REVIEW OF THE LITERATURE

TERMINOLOGY

The descriptions of glucagon and the glucagon-like peptides (GLPs) are varied throughout the literature and can cause much confusion. Many of the discrepancies have stemmed from the evolving precision of the methodologies used to analyze these hormones. Further complicating the situation is that glucagon and the GLPs are encoded on the same mRNA and protein and these hormones become distinct separate hormones only after post-translational processing, as discussed later. Early immunological techniques used a side-viewing assay that could only detect "glucagon-like immunoreactivity" (GLI), but could not distinguish between glucagon and the GLPs. This terminology is still used to some extent currently, where "total glucagon-like immunoreactivity" is measured and a specific C-terminus-viewing assay measures glucagon of pancreatic origin; the difference between the two is designated "gut glucagon-like immunoreactivity", which is presumably attributable to the GLPs. This glucagon-like immunoreactivity that can be attributed to gastrointestinal origin (*entero*) is also known as enteroglucagon. A third term used is the proglucagon-derived peptides (PGDPs), which accounts for the fact that the proglucagon gene encodes multiple peptides, including glucagon, glucagon-like peptide-1 (GLP-1), and glucagon-like peptide-2 (GLP-2). The full complement of PGDPs can be seen in Figure 2.1.

EARLY LITERATURE

Glucagon was first identified in 1923 as a hormone present in the pancreas that was capable of raising blood sugar (23). However, a second source of glucagon-like immunoreactivity was identified as originating in the duodenal and ileal mucosa of the canine gastrointestinal tract in 1948 (24). Unlike glucagon of pancreatic origin, this glucagon-like immunoreactivity (or GLI) was not able to cause glycogenolysis in isolated liver slices. Later research showed substantial amounts of glucagon-like activity in

extracts of human stomach and small bowel, and although this substance could not be conclusively identified as glucagon, it also could not be differentiated from glucagon (25). At this time, there was substantial confusion as to whether glucose administration increased or decreased blood concentrations of glucagon-like immunoreactivity; different laboratories had observed both increases and decreases in response to glucose, but this effect was dependent on the route of administration (26). In 1968, a report by Unger et al. demonstrated the glucagon-like immunoreactivity contained in canine jejunum was of a different size than pancreatic glucagon and, as found before, did not stimulate hepatic glycogenolysis but did stimulate insulin release. Furthermore, they found that glucose administered intravenously did not increase GLI concentrations in the vena cava; however, glucose administered intraduodenally more than doubled GLI concentrations in the vena cava (26). Moreover, glucagon-like immunoreactivity concentrations in the vena cava continued to increase in response to duodenal glucose even when the vascular flow from the pancreas was prevented, demonstrating extra-pancreatic origin of the GLI. To further define the source of GLI, dogs were catheterized in the mesenteric vein, pancreaticoduodenal vein, and vena cava. The rise in GLI concentrations in response to intraduodenal glucose was greater in the mesenteric vein than either the pancreaticoduodenal vein or vena cava (26). These data demonstrated that GLI secretion was stimulated by luminal nutrients and strongly suggested that GLI was secreted by the gastrointestinal tract. This report was critical to set the groundwork for future investigations into the glucagon-like substance found in the gut, or enteroglucagon.

Concurrent with this research, other researchers observed that intestinal resection stimulated growth of the intestinal tract, particularly of the jejunum and ileum (27). They suggested that a growth factor was released in response to the loss of intestinal tissue and this factor, termed “intestinal epithelial growth hormone”, accelerated the rate of epithelial proliferation in the small intestine. It was not until a research report in 1971 that more definitive information about this growth factor was determined. A woman harboring a tumor on her kidney had severely reduced intestinal motility in addition to small bowel hypertrophy, with an increased diameter of the small intestine, especially in the jejunum (28). The height of the villi in the jejunum was approximately 1,150 μm, whereas the normal range is 350 to 800 μm. These symptoms were reversed upon

removal of the tumor. Histological examination of the tumor revealed the presence of a number of secretory granules, suggesting an endocrine origin; furthermore, tumor cells were strongly reactive to a glucagon antibody. Their conclusion to the histological findings was "that the tumour was producing glucagon, either alone or in combination with another polypeptide hormone (or at least, that it was secreting a polypeptide which cross-reacted with anti-glucagon serum)" (28). This report established the link between intestinal growth and a glucagon-reactive substance.

Although the research by Gleeson et al. (28) strongly suggested an endocrine mechanism linking the glucagon-reactive substance and intestinal growth, for several years this was hotly debated. In parabiotic rats in which the jejunum and ileum of one pair member was irradiated, causing a transitory increase in duodenal and colon proliferation, no effect on proliferation was observed in the other pair member, leading the researchers to conclude that the growth factor did not act via an endocrine mechanism but more likely paracrine (29). Bloom (30) attempted to sort out the local versus circulating debate. Three groups of rats were used; orally-fed rats with a jejunal transection (controls), orally-fed rats with a Thiry-Vella fistula to bypass 75% of the proximal small intestine, or intravenously-fed rats with the same Thiry-Vella fistula. Crypt cell proliferation rate in the terminal ileum of control (transected) rats and the IV-fed fistulated rats was similar and much lower than proliferation rates in the orally-fed fistulated rats, demonstrating a dependence (direct or indirect) of the proliferation effect on luminal nutrient presence. However, the same treatment effects were observed on crypt cell proliferation rates in the Thiry-Vella fistula itself, and because the fistulated intestine was excluded from luminal nutrition and pancreaticobiliary secretions, this suggested a humoral component to the proliferation effect as well (30). Although Bloom (30) concluded that only the gut glucagon system correlates well with gut growth and the enteroglucagon gene structure had already been reported in 1983 (31), it would not be until 1996 when the individual secretory products of the enteroglucagon gene were tested that it was found that glucagon-like peptide-2 was the component that induced the small intestinal growth observed in previous experiments (4).

GLUCAGON-LIKE PEPTIDE-2

Location in GI Tract and Other Organs

Glucagon-like peptide-2 (GLP-2) is secreted from the enteroendocrine L-cell. Cells with gut-type GLI are observed in the duodenum and jejunum but greater numbers exist in the ileum and proximal colon, with few species differences between pig, dog, cat, and rat (32). Although there are few differences among species, rodents (rats and mice) appear to have a slightly greater number of L-cells in the colon than some other species, including pigs and man (33, 34). In the more proximal gastrointestinal tract, L-cells have been detected in the stomach at very low frequency (33), and are present in the duodenum (35, 36). However, the consensus is that the greatest numbers of L-cells are found within the distal jejunum and ileum (33, 35).

Proglucagon Gene Structure and Regulation

The structure of proglucagon was first reported in 1983 (31, 37). The gene consists of four exons in which the signal peptide, glucagon, GLP-1 and GLP-2 are encoded, respectively. Exons 2 and 3 also each encode an intervening peptide after glucagon and GLP-1, respectively. The amino acid homology between the human and hamster forms for proglucagon is 94.3%, with homology for individual components ranging from 91.2% homology for GLP-2 to 100% homology for glucagon and GLP-1 (17, 31). The bovine proglucagon sequence is also highly homologous to the human form (37). Although the proglucagon mRNA sequence was first described in the pancreas (31, 37), the proglucagon mRNA is identical in the pancreas and gut (38, 39). Like the previously described distribution of the L-cell, mRNA transcripts for proglucagon have been described in the ileum, colon, and rectum (38). Additionally, although the significance is unknown, preproglucagon mRNA is also expressed in a population of neurons within the brain of rats (40).

All proglucagon mRNA sequences in mammals contain one glucagon, GLP-1, and GLP-2 sequence (41). Within human, mouse, rat, dog, and cow, there is high sequence homology (> 50% up to 100%) near and within the proglucagon gene, including large regions of noncoding sequence (41), as demonstrated in Figure 2.2. The highest areas of

identity are immediately upstream (5 kb) of the mRNA start site, the promoter region, the region surrounding the 3' end of intron 1, and the middle of intron 3 (42). Generally, gaps in alignment are due to insertion or deletion of bases rather than actual base changes (42). On the promoter sequence of the proglucagon gene there are five elements (G1, G2, G3, G4, and G5) that have been identified as binding regions for binding proteins; G1 is the minimum promoter region and G2 to G5 are enhancer regions (43). Among a diverse group of mammals (human, rhesus monkey, mouse, rat, hamster, cow, dog, and opossum), the G1 element is almost completely conserved whereas the G3 enhancer shows the greatest sequence variance (44).

Although there is considerable information about control of pancreatic proglucagon mRNA expression, there is evidence that intestinal and pancreatic expression are regulated differently. The transcription factors HNF-3-α and -β can bind the G1 element of the glucagon gene promoter, but the binding activities differ between pancreatic and enteroendocrine cells (45). The Wnt signaling pathway increases proglucagon mRNA expression in intestinal but not pancreatic cells (46), and insulin increases proglucagon mRNA expression in intestinal cells but decreases proglucagon expression in pancreatic cells (47). Therefore, reports of transcriptional control of proglucagon expression need to be carefully interpreted, and only intestinal-specific transcriptional control is presented below.

There is substantially less research reporting intestine-specific transcriptional control of proglucagon mRNA expression. The proglucagon gene structure and putative transcriptional control factors affecting proglucagon mRNA expression in the intestine are shown in Figure 2.3. *Pax*6, a critical determinant of islet cell development and proglucagon gene expression in islet α-cells, is also essential for proglucagon gene transcription in the small and large intestine. *Pax*6 is expressed in enteroendocrine cells, binds to the G1 and G3 elements in the proglucagon promoter, and activates proglucagon gene transcription (48, 49). Mice with a dominant negative *Pax6* mutation demonstrate a total absence of glucagon-like peptide-1 and -2 immunoreactivity in the intestine but do have other endocrine cells containing PYY, cholecystokinin, serotonin, and secretin (48). The existence of one or more cAMP-response elements (CRE) is an additional important regulatory component of the proglucagon gene promoter (50, 51). These regions of DNA

in the gene promoter are bound by specific binding proteins in response to upstream production of cAMP and activation of protein kinase A (PKA). However, cAMP can also stimulate a second, PKA-independent pathway via the Epac pathway, which stimulates activation of the Ras/Rap-Raf-MEK-ERK signaling cascade (52). Therefore multiple elements within the proglucagon gene promoter are responsive to changes in intracellular cAMP. A third signaling pathway is the Wnt signaling pathway, which increases proglucagon expression through β-catenin and TCF-4 binding at the G2 element of the proglucagon promoter in intestinal endocrine cell lines (47); furthermore, insulin also appears to upregulate proglucagon mRNA expression in intestinal cells by this pathway (47).

Substantial evidence demonstrates that proglucagon gene expression is related to nutrition, although the link between nutrients and the aforementioned mechanistic signaling pathways is unknown. Expression of enteroendocrine proglucagon mRNA is stimulated by nutrients, as evidenced by a decrease of ileal proglucagon expression with fasting and a subsequent increase of proglucagon mRNA with refeeding (53, 54). In addition to changes in total nutrient intake, specific nutrients can also affect proglucagon mRNA expression. Protein hydrolysates increased the levels of proglucagon mRNA by activating a response element close to the transcription start site (< -350 bp) that increased the rate of transcription in two cell lines of intestinal origin (STC-1 and GLUTag); interestingly, this effect was not observed in cells of pancreatic origin (RINm5F or INR1G9), demonstrating tissue-specific regulation of the proglucagon gene (55). In agreement, peptones from meat hydrolysate activated the proglucagon promoter region via a previously described CRE and possibly by an additional CRE-like element (50) and high protein diet increased proglucagon mRNA expression (56). Fiber feeding significantly increased levels of proglucagon mRNA in the jejunum, ileum, and colon of mice (53), and dietary resistant starch increased proglucagon in the cecum and colon of rats (57). However, these effects on proglucagon mRNA expression may be related to fermentation products of these substrates (short-chain fatty acids, SCFA), as proglucagon expression was dose-dependently increased by infusion of (in decreasing order of potency) butyrate, propionate, and acetate (57, 58) and intravenous SCFA

supplementation of total parenteral nutrition (TPN) increased ileal proglucagon mRNA (59).

Nutritionally-regulated hormones can also affect proglucagon mRNA expression in the intestine. Insulin increased the expression of proglucagon in the GLUTag intestinal cell line (47), a mouse enteroendocrine cell line that secretes the GLPs in a physiological manner (60). In contrast, epidermal growth factor (EGF) reduced ileal GLI concentrations in mice (61). However, ileal GLI was unaffected in mice treated with the insulin-like growth factors-I and –II (IGF-I and –II) or growth hormone (61), which are well known local and systemic growth factors (62). Perhaps most importantly, GLP-2 itself, either in its native state or as a long-acting analog, does not affect the amount of proglucagon mRNA, ileal GLI, or GLP-2 protein expression in the duodenum, ileum, or colon of mice or rats (2, 61, 63), demonstrating that GLP-2 does not appear to downregulate its own expression.

GLP-2 Protein Structure and Processing

In intestinal and pancreatic cells, the entirety of the proglucagon mRNA sequence is transcribed into protein (39). Tissue-specific hormone production is dependent on the complement of cleavage enzymes present. Prohormone convertases are a family of serine proteases that process protein precursors within the secretory pathway of cells (64). Expression of prohormone convertases (PC) 1/3 and 2 is restricted to the neuroendocrine system and brain, where they function to cleave precursors of a variety of prohormones and neuropeptides within the vesicles of the regulated secretory pathway (64). Both PC1/3 and PC2 are expressed in cells that also express proglucagon; however, the intestinal L-cell expresses PC1/3 but not PC2 (65), whereas the pancreatic α-cell expresses PC2 but not PC1/3 (66). The tissue specific processing of proglucagon by the prohormone convertases and the resulting products are shown in Figure 2.1. The classical motif for processing by the PCs is Lys-Arg or Arg-Arg (67); these basic amino acid pairs link each of the components of the proglucagon gene (GRPP, glucagon, IP-1, GLP-1, IP-2, GLP-2) and thus make them susceptible to PC action.

Thus, in the pancreas, the presence of prohormone convertase PC2 catalyzes the cleavage of proglucagon protein into glucagon, an intervening peptide, and the major proglucagon fragment from the proglucagon protein (67-69); in contrast, glucagon-like peptides (1 and 2), glicentin, a second intervening peptide, and oxyntomodulin are released from proglucagon by the action of PC1/3 in the intestinal L-cell (67-70). The action of PC1/3 is certainly critical to the release of processed glucagon-like peptides, as PC1 null mice exhibit little to no secretion of GLP-1 and GLP-2; however, this experiment also highlighted the relative unimportance of PC1/3 to pancreatic processing, as glucagon production in null mice was unaltered (71). Interestingly, the transcription of proglucagon mRNA and PC1/3 mRNA appear to be co-regulated via activation of the PKA pathway and 2 CRE located in the promoter region of the PC1 gene (72); as discussed previously, the proglucagon gene promoter also contains at least one CRE (50, 51).

After the action of PC1/3, the final secretory products, GLP-1 and GLP-2, are produced. The final cleavage of proglucagon protein to GLP-1 and GLP-2 by PC1/3 likely occurs within the secretory granules of the L-cell as GLP-1 and GLP-2 are co-secreted in equimolar amounts (73). Indeed, GLP-1 and -2 immunoreactivities are colocalized within single secretory granules in human colorectal enteroendocrine cells (74). These hormone products are stored in the secretory granule of the L-cell until secretion is stimulated.

STIMULATION OF GLP-2 SECRETION

The secretory granules of the L-cell are located adjacent to the basolateral membrane (33, 75). Upon stimulation of the cell by a variety of factors discussed below, fusion of the secretory granule with the plasma membrane is triggered and the granule contents are released (76). Secretion of the GLPs is pulsatile, with approximately 7 to 9 pulses/min in humans and a greater amplitude but not frequency of pulses with additional stimulation (77). The ability of the cell to sense the substances present in the lumen of the intestine is provided by the “open type” nature of the intestinal L-cell (33), meaning that the cell extends to the mucosal surface and has microvilli at the apical end of the cell

(78). Indeed, GLP-2 secretion is stimulated by both the amount and composition of nutrients (79-82). With a mixed meal, GLP-2 concentrations in plasma increase within the first hour postprandially, and the ratio of GLP-2 (1-33) to its inactive cleavage product GLP-2-(3-33) decreases from approximately 1:2 to almost 1:1 (80), demonstrating an increase in the proportion of active circulating GLP-2 and suggesting the hormone response is due to secretion of preformed secretory vesicles from the L-cell. However, feed intake does also increase proglucagon mRNA expression and there is a positive correlation between proglucagon mRNA and plasma enteroglucagon (79, 83, 84), so that feed intake stimulates secretion of fully processed GLP-2 as well as increasing proglucagon mRNA expression.

The responsiveness of GLP-2 is dependent on luminal nutrition rather than whole body sensing of nutrient intake because infusion of a TPN solution intravenously is ineffective at completely restoring ileal proglucagon mRNA expression and plasma GLP-2 concentrations to fed levels after fasting; in contrast, intragastric infusion of the same TPN solution partially restored ileal proglucagon mRNA expression and fully restored plasma GLP-2 concentrations to fed levels, and ad libitum feeding was even more effective than intragastric TPN infusion (84). In piglets, plasma GLP-2 concentrations did not increase until at least 40-60% of total intake was provided enterally, with the remaining intake provided by TPN (82), again underscoring the dependence of GLP-2 secretion on luminal nutrient presence. Furthermore, nutrients seem to potentiate the GLP-2 response to intestinal injury, as resection increases GLP-2 independent of the presence of luminal nutrients, but for a sustained increase in circulating GLP-2 luminal nutrients are necessary (83).

There appears to be a caloric threshold for a meal to effectively stimulate secretion of the GLPs. Intraduodenal glucose infusion at 1.1 kcal/min did not stimulate GLP-1 release but the same infusion at 2.2 kcal/min was effective at stimulating GLP-1 release (85). Likewise, consumption of mixed meals > 400 kcal were able to increase plasma GLP-2 concentrations but mixed meals < 400 kcals were not (80), and similar results were observed by Enç et al. (86). There has been some disagreement on what the caloric threshold actually is, as intraduodenal infusion of glucose at 1 kcal/min transiently increased plasma GLP-1 in one experiment (87), but infusion at 1.1 kcal/min did not

affect plasma GLP-1 in another (85). It is likely that this caloric threshold changes to some extent in response to the prevailing nutritional environment. Although it is not known what the sensing mechanism is for this apparent threshold, it appears that the sensing mechanism is located in the proximal intestine, likely the duodenum, as the response to nutrient intake is fairly rapid (80, 85, 87).

The response of GLP-2 to nutrient ingestion is bi-phasic. There is an initial rapid induction of secretion within 30 minutes of meal ingestion, and a longer, more sustained release of GLP-2 at 90 to 120 minutes (80). Proximally-located L-cells may contribute to the early rise in GLP secretion in response to duodenal nutrients (88), as L-cells are located in the duodenum (36). However, the L-cell is predominately located in the distal intestinal tract (33, 35), and nutrients from a consumed meal do not reach the distal tract within 30 minutes of ingestion (89). Furthermore, infusion strategies in which nutrients are infused into the duodenum but prevented from moving distally are effective at increasing secretion of the GLPs (85), strongly suggesting a feedforward mechanism to the L-cells of the distal intestinal tract. These observations lead to the idea that there may be both direct and indirect mechanisms by which GLP-2 secretion is induced by nutrients.

Direct Mechanisms by Nutrients

Non-structural carbohydrates

Non-structural carbohydrates effectively induce secretion of the GLPs. A liquid dextrose meal increased intact GLP-2 concentrations within 10 minutes and peaked at 45 minutes at 560% of basal concentrations (80). After feeding a high carbohydrate meal, similar results were seen by Raben et al. (90), who observed that GLP-2 concentrations seemed to plateau after 1 h, a similar observation to that of Xiao et al. (80). Infusion of glucose into the duodenum increased plasma GLP-1 secretion in pigs (91), but in humans greater than 60 cm of the duodenum needed to be exposed to glucose concentrations to raise plasma GLP-1 concentrations (92). Likewise, mean GLP-1 concentrations were higher after jejunal glucose infusion than duodenal glucose infusion in humans (93). Thus, although sugars are stimulatory to GLP-1 secretion, a long segment of proximal

intestine may need to be exposed in order to induce GLP secretion, especially in humans who seem to be less sensitive to duodenal sugars than other species.

The way in which the L-cell directly senses the luminal carbohydrate load is not fully understood. In the vascularly-isolated perfused rat ileum, GLP-1 concentrations in the venous effluent were increased by substrates of the luminal Na^+-dependent glucose cotransporters (glucose, galactose, methyl-α-glucoside, and 3-*O*-methylglucose) and fructose (substrate of the luminal Na^+-independent fructose transport mechanism), but not 2-deoxyglucose or N-acetylglucosamine (94). Likewise, colonic glucose infusion increased enteroglucagon concentrations in plasma compared to mannitol infusion (95). These results suggest that the stimulatory effect of luminal sugars on GLP secretion requires transport of the sugar across the apical membrane but does not require metabolism or basolateral transport. The mechanism of this was suggested to be two-fold by Gribble et al. (96). Using GLUTag cells, they found that low concentrations of metabolizable sugars such as glucose and fructose can induce depolarization of the L-cell via closure of the ATP-sensitive K^+-channels (Kir6.2/SUR1) and thus cause GLP secretion (97). However, GLP-1 secretion can also be induced by depolarization of the cell due to the electrogenic action of the SGLT transporters themselves because of the inward current generated from the Na^+ that is co-transported with the sugar molecule, especially when the substrate concentrations are high or the outward currents are already reduced by closure of the ATP-sensitive K^+-channels (96). These results agree with those of Ritzel et al. (94) and collectively demonstrate that sugars transported by the SGLT transporters or fructose stimulate GLP release, and it is not the metabolism of these sugars that stimulates release but rather the transport itself that is the sensing mechanism in the L-cell.

Structural carbohydrates

In addition to the effect of non-structural carbohydrates on GLP secretion from the L-cell, structural carbohydrates also stimulate secretion of GLP, although likely not directly. In dogs fed diets with similar amounts of fiber but differing in the fiber fermentability, a highly fermentable fiber diet increased GLP-1 secretion and as well as proglucagon and GLP-1 protein content in the ileum and colon compared to diets

containing a lowly fermentable fiber (98). Likewise, feeding a resistant starch to rats increased plasma GLP-1 concentrations compared to an isoenergetic cornstarch-based diet (58). This stimulatory effect could be due to fermentation substrates, primarily SCFA, produced as a result of fermentation as opposed to fiber per se, as evidenced by increased proglucagon mRNA expression with SCFA (57, 58). Additionally, supplementation of TPN with SCFA increased plasma GLP-2 concentrations (99), suggesting that the luminal presence of SCFA is not required for GLP-2 stimulation. However, colonic infusion of 0.5 M SCFA mixture did not increase GLP-1 concentrations in rats (100), and similar results were observed with a 150 mM SCFA mixture infused into the pig ileum (101) or individual infusions of acetate, propionate, or butyrate into isolated rat colon (102). In contrast, infusion of 20 mM butyrate or propionate in the isolated rat ileum did increase GLP-1-like immunoreactivity (103). Mixed results from experiments may indicate a role for fiber and resistant starch in GLP secretion beyond that of SCFA, or may implicate the importance of the SCFA concentration or profile in stimulating secretion from the L-cell.

Fats

Fats are also potently stimulatory to the L-cell. Plasma enteroglucagon concentrations in humans are significantly increased by ileal infusions of oleic acid, triolein, and medium-chain triglycerides trioctanoin and tridecanoin (104). Likewise, a fat meal increases plasma concentrations of intact GLP-2 by 3- to 6-fold (80, 90). A stimulatory effect of intrajejunal infusion of long-chain triglycerides on jejunal proglucagon mRNA in rats has also been observed, although increased plasma concentrations of enteroglucagon were not observed (79). The length of time for fat to increase GLP secretion is slightly longer than that of glucose, about 60 minutes after duodenal entry (80, 91, 105, 106). The stimulatory effect of fat on L-cell secretion seems to be dictated by chain length, as duodenal infusion of lauric acid (C12) increased GLP-2 concentrations in plasma but infusion of decanoic acid (C10) did not (105, 106). Saturation of fatty acids may also dictate the responsiveness of the L-cell to luminal fats. Although differing degrees of fat saturation in a meal (saturated, monounsaturated, or polyunsaturated from butter, olive oil, or corn oil, respectively) did not affect GLP-1

secretion in one experiment (107), another experiment showed that monounsaturated fats increased GLP-1 secretion more than saturated fats (108). In agreement, Rocca and Brubaker (109) reported that GLP-1 secretion by fetal rat intestinal culture was increased by monounsaturated fats to a greater extent than polyunsaturated or saturated fats, and chain length also affected GLP-1 secretion as fatty acids less than 16 carbons were unable to stimulate secretion. An additional component to the stimulatory effect of fats on GLP secretion may be that of the bile acids released from the gallbladder upon fat ingestion; colonic infusion of deoxycholate or hyodeoxycholate increased plasma enteroglucagon and GLP-1 concentrations, respectively (102, 110).

Proteins

Although carbohydrates and fats have generally been considered more stimulatory to GLP secretion than proteins (111), there is some evidence to suggest that proteins, or perhaps their digestion products, can stimulate secretion from the L-cell. Luminal administration of peptones into either the jejuno-ileum or colon dose-dependently stimulated GLP-1 secretion in an isolated intestinal perfusion model (55, 103). In humans, dietary protein has increased GLP-1 or -2 concentrations in plasma in some (90, 112) but not all (80) experiments; however, type of protein may also have an effect as GLP-1 concentrations increased more after a whey than casein preload meal (112). Dietary protein also increases GLP secretion in other species, including mice and pigs (56, 113). However, it is possible that the effect observed in vivo is due to the protein digestion products, peptones or amino acids, rather than whole protein per se. In vitro, increasing concentrations of hydrolysates of meat or egg albumin linearly increased GLP-1 release into the medium, but varying concentrations of bovine serum albumin or an amino acid mixture did not (55), and similar responses were observed with an isolated intestinal (ileum or colon) perfusion model (55, 103). Although amino acids did not increase proglucagon mRNA expression or GLP-1 secretion in the experiments of Cordier-Bussat et al. (55), Reimann et al. (114) demonstrated secretion of GLP-1 from GLUTag cells in response to individual amino acids (asparagine, methionine, leucine, alanine, serine, glutamate, and glutamine); in particular, glutamine dose-dependently increased GLP-1 secretion up to 7-fold (at 10 mM), whereas 10 mM glucose increased

GLP-1 secretion by only 3-fold (114). Thus, protein ingestion does appear to stimulate GLP secretion from the L-cell, but is perhaps less potent in vivo than fat or carbohydrate stimulation.

There seems to be synergy among nutrients in the efficacy of L-cell stimulation. Although intraduodenal infusions of glucose and fat both independently stimulated secretion of GLP-1, the effect was greater with both nutrients infused together (91). Likewise, a combination of a high fat and high protein diet increased plasma GLP-1 concentrations and proglucagon mRNA expression in the colon to a greater extent than either the high fat or high protein diets separately (56). Therefore it is likely that the GLP response to a variety of nutrients will be more stimulatory than some of the observations made using single nutrient infusions; however, it is clear that secretion from the L-cell is highly responsive to the nutrient profile in the intestinal lumen.

Indirect Mechanisms by Nutrients

Because nutrient ingestion rapidly stimulates GLP release from the L-cell, presumably before nutrients are present to directly stimulate the L-cells of the distal small intestine, other nutritionally regulated factors have been proposed. There are a number of different hormonal and neural mechanisms that have been demonstrated to affect secretion of GLP from the L-cell. These are discussed in detail below and include hormonal and neural regulation.

Hormonal regulation

There are several hormones produced within the gastrointestinal tract that have been identified as mechanisms regulating the secretion of GLP. Perhaps the most well-known of these is glucose-dependent insulinotropic polypeptide (GIP). The duodenally-produced GIP is, in addition to GLP-1, a potent insulin secretogogue that is responsive to nutrient ingestion (115). In rat intestinal cell culture, GIP dose-dependently stimulated GLI release (116). Roberge and Brubaker (117) observed that increases in gut GLI in response to fat or glucose in the duodenum was associated with a preceding increase in GIP concentrations, whereas fat or glucose in the ileum raised gut GLI without affecting

GIP concentrations. Further investigation showed that intravenous administration of a physiological dose of GIP increased gut GLI concentrations, demonstrating response of the L-cell to GIP in the rat (117). Total GLP-1 secretion was dose-dependently increased by a range of physiological doses of GIP (0.25 to 1 nM) in an isolated perfused rat colon model (118), but in an isolated perfused rat ileum model, pharmacological doses of GIP (3 nM) could increase GLP-1 secretion but not physiological doses of GIP (0.5 nM) (119). However, not all species may exhibit L-cell responsiveness to GIP. Although rat and canine L-cells appear to respond to GIP, human L-cells may not. Schirra et al. (85) noted no correlation between postprandial GIP and GLP-1 concentrations and suggested that human L-cells might not be responsive to GIP. Glucose infusion into the proximal 0 to 60 cm of the duodenum failed to increase plasma GLP-1 concentrations, whereas GLP-1 concentrations increased when glucose was allowed to pass to the distal intestine, and because GIP concentrations were similar with both infusion protocols, the researchers concluded that human L-cells were not responsive to GIP but may require direct stimulation by nutrients (92).

Less data exists on the effects of other gastrointestinal hormones on GLP secretion. Cholecystokinin (CCK) is a nutrient-responsive hormone secreted from the I-cells of the duodenum and jejunum (120). However, CCK did not affect GLP-1 secretion in the isolated perfused rat ileum or colon (119, 121), nor did it affect GLP secretion in vivo in rats (117). Likewise, secretin, a hormone secreted from the S-cells of the duodenum and proximal jejunum (120), did not change GLP-1 secretion in isolated perfusion models of rat ileum or colon (119, 122). In agreement, in fetal rat intestinal cell cultures, GLI secretion was not induced by cholecystokinin, glucagon, glucagon-like peptide-1, glucagon-like peptide-2, neurotensin, or peptide YY (116).

Two other hormones that do not originate in the gastrointestinal tract also affect GLP secretion. Insulin stimulated GLP-1 secretion from intestinal cell lines from mice, humans, and rats (123). Leptin, a hormone produced by adipose tissue, dose-dependently increased GLP-1 secretion from three different intestinal cell lines and increased GLP-1 secretion in vivo when administered to rats (124). Both insulin and leptin are responsive to alternations in nutrition (125), suggesting that nutrition could alter L-cell secretion by effects on systemic hormones in addition to those produced in the gastrointestinal tract.

One of the few negative regulators of GLP secretion is the hormone somatostatin. Somatostatin is produced in two forms, SS14 and SS28, from enteric neurons and enteroendocrine D cells, respectively (120). Inhibition of GLI release from fetal rat intestinal culture occurred with both SS28 and SS14, although SS28 was more potent in inhibiting GLI release (116). Likewise, infusion of antibodies to somatostatin increased secretion of GLP-1 and -2 in the isolated perfused pig ileum, and SS28 dose-dependently inhibited GLP-1 and -2 secretion whereas SS14 generally had weak and inconsistent effects on GLP secretion (126). This suggests that there is a basal level of SS28 from paracrine enteroendocrine cells in the intestine to inhibit GLP secretion, and both experiments demonstrate the inhibitory effect of somatostatin on the L-cell.

Neural regulation

Part of the regulation of GLP secretion could also occur through an indirect mechanism involving the vagal nerve. Rocca and Brubaker (127) observed that left cervical vagotomy reduced basal gut GLI in plasma by approximately half whereas stimulation of the celiac branch of the vagus increased gut GLI. Moreover, the increased gut GLI secretion in response to duodenal fat was abolished with subdiaphragmatic vagotomy. Interestingly, supraphysiological but not physiological doses of GIP were able to increase gut GLI in hepatic branch vagotomized rats, suggesting that the stimulatory effect of GIP on L-cells is mediated by the vagus at low concentrations but may be a direct effect at high concentrations (127), demonstrating interactions between the humoral and neural components of L-cell stimulation.

Additional evidence of regulation of the L-cell by the parasympathetic nervous system is evidenced by use of parasympathetic nervous system neurotransmitter agonists and antagonists. Acetylcholine infusion increased GLP-2 secretion from the isolated pig ileum (126) and a parasympathomimetic muscarinic receptor agonist, bethanechol, stimulated GLP-1 secretion from the isolated vascularly perfused rat ileum and colon as well as fetal rat intestinal cell culture (116, 119, 128). Additionally, atropine, a non-selective muscarinic receptor antagonist, reduced the increase in plasma GLP-1 in response to glucose or fat in the intestine (77, 129). Acetylcholine also acts via nicotinic receptors, and these nicotinic receptors participate in the neural stimulation of GLP

secretion because a nicotinic receptor antagonist abrogated the GLP-1 release in response to duodenal nutrients (100). Also, an antagonist to the nicotinic receptors prevented meal-induced GLP-1 secretion in macaques but a muscarinic antagonist did not (130). These results suggest that parasympathetic activation of L-cell secretion is acetylcholine-dependent, but the individual receptors involved (muscarinic or nicotinic) may be more species-specific.

The sympathetic nervous system also regulates L-cell secretion. The synthetic catecholamine isoproterenol, a general β-adrenergic receptor agonist, transiently increased GLP-1 secretion from the isolated vascularly perfused rat ileum but was more effective in inducing GLP-1 secretion from the isolated vascularly perfused rat colon at similar concentrations (119, 131). Also using an isolated perfused rat ileum model, Claustre et al. (132) demonstrated that the L-cell is positively stimulated by activation of β_2-adrenergic receptors, and α_1-adrenergic receptor activation may synergistically stimulate GLP secretion. In contrast, α_2-adrenergic receptor activation may act to inhibit GLP secretion from the L-cell (132).

It has also been suggested that the enteric nervous system is involved in the indirect stimulation of the L-cell. Rocca and Brubaker (127) hypothesized that in the rat the enteric nervous system was related to the responsiveness of the L-cell to duodenal fat, because increases in gut GLI with duodenal fat were substantially slower when the gut was transected. Secretion of GLI from fetal rat intestinal culture was dose-dependently increased by the neurocrine peptides gastrin-releasing peptide (GRP), and calcitonin gene-related peptide (CGRP), and this effect has also observed in GLUTag cells (116, 124). Both GRP and CGRP are produced in nervous tissue and are used within the intrinsic enteric nervous system (133). Moreover, GRP and CGRP increase GLP-1 secretion from the isolated vascularly perfused rat ileum and colon (119, 134), and neuromedin C (from GRP proprotein) infusion increased GLP-2 secretion from the isolated perfused pig ileum (126).

Thus, an integrated view of how duodenal nutrients can stimulate GLP secretion from the distal L-cell was initially proposed by Rocca and Brubaker (127) and further expanded by Anini et al. (129) and Brubaker and Anini (111). These researchers suggest that, in the rat, duodenal nutrients stimulate GIP secretion by the K cell, which stimulates

the afferent vagus nerve and via the central nervous system activates the efferent celiac branch of the vagus nerve, sending the signal to the ileum. Activation of the intrinsic enteric neurons by a nicotinic receptor induces release of GRP, which stimulate cholinergic neurons in the enteric nervous system to release acetylcholine, which binds with muscarinic receptors on the intestinal L-cell to induce secretion. This model may not fully apply to some species, as in humans atropine failed to affect the secretory response of GLP-1 in the first hour after oral glucose but was able prevent the increase in GLP-1 secretion after 60 min, when glucose presumably reached the distal intestine (77). This result suggests vagal cholinergic innervation does not play a major role in regulating GLP-1 secretion in humans (115). However, very little research has been done in non-rodent species examining the possibly indirect control of L-cell secretion by neural components.

Regulation of Secreted GLP-2

Once GLP-2 is secreted from the L-cell into the bloodstream, its circulating lifetime will be dictated by the ubiquitous serine protease dipeptidyl peptidase IV (DPPIV). The substrate specificity for DPPIV is a proline or alanine as the second amino acid from the N terminus with any amino acid except proline (but preferentially hydrophobic or basic) at the adjoining third position (135). The N-terminus sequence for both the GLP-1 and GLP-2 is His-Ala-Asp which makes them substrates for DPPIV activity, and indeed both are inactivated by DPPIV activity (3, 136), yielding a N-terminal His-Ala dipeptide and the truncated hormone. Because of the activity of DPPIV, GLP-2-(1-33) has a half life of only 7 minutes before it is degraded to GLP-2-(3-33), which has a longer half life of 22 to 27 minutes (81, 137).

Dipeptidyl peptidase IV is present along the brush border membrane of the intestinal tract (138) as well as in kidney, hepatocytes, and as a soluble form in plasma (115, 135, 139). Additionally, DPPIV is present on the endothelial lining of the blood vessels (140), and the cells lining the capillaries supplying the L-cells may be most responsible for GLP-2 cleavage as the DPPIV-positive capillaries are located in close proximity to the L-cells (141). To illustrate this point, a large proportion of GLP-1 secreted from the ileum is already degraded to the truncated form (54% and 33% intact for in vitro and in vivo

preparations, respectively), and addition of a DPPIV-inhibitor increased the proportion of intact GLP-1 to almost 100% (141).

The inactivated hormone, as well as intact hormone, is cleared primarily by the kidney. Distribution of ^{125}I-GLP-2 was detected in the liver and kidney; in the kidney radioactivity was localized to the proximal tubules, but since neither liver nor kidney uptake was affected by non-radioactive GLP-2, it was suggested that the uptake of GLP-2 by these organs was due to non-specific metabolism (likely clearance) rather than receptor-mediated uptake (142). Nephrectomized rats had substantially slower clearance curves of GLP-2 than intact rats, but because nephrectomy did not completely prevent GLP-2 clearance, the authors suggested that other organs may contribute (143). In the pig, significant extraction of GLP-2-(1-33) was detected across the kidney as previously reported, but also across the hindleg and splanchnic bed (~20 to 25% extraction for each tissue bed), whereas extraction (~15%) of the truncated form GLP-2-(3-33) occurred only across the kidney (137). However, in contrast to the distribution of GLP-2 to the rat liver observed by Thulesen et al. (142), neither GLP-2-(1-33) nor GLP-2-(3-33) were removed by the pig liver (137). Whether this is species-specific differences in site of clearance or due to differences in methodology is unknown.

The importance of DPPIV for in vivo regulation of GLP-2 activity is apparent. Inhibition of DPPIV increases the amount of intact circulating GLP-2 in pigs (137). Also, DPPIV-deficiency or inhibition increases intestinal weight (3). There are apparent species-specific differences in the ability of DPPIV to inactivate GLP-2; rats have higher plasma DPPIV activity than mice and have a reduced intestinotrophic response to GLP-2 than mice (3, 144). The understanding of the role that DPPIV plays in GLP-2 metabolism has led to the development of analogs of GLP-2 that resist DPPIV degradation by altering the second amino acid, generally to a Gly to yield [Gly^2]GLP-2. Administration of [Gly^2]GLP-2 increases intestinal weight substantially (3, 61, 143), and the dose of [Gly^2]GLP-2 that maximally activated the endogenous rat GLP-2 receptor was similar to circulating concentrations of GLP-2 (145). The development of these analogs has proven to be beneficial for disease conditions such as short-bowel syndrome, where administration of [Gly^2]GLP-2 can improve intestinal function in patients (146, 147).

GLUCAGON-LIKE PEPTIDE-2 RECEPTOR

The GLP-2 receptor (GLP2R) was identified in 1999 as a 7-transmembrane G-protein coupled receptor, similar to the receptors for other members of the glucagon-secretin superfamily of hormones (148). The C-terminal end of the GLP2R protein appears to be important for cell surface expression of the receptor in the plasma membrane (149). This receptor is highly specific for GLP-2, with cells exhibiting a 4-fold induction in cAMP accumulation with GLP-2 but no ability to increase cAMP production in response to GLP-1, glucagon, GIP, parathyroid hormone, secretin, pituitary adenylate cyclase-activating polypeptide-27 or -38, vasoactive intestinal peptide, GRF, corticotropin releasing factor, or exendin-3 (148, 150). The EC_{50}, or dose of GLP-2 effective to cause half the maximal accumulation of cAMP, ranges from 31 pM to 0.58 nM depending on the cell culture expression of the GLP2R (148, 151, 152). Regulation of GLP2R mRNA is not well described, but unlike proglucagon mRNA the GLP2R mRNA does not appear to be nutritionally regulated, as fasting and refeeding did not affect GLP2R mRNA expression in mice (54).

Localization of the GLP2R

Expression of GLP2R mRNA was first reported using an RNase protection assay in several tissues of the gastrointestinal tract, including (in descending order of expression levels) jejunum, duodenum, ileum, colon, and stomach, with low or no detection of GLP2R mRNA in brain, heart, kidney, liver, lung, muscle, or spleen in the rat (148). This localization has been corroborated by Northern blot analysis, and using a more sensitive RT-PCR assay the GLP2R was also detected in the hypothalamus, brainstem, and lung, although expression was 20-30 fold greater in the jejunum than in the brain (153). More recently, GLP2R expression on pancreatic α-cells has been noted (154). Using ^{125}I-GLP-2 distribution, the relative distribution of radioactivity along the rat gastrointestinal tract was greatest in the proximal jejunum, with decreasing distribution distally down the tract (142), and GLP2R was expressed throughout the small intestine in

rat, mouse, and man, but the most intense staining was in the proximal small intestine in all species (5). There is substantial agreement that GLP2R expression in the gastrointestinal tract is highest in the proximal small intestine, particularly the jejunum.

The GLP2R has been localized to several distinct cell populations within the gastrointestinal tract. One of these subpopulations is the enteroendocrine cell. In humans, pigs, and rats, the GLP2R is localized in cells of the villus epithelium expressing chromogranin A, a general enteroendocrine cell marker (14, 63, 153). Colocalization of GLP2R with cells positive for serotonin, GLP-2, PYY, and GIP in the human gastrointestinal tract has also been reported (153). Additionally, GLP2R localizes with enteroendocrine cells expressing 5-HT (14, 63), a neurotransmitter in enteroendocrine cells that activates extrinsic and intrinsic enteric afferent neurons (155). This suggests that activation of the GLP2R in enteroendocrine cells could stimulate 5-HT secretion to activate the enteric nervous system. However, GLP-2 could also activate the extrinsic and intrinsic enteric nervous systems directly. The GLP2R is expressed by vagal afferent neurons and can stimulate activity of these neurons (156). The intrinsic enteric nervous system also expresses the GLP2R on submucosal and myenteric plexi in rat, pig, and human small intestine (5, 14, 156). These GLP2R-expressing intrinsic enteric neurons also express endothelial-isoform nitric oxide synthase (eNOS) and vasoactive intestinal peptide (VIP) (14, 63, 157), and both eNOS and VIP are involved in vasodilatory response and could be involved with observed GLP-2 effects on blood flow, as discussed below. Finally, within the gastrointestinal tract, GLP2R has also been localized to subepithelial myofibroblasts (5, 156), cells that are located in the subepithelium along the gastrointestinal tract, existing as a syncytium extending into the lamina propria and even circling the capillaries within the lamina propria (158). Because myofibroblasts produce a number of growth factors including IGF-I and –II, keratinocyte growth factor, hepatocyte growth factor, platelet-derived growth factor, and transforming growth factor-β (158), they could have a role in the GLP-2-induced growth of the intestinal tract, as discussed below. Despite the localization of GLP2R to enteroendocrine cells, enteric neurons, and subepithelial myofibroblasts, it is importantly not localized to enterocytes (153), demonstrating that the effects of GLP-2 are not directly mediated.

GLP-2 Receptor Activation and Signaling Cascade

In testing the binding and signaling activity of the GLP2R, Munroe et al. (148) found that GLP-2 analogs with extensions on the carboxyl-terminal retained binding and activation and in vivo activity (small intestinal growth response) whereas extensions on the amino-terminal caused loss of binding and activation and in vivo growth response. In contrast, truncation of both the carboxyl- and amino-terminals reduced but did not fully abrogate the binding or activation (in vivo or in vitro) of the GLP2R (148). Truncation of 1 or 2 amino-terminal amino acids, yielding GLP-2 (2-33) or (3-33), respectively, did not cause small intestinal weight increases in vivo but these analogs were able to bind and activate the GLP2R in vitro, albeit much more weakly than GLP-2 (1-33) (148). Similarly, although the first two amino acids (His and Ala) in the GLP-2 sequence are not critical for receptor binding, the amino terminus is necessary for GLP2R activation of adenyl-cyclase signal transduction (150). In the mouse, GLP-2-(3-33) functions as a weak agonist with properties of a competitive antagonist at very high concentrations (151), and similar antagonistic properties of GLP-2-(3-33) have been observed in other experiments with mice or rats (54, 84). However, injection of GLP-2-(3-33) into mice for 10 days did not affect intestinal weight compared to PBS-injected controls, suggesting that the truncated form of GLP-2 has little effect in vivo (143). Likewise, the truncated metabolite also did not affect mucosal growth in rats (144). This suggests that despite the longer half life of GLP-2-(3-33), there is little effect in vivo of the truncated metabolite but at high enough concentrations it could act as a competitive GLP2R antagonist. As mentioned previously, the GLP2R is highly specific for GLP-2 (148), thus only upon binding of GLP-2-(1-33) will activation of the signaling cascade occur.

Activation of the GLP2R stimulates several cellular signaling pathways. These have been summarized in Figure 2.4. One of these is activation of adenylate cyclase to increase cAMP production (145, 148, 150-152). Activation of protein kinase A (PKA) occurs with GLP2R activation (145, 159-161) because increased cAMP binds to the PKA regulatory subunits, allowing release of the catalytic subunits which then activate, among other downstream effectors, the CREB protein (161) to allow DNA binding and transcription of genes. A second signaling pathway activated by GLP2R is the Ras/Raf/MEK/ERK pathway. Dose-dependent activation of this pathway by GLP-2

resulted in an increase in the activation of MEK and ERK1/2 and increased cell proliferation (160-162). Increased expression of the downstream effectors of this pathway, including cFOS, p706K, and p90Rsk has also been observed with GLP-2 administration (159, 161). A third GLP2R signaling pathway is activation of activation of Akt/PKB by PI3K activity (8, 161-164). This pathway has several downstream effectors, including CREB, mTOR, eNOS, and GSK-3, all of which have been observed to be activated by GLP-2 (8, 161, 163, 164). In contrast, activation of the GLP2R does not appear to affect intracellular Ca^{2+} accumulation (145, 159), and one report suggests that GLP-2 can reduce cAMP to increase cell proliferation via a non-GLP2R-mediated mechanism (165).

Adding to the complication of this signaling pathway is the observation that multiple pathways may or may not be activated upon GLP2R stimulation. The activation of p70S6k, p90Rsk, and AP-1 dependent signaling pathways was partially dependent on PKA activation but was not dependent on MAPK or PI3K activation, suggesting that GLP-2 acts through additional, non-cAMP-dependent pathways (145, 159, 166). In contrast, increased proliferation of cells with GLP-2 was associated with activation of both the Ras/Raf/MEK/ERK1/2 and PI3K pathways (162). Additionally, although GLP-2 activated the Ras/Raf/MEK/ERK1/2 pathway to stimulate proliferation, the anti-apoptotic action of GLP-2 is not through the ERK1/2 pathway but is dependent on the PKA pathway (160).

In agreement, GLP-2 induced survival was via a PI3K-independent but PKA-dependent pathway because GLP-2 was able to improve cell survival, reduce apoptotic markers, and stimulate GSK-3 phosphorylation similar to forskolin despite inhibition of PI3K, but this action was abrogated by the addition of a PKA inhibitor (166). In contrast to the results of Yusta et al. (166), in vivo dose-dependent inhibition of apoptosis with increasing infusate concentrations of GLP-2 was associated with a reduction in caspase-3 and caspase-6 activities (pro-apoptotic factors), increased Bcl-2 expression (a anti-apoptotic factor), and increased Akt/PKB phosphorylation, suggesting that activation of the PI3K pathway is important in the anti-apoptotic properties of GLP-2 (8). However, increased protein synthesis and phosphorylated GSK3 expression with GLP-2 (8) also suggest that the PI3K pathway can mediate some of the proliferative effects of GLP-2, as

phosphorylation of GSK3 allows β-catenin translocation to the nucleus to activate transcription and protein synthesis.

Likewise, increased protein synthesis and crypt cell proliferation and reduced apoptosis was associated with increased expression of phosphorylated PKA (at 1 h) and increased phosphorylated CREB (at 1 and 4 h), indicating activation of the PKA pathway, but PKB and GSK phosphorylation were also upregulated with GLP-2, indicating upregulation of the Akt/PKB pathway (161). Finally, phosphorylated ERK1/2 increased with GLP-2, indicating upregulation of the 3rd putative GLP2R signaling pathway (161). Additionally, downstream effectors of the PKA and MAPK pathways, such as c-Fos and p90RSK (MAPK effector) were also upregulated (at 1 and 4 h for c-Fos and at 48 h for p90RSK (161). These results suggest that all three putative signaling pathways of the GLP2R were activated.

The overall consensus for the signaling pathways involved in GLP2R signaling is that there is little consensus, but that there are likely multiple signaling pathways involved, some or all of which may be activated upon GLP-2 binding to its receptor. Some of the conflict in research results may also be a result of the methodologies employed, as Ras can mediate its actions different ways depending on cell type (167); therefore research in a homogeneous cell culture may yield different results than research utilizing heterogeneous tissue samples. Receptors such as the GLP2R can couple with multiple downstream effectors, allowing flexibility and integration to determine the ultimate cellular response. The stimulation of proliferation with GLP2R activation appears to commonly involve the Ras/Raf/MAPK pathway, a well-known mitogenic pathway (168). In contrast, the GLP-2-mediated inhibition of apoptosis often involves the PKA and PI3K pathways, which are well-known anti-apoptotic pathways (169). Two important notes must be made about GLP2R signaling pathways. First, these signaling pathways, while often viewed as independent, are highly interactive. Second, the coupling of GLP2R signaling with downstream effects such as proliferation or inhibition of apoptosis is almost certainly intercellular, as activation of c-Fos and caspase-3 (downstream effectors) was observed in mucosal enterocytes (161) but the GLP2R is not (153).

GLP2R Desensitization

Desensitization of the GLP2R has been observed in response to GLP-2, a process known as homologous desensitization. Pretreatment of rat isolated primary small intestinal cells with GLP-2 diminished the ability of the GLP2R to be activated with a subsequent GLP-2 dose (145). Likewise, GLP-2 pretreatment of cells stably transfected with the GLP2R dose-dependently reduced the cAMP increase in response to a 2nd dose of GLP-2; this effect was exacerbated by longer pretreatment times, and recovery of cAMP response improved with increasing recovery time (170). Also observed was a rapid agonist-induced internalization of the GLP2R with a slow reappearance of receptors on the cell surface, recovering original values by approximately 200-300 minutes, with apparent recycling through the endosome but without any apparent degradation of the receptor within the cell (170).

Despite the in vitro observations of GLP2R desensitization, in vivo results are not apparent. In rats subjected to intestinal transection or resection, administration of exogenous GLP-2 increased GLP2R mRNA expression in the jejunum (transection and resection) and ileum (resection) (63), suggesting that GLP-2 does not downregulate GLP2R transcription in vivo. However, increased small bowel weight with GLP-2 administration plateaus after approximately 14 d despite continued administration, demonstrating a degree of downregulation of the response (6, 12); whether this is related to GLP2R expression is unknown. Additionally, because the effects of GLP-2 are mediated by other factors, in vivo downregulation of GLP2R with high GLP-2 concentrations may not significantly alter intestinal growth effects because other downstream pathways are already activated. Indeed, even a single dose of GLP-2 increases in vivo and in vitro proliferation (6, 162).

ACTIONS OF GLP-2: A TRIPARTITE EFFECT

GLP-2 and Gastrointestinal Growth

Drucker et al. (4) reported that mice with enteroglucagon-producing tumors had greater small bowel weight, crypt-villus height, and intestinal epithelium proliferation rate than normal mice. Upon testing the various peptide components of enteroglucagon (glicentin, oxyntomodulin, intervening peptide 2, GLP-1, and GLP-2), they found that GLP-2 consistently increased small bowel weight by 1.5 to 2-fold. Mice injected with GLP-2 had greater intestinal crypt-villus heights and mucosal thickness, as well as greater rates of intestinal epithelial proliferation compared to saline-treated control mice. This report led to a rapid increase in research investigating the role of GLP-2 in intestinal growth, and overall the effect of GLP-2 in normal mice or rats is a remarkably consistent 1.35 to 2-fold increase in small bowel weight (2, 5, 6, 9, 10, 12, 61). The greatest effects of GLP-2 on intestinal growth are seen in the proximal and middle intestine (6, 7, 12, 144), but increased colonic weight due to GLP-2 administration has been seen in some (5, 9, 10, 171) but not all experiments (7, 172, 173). Although increased stomach mass has been observed (9), in other experiments GLP-2 has not affected stomach mass (7, 8, 173), and stomach protein synthesis was reduced by GLP-2 (8). However, increases in whole body weight or weights of other non-gastrointestinal organs are rarely observed (6, 7).

The mechanism of action of GLP-2-induced growth of intestinal mucosa is a trophic effect on the gastrointestinal tract rather than systemic growth or stimulation of feed intake (6, 144). The increase in small bowel weight upon GLP-2 administration occurs within 6 d (4, 12), although increases in protein mass can be observed in just 4 h (13). The increase in small bowel weight with GLP-2 appeared to plateau after approximately 14 d despite continued administration, demonstrating a degree of downregulation of the growth response (6, 12), and cessation of GLP-2 administration resulted in similar small bowel weights between treated and control mice after 10 d (6). The presence of enteral nutrients is not required for GLP-2 to stimulate proliferation because TPN-fed animals exhibit intestinal growth with GLP-2 administration despite the atrophic effect of TPN on intestinal tissue (7, 8, 174). However, a role of enteral nutrients does exist; besides stimulating release of GLP-2 as previously discussed, the combination of supplemental

enteral nutrition and GLP-2 increased intestinal mass, DNA and protein accretion, and villus height to a greater extent than GLP-2 alone in TPN-fed rats (174).

Increased small intestinal weight with GLP-2 results from preferential expansion of the mucosal compartment rather than the muscularis compartment (4, 6, 8, 9, 144). Importantly, GLP-2 does not change the proportion of absorptive cells, goblet cells, Paneth cells, and endocrine cells in the small intestine (4). The mucosal growth induced by GLP-2 is the result of increased height of the crypt-villus complex, due to increases in both villus height (7, 144, 151, 175, 176) and crypt depth (7, 144, 175). This increase in small bowel weight is the result of both an increase in epithelial proliferation and a reduction in apoptosis, although effects on both mechanisms are not always observed in the same experiment (4, 7, 8, 11, 12).

Proliferation

In vitro, GLP-2 dose-dependently increased the [^{3}H]-thymidine incorporation into Caco-2 or T84 human intestinal epithelial cell lines, and similar to in vivo observations of GLP-2 effects on intestinal weight, the proliferative effect of GLP-2 could be detected as early as 1 d, was maximal at 5 d, and returned to baseline by 7 d (177). Even a single dose of GLP-2 was sufficient to increase [^{3}H]-thymidine incorporation and the cellular protein concentrations of cyclin E, A, and D_1 (177), which are proteins that allow transition from the quiescent G_0 phase through the S phase (DNA replication) and into the G_2 phase, where cell growth and protein synthesis occur.

In vivo, the proliferative effect of GLP-2 has been demonstrated using a variety of cellular markers, including bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA). Bromodeoxyuridine is a derivative of thymidine that is incorporated into DNA during DNA synthesis, whereas PCNA is a cyclin protein that increases during the DNA replication phases of the cell cycle. Increased small intestinal weight due to GLP-2 treatment was associated with an increased proportion of intestinal crypt cells staining positive for PCNA (4, 6) or BrdU (8). Similar to in vitro results, even a single GLP-2 injection (5 μg for mice) was able to increase the jejunal crypt cell proliferation rate by 40% after only 2 h (6). Increases in protein synthesis are also observed with GLP-2 administration to TPN-fed piglets (8, 13, 161). However, GLP-2-mediated

increases on cell proliferation and intestinal protein and DNA accretion may only occur at high pharmacological GLP-2 concentrations (8), whereas lower physiological GLP-2 concentrations may increase intestinal mass by inhibiting apoptosis.

Apoptosis

In vivo, the proportion of apoptotic cells in the jejunal villi was decreased by up to 50% with GLP-2 treatment in mice or TPN-fed piglets as determined by TUNEL assay, an assay that detects the nicked ends of DNA that occurs during apoptosis (6, 7). In TPN-fed piglets, GLP-2 dose-dependently reduced apoptosis in both villus (8, 161) and crypt cells (8). Depending on the dose and length (acute or chronic) of GLP-2 treatment, GLP-2 has either not affected rates of proteolysis or protein oxidation (acute, high dose; (13)) or has suppressed proteolysis in the jejunum (chronic, lower dose; (7)). Increasing rate of GLP-2 infusion markedly reduced the activity of two of the primary effector caspases in the apoptotic cascade, caspase-3 and -6, and decreased the ratio of active:pro-caspase-3, demonstrating a reduced activation of caspase-3 to its active form with GLP-2 (8), and a similar effect was observed with increased time of GLP-2 administration (up to 48 h, (161)). Additionally, the expression of the anti-apoptotic factor Bcl-2 increased dose-dependently with GLP-2 concentrations, especially within the physiological range (< 200 pM) (8), and this effect was again observed with increased time of GLP-2 administration (up to 48 h, (161)). These results suggest that increased Bcl-2 protein prevents cytochrome c release from the mitochondria to inhibit apoptosis, a result observed in vitro; GLP-2 reduced activation of activating caspase-8 and -9 and effector caspase-3 and prevented release of mitochondrial cytochrome c in chemically-induced apoptotic cells (178). Furthermore, GLP-2 increased the amounts of XIAP and cIAP-2 proteins, which are inhibitors of the effector caspases (161). Thus, in TPN-fed piglets, the proliferative effect is primarily due to multiple anti-apoptotic actions in the intestine.

Indirect mechanism of GLP-2

Although GLP-2 potently stimulates increases in small intestinal weight, localization of the GLP2R suggests that the effects of GLP-2 are indirect and other growth factors are involved. Although there are a variety of growth factors in the intestine, several have

emerged as potential mediators of the GLP-2 growth response. There is compelling evidence to suggest that IGF-I is a primary component of the GLP-2-induced growth response in the small intestine. Intestinal IGF-I is expressed in the subepithelial myofibroblasts (158), a cell type demonstrated to express the GLP2R (5, 156). The ability of GLP-2 to increase putative factors involved in cell proliferation was dependent upon IGF-I signaling (179). Additionally, of several intestinal growth factors only IGF-I was able to increase small intestinal weight in mice to a level comparable to GLP-2 administration (61). Notably, IGF-I also increases villus height in the jejunum (61), an effect observed with GLP-2.

Incubation of fetal rat intestinal cells with GLP-2 increased IGF-1 mRNA expression and secretion, and similar results were observed in vivo in mice (180). Normal mice given GLP-2 had greater jejunal and ileal villus height and crypt depth than controls, but IGF-I knockout mice did not exhibit any changes in villus height or crypt depth in response to GLP-2. However, small intestinal weight as a percent of BW was increased by the highest dose of GLP-2 in IGF-I knockout mice (180), suggesting that although IGF-I may be an important mediator of GLP-2-induced growth, there are possibly other growth factors involved.

Mice with knockout of IGF-II show a mixed response to GLP-2. Effects of GLP-2 on villus height and crypt depth were similar in IGF-II knockout mice and normal mice, but small intestinal mass was lower for the IGF-II knockout mice compared to normal mice (180), suggesting that IGF-II may have a small role in mediating GLP-2-induced growth, In vitro, release of VEGF and TGF-β growth factors from subepithelial myofibroblasts was dose-dependently increased by incubation with GLP-2 (181). Another factor produced in the subepithelial myofibroblasts, keratinocyte growth factor (KGF), may mediate the increase of colonic weight with GLP-2 as KGF antibodies partially inhibited the ability of GLP-2 to increase colonic weight; however, small intestinal weight was similar between the two groups (5). The ability of GLP-2 to prevent TPN-induced intestinal atrophy was reduced when intestinal polyamine concentrations were depleted (173). The aforementioned experiments demonstrate several key points. Different regions of the small intestinal mucosa differ in their sensitivity to growth factors, either alone or in combination with other growth factors (61). Furthermore, although IGF-I is a

primary candidate for the majority of the growth effects observed with GLP-2 administration, it is probable that GLP-2 acts through multiple different mechanisms to induce growth of the small intestine. A neural component of GLP-2 action may exist, as the GLP2R is expressed by vagal afferents and can activate vagal afferent signaling; however, ablation of this signaling did not prevent GLP-2-induced intestinal growth in a TPN model (156), demonstrating the importance of other mechanisms.

An important aspect of gastrointestinal growth is control; although growth of the intestinal epithelium can be beneficial, exquisite control is necessary or cancerous growth can result. Mice bearing chemically-induced tumors had a greater number of polyps in the colon when given GLP-2 or [Gly^2] GLP-2 (a DPPIV-resistant GLP-2 analog) than control mice, but only after a month of treatment; animals given GLP-2 for only 10 days did not exhibit any differences in the number or size of colonic polyps than control animals (182). In vitro, GLP-2 dose-dependently increased the migratory activity and proliferation rate of two different lines of human colon carcinoma cells, an effect that is potentiated by inhibition of DPPIV (183). However, expression of the GLP2R is rarely found in human colon cancer cells, and GLP-2 did not affect the growth or survival rates of colon cancer cell lines and did not affect growth of colon cancer xenografts in mice (184). Therefore, no evidence suggests that GLP-2 has a causal role in cancerous growth in the intestinal tract, although GLP-2 may accelerate growth of already cancerous tissue. This proves important for the potential use of GLP-2 in patients with intestinal disease or dysfunction.

GLP-2 and Blood Flow

A second important effect of GLP-2 administration is a rapid and significant increase in blood flow. Infusion of GLP-2 into piglets fed by total parenteral nutrition increased portal blood flow by approximately 25% (13). Likewise, superior mesenteric artery blood flow in TPN-fed pigs, anesthetized rats, and conscious humans was dose-dependently increased by GLP-2 infusion (14, 16, 185). Importantly, the responsiveness of blood flow to GLP-2 was greatest at physiological concentrations (62.5 to 250 $pmol \cdot kg^{-1}h^{-1}$ (14)). This stimulation of blood flow is rapid (within 10 minutes of GLP-2

infusion) and abates within 20 minutes of infusion cessation (13, 15, 16). Increased blood flow in response to GLP-2 is not accompanied by changes in arterial pressure (14, 16, 185).

The effect of GLP-2 on blood flow is restricted to "intestinal" vessels, as GLP-2 rapidly increases blood flow of the superior mesenteric artery and portal vein (14-16) but does not affect blood flow at "non-intestinal" vessels such as the celiac, renal, or gastric arteries (15, 16). Blood flow to the distal colon, spleen, left and right kidneys, and brain was unchanged or very minimally affected by GLP-2 infusion, whereas blood flow to the duodenum and pancreas increased at extremely high concentrations (2000 $pmol \cdot kg^{-1}h^{-1}$) and blood flow to the stomach and proximal colon decreased by 12 to 15% (15).

The mechanism by which GLP-2 increases blood flow is dependent on nitric oxide as the use of L-NAME, a nitric oxide synthase (NOS) inhibitor, can at least partially abolish the GLP-2-induced increase in blood flow (13, 14, 16). Vasodilation occurs because NO increases cGMP accumulation which, in turn, reduces intracellular Ca^{2+} concentrations; because the contractile apparatus requires Ca^{2+} to contract, NO signaling ultimately causes relaxation of the vascular smooth muscle. Infusion of GLP-2 increased the activity of total NOS by increasing constitutive NOS activity and not affecting inducible NOS activity (13), likely because jejunal protein abundance of endothelial NOS (eNOS), but not inducible NOS or neuronal NOS, was increased by GLP-2 infusion (13). Infusion of GLP-2 increased eNOS protein after 1 h, and eNOS mRNA and protein (both total and phosphorylated active) were increased after 4 h (14). The hemodynamic alterations induced by GLP-2 seem to be mediated at least in part, by NO with the involvement of the enteric non-cholinergic neurons, as muscarinic, GLP-1, and CCK receptor inhibitors as well as prostaglandins did not change the blood flow response to GLP-2 infusion (16). At least three possibilities exist as to how GLP-2 may increase blood flow, discussed in detail by Guan et al (14). First, because the GLP2R colocalized with VIP-containing enteric neurons (14), activation of the GLP2R could cause vasodilation by activation of the non-cholinergic secretomotor/vasodilator neurons to release VIP and cause vasodilation. Second, because GLP2R was colocalized in enteroendocrine cells expressing 5-HT (13), receptor activation could cause release of 5-HT to directly increase vasodilation by effects on vessel endothelial cells (which secrete vasodilatory substances

such as NO; (186)) or indirectly increase vasodilation by activating the nervous system. Third, because GLP2R was colocalized with eNOS-containing enteric neurons (14), production of NO in the neuron could possibly diffuse to local vessels and cause vasodilation in the vascular smooth muscle.

Consistent with the observed localization of the GLP2R (5, 142, 148, 153), blood flow increases in response to GLP-2 are greater in the jejunum than ileum (15), and because the mechanism of blood flow changes due to GLP-2 are NO-dependent, these observations agree with those of Guan et al. (13) that increased activity and protein abundance of eNOS was detected in the jejunum but not ileum. Coupled with the observations that GLP-2 has the greatest epithelial growth effects in the jejunum (6, 7, 12, 144), the possibility that nutrient uptake in the jejunum could be increased by GLP-2 is enticing.

GLP-2 and Nutrient Transport

The third mechanism by which GLP-2 could increase nutrient absorption is by effects on digestive enzymes and nutrient transporters in the intestine.

Increases in the disaccharidases maltase-glucoamylase, sucrase-isomaltase, and lactase mRNA and/or activities have been observed in response to GLP-2 administration (63, 171, 187, 188). Likewise, GLP-2 increased aminopeptidase N activity in jejunum of TPN-fed piglets (187). However, GLP-2 did not affect uptake of glucose, leucine, or proline in premature, TPN-fed piglets (189). Many experiments observing positive effects on digestive capacity have used a TPN model (171, 187, 190) or an intestinal transection/resection model (63). In models that do not induce intestinal injury or atrophy, GLP-2 effects on digestive enzyme activity are not particularly strong. A DPPIV-resistant GLP-2 analog did not affect the maltase activity in mouse duodenum (61). Although GLP-2 administration increased activities of maltase, sucrase, lactase, glutamyl transpeptidase, and DPPIV in mouse duodenum when expressed as units of activity/5 cm, increases due to GLP-2 were abolished when activities were normalized to g of protein (2), in contrast to the increased specific activities of digestive enzymes in the TPN model (171, 187, 188). This indicates that in non-injured animals GLP-2 may

increase overall enzyme activity in the intestine by increasing mucosal growth but does not affect the activity or concentration of the individual enzymes per se.

Nutrient transporters are a second critical step to transport nutrients out of the lumen and into the blood to be distributed to the rest of the body. In vitro, glucose uptake across the apical membrane is increased with GLP-2 because of an increase in SGLT-1 protein abundance and activity (191). Likewise, fructose uptake across the apical membrane is increased with a concurrent increase in apical GLUT2 expression with GLP-2 (192). Basolateral upregulation of GLUT2 by GLP-2 also been observed (193), and although the amount of surface GLUT2 at the basolateral membrane was not upregulated by GLP-2 in another experiment, basolateral transport of glucose transport was increased with GLP-2 (194).

In vivo, administration of GLP-2 to TPN-fed piglets increased GLUT2 protein in the ileum compared to TPN alone (13), and GLP-2 administration to TPN-fed piglets was able to restore intestinal glucose transport activity (V_{MAX}) and jejunal brush border membrane SGLT1 (but not GLUT2) protein to the level of enterally fed-piglets compared to those fed TPN (188). Although GLP-2 did not affect expression of SGLT1 mRNA in TPN-fed piglets (189), in a different experiment GLP-2 increased SGLT1 protein by 3-fold and increased apical membrane localization of SGLT1 in orally-fed rats despite a lack of increased SGLT1 mRNA (175). Additionally, GLP-2 can reverse the effects that TPN has on several amino acid and peptide transporters in rats, including PEPT1, ASCT1 and 2, and EAAC1 (195). Despite the positive effects of GLP-2 on nutrient transport in TPN-fed animals, fewer positive results are observed with orally-fed animals. Jejunal expression of GLUT2 and GLUT5 mRNA was not affected by GLP-2 administration in rats (175). Administration of GLP-2 for 10-d reduced mRNA expression of GLUT2 and SGLT1 in mouse jejunum, and despite increased growth of the intestine with GLP-2, nutrient uptake of duodenal glucose or maltose was not increased (2). However, in the same experiment GLP-2-treated rats had greater uptake of leucine and triolein (2), and greater concentrations of plasma free fatty acids and triglycerides after a meal in GLP-2 treated humans might suggest that lipid absorption was improved with GLP-2 (196). Additionally, 14-d infusion of GLP-2 increased glycine and galactose uptake in rat isolated mid-small intestine (197). Thus, in healthy animals, GLP-2 may improve

digestive capacity, but this may be more related to overall increases in intestinal epithelial growth and blood flow than alterations in the digestive enzymes and transporters per se.

POTENTIAL IMPORTANCE OF GLP-2 IN RUMINANTS

Ruminants have a uniquely complex gastrointestinal tract that undergoes substantial changes during the animal's lifetime. Following weaning, the rumen dramatically increases in proportional size with a concurrent decrease in the proportional size of the abomasum (18). Intestinal development also occurs rapidly during this time. A second period of growth of the ruminant gastrointestinal tract occurs upon initiation of lactation, as one necessary adaptation of maternal tissues to meet metabolic demands of the periparturient animal is hypertrophy of the gastrointestinal tract (19) and is likely concurrent with increased feed intake during early lactation (198, 199). Additionally, altering the diet causes changes in intestinal and forestomach growth in adult animals. It has been well documented across species, including cattle, that increased intake is associated with greater ruminal and intestinal mass (20, 200, 201). In ruminants, increased gastrointestinal mass occurs by increased epithelial layer mass in the intestines (21) and increased epithelial and muscle layer masses in the rumen (202). The increases in small intestinal and rumen epithelial mass are most commonly observed to be due to cellular hyperplasia (21, 202) but the mechanism(s) by which intake and epithelial hyperplasia are linked have not been elucidated. However, because GLP-2 is a hormone that seems to link intake and epithelial growth in nonruminants, it provides a potential mechanism to explain the diet-induced growth of the bovine gastrointestinal tract.

Although there are no descriptions of GLP-2 in the bovine, some research has investigated ruminant enteroglucagon and GLP-1. Early evidence that enteroglucagon may be important in ruminants was found in 1972, when it was observed that GLI, primarily of gut origin, increased substantially in the early stages of lactation in cattle (203). Changing the diet of feedlot steers from 70% hay to 80% barley or sheep from alfalfa to oats increased the plasma concentrations of gut GLI, and duodenal glucose infusion into sheep increased gut GLI concentrations (204). Moreover, glucagon-immunoreactive cells are observed in all segments of the intestine of adult ruminants,

with a small number of cells in the duodenum, relatively high numbers in the jejunum and ileum, and some cells in the colon and rectum (205).

Because GLP-1 and GLP-2 are co-secreted from the L-cell, experiments investigating how dietary changes affect GLP-1 concentrations in ruminants can be used to give insight into how diet affects secretion from the L-cell. Plasma GLP-1 concentrations decreased with fasting and increased upon refeeding of cattle, with a similar trend, although not statistically significant, on ileal proglucagon expression (206). In parallel with increased feed intake, plasma GLP-1 concentrations linearly increased after calving (207), similar to the rapid increase in GLP-1 concentrations observed within the first week following parturition in goats (208). Abomasal oil infusion increases plasma concentrations of GLP-1 in cows (209-212), with unsaturated fatty acids (either mono- or poly-unsaturated) having a greater effect than saturated fatty acids or triglycerides (210, 211). Additionally, abomasal casein infusion increases plasma GLP-1 concentrations in cows (212). These results demonstrate that GLP-1 secretion in ruminants is stimulated by similar nutrients as that in nonruminants, giving credence to the possible importance of GLP-2 in ruminants. Likewise, although almost no research has been done investigating the regulation of GLP secretion in ruminants, somatostatin-28 was observed to inhibit GLP-1 secretion in sheep (213), suggesting that some regulatory elements could be similar in ruminants and nonruminants.

This indirect evidence suggests that GLP-2 increases concurrently with times of observed growth of the gastrointestinal tract, and may be a mediator of this growth. Given the importance of this peptide hormone to gastrointestinal tract maintenance in other species studied (human, pig, rat, mouse), it can be speculated that this hormone would likely play an important role in ruminants as well. Furthermore, the GLP-2 amino acid sequence is highly conserved among mammals; there is 89 to 97% homology between human, mouse, rat, bovine, and pig sequences, suggesting that this sequence is critical to mammalian metabolism (17). This evidence suggests that GLP-2 could be a key participant in growth and adaptation of intestinal tissues in cattle. However, the only direct information regarding the potential role of GLP-2 in ruminants is that proglucagon mRNA and GLP-2 protein were identified in ruminant ileum (214).

Therefore, the objectives of this dissertation are

1) Identify the existence of mRNA for proglucagon and the GLP-2 receptor in the ruminant gastrointestinal tract
2) Identify the existence of circulating intact GLP-2 in the ruminant
3) Examine the tissue distribution of mRNA for proglucagon and the GLP-2 receptor in the ruminant gastrointestinal tract
4) Identify response of GLP-2 to dietary manipulation using dietary changes in total energy intake and site of starch infusion (ruminal or abomasal)
5) Demonstrate the effect of GLP-2 on splanchnic blood flow and nutrient flux in the ruminant
6) Demonstrate the effect of GLP-2 on gastrointestinal mass in the ruminant

Figure 2.1. Tissue-specific processing of the proglucagon protein by the prohormone convertases 1/3 and 2. From Estall and Drucker (215).

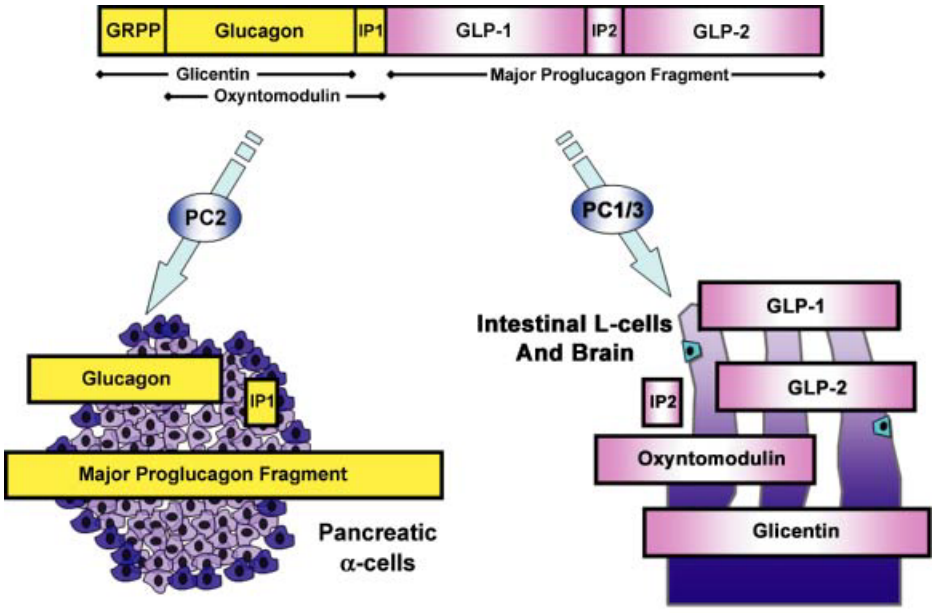


Figure 2.2. Proglucagon amino acid sequence homology from seven mammalian species (216). Major proglucagon products are indicated by bars; GRPP, glicentin-related pancreatic peptide; IP-1 and IP-2, intervening peptides; GLP-1 and GLP-2, glucagon-like peptides. Shaded residues are those conserved across species relative to the human sequence.

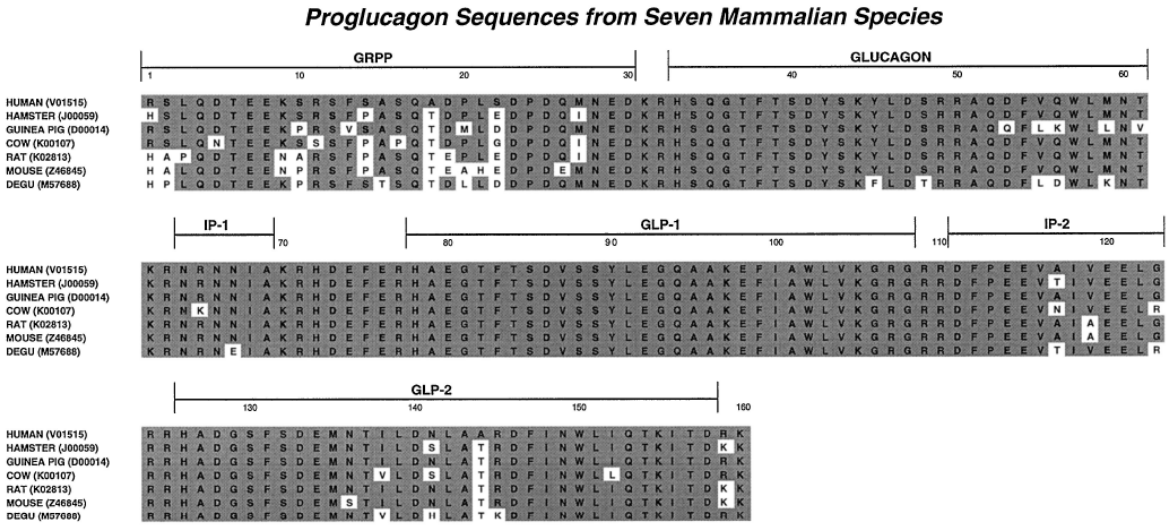


Figure 2.3. Interactions of transcription factors responsible for regulation of the proglucagon gene promoter in intestinal L-cells. From Jin (43).

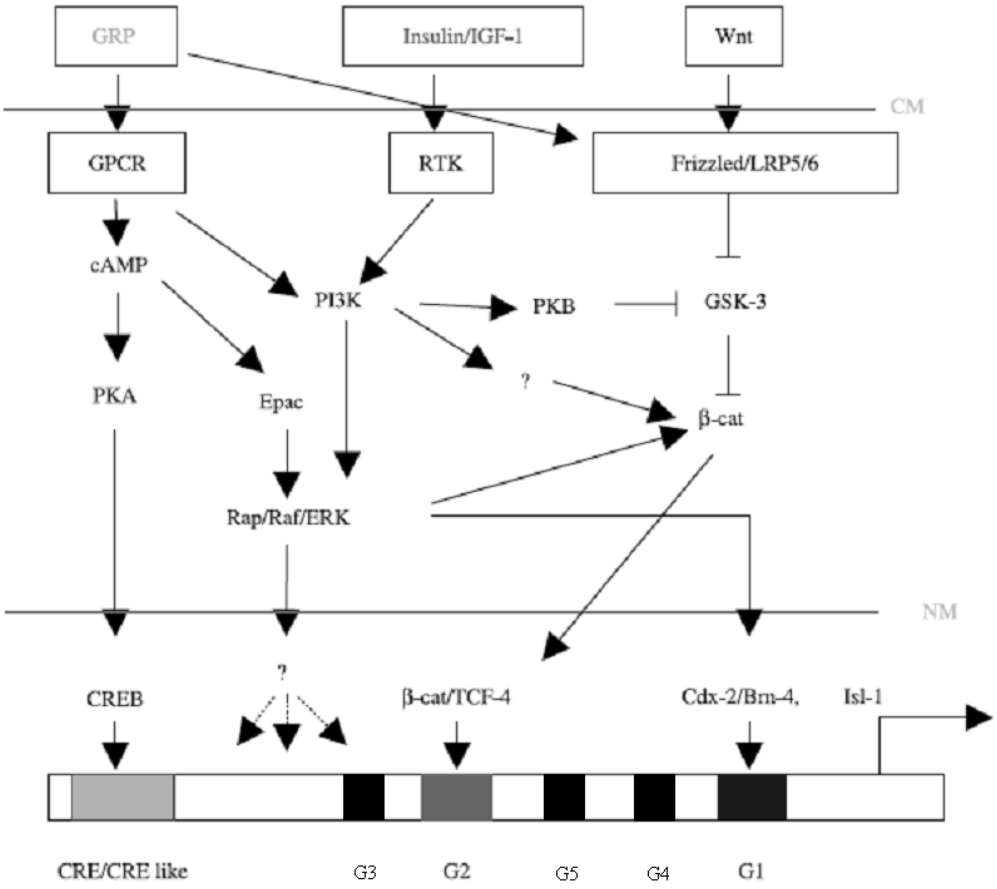
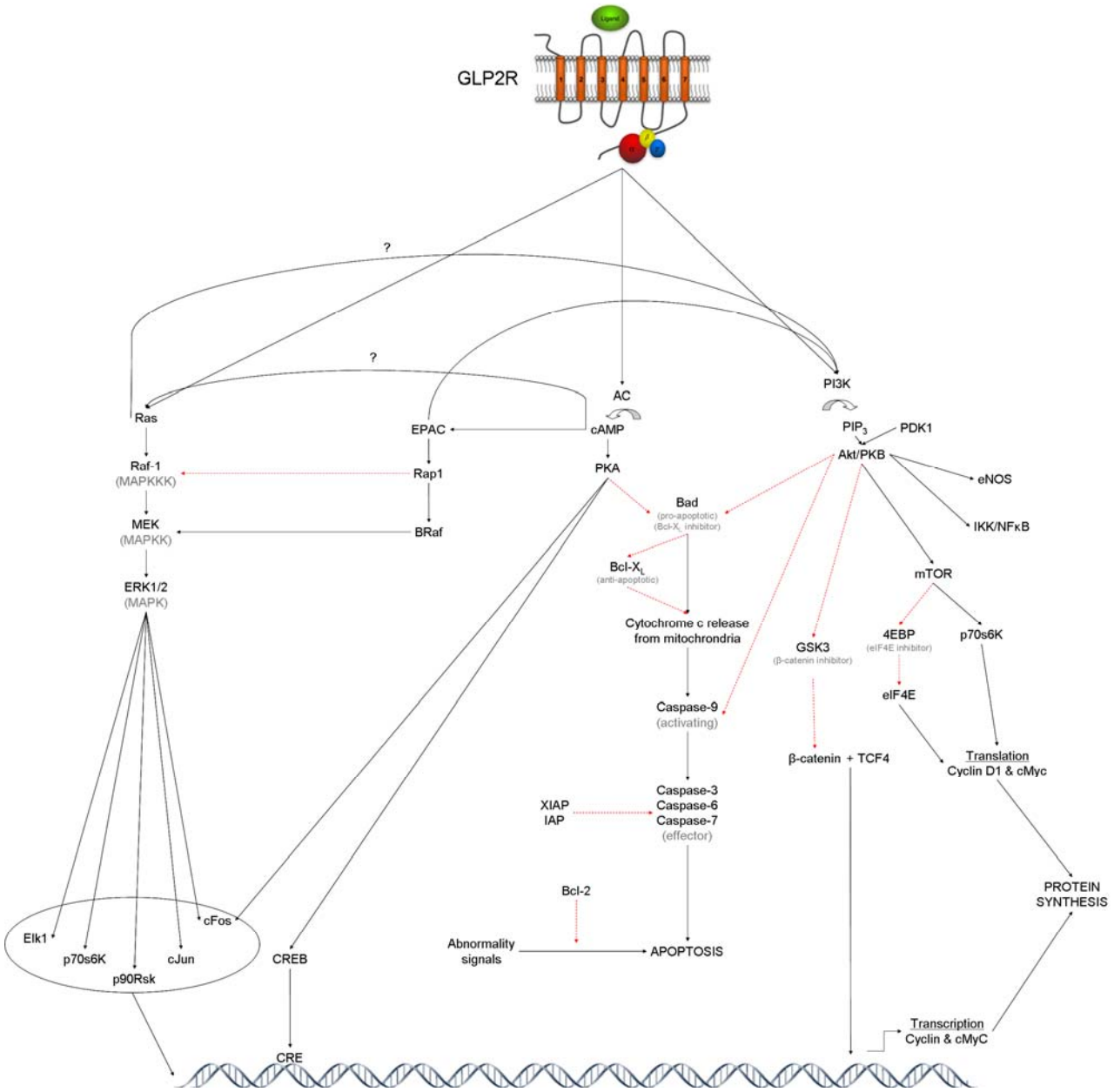


Figure 2.4. Putative signaling pathways activated by the glucagon-like peptide-2 receptor.



CHAPTER 3: ENERGY INTAKE AFFECTS PLASMA GLUCAGON-LIKE PEPTIDE-2 (GLP-2) AND MRNA FOR PROGLUCAGON AND THE GLP-2 RECEPTOR IN THE GASTROINTESTINAL TRACT OF CATTLE

INTRODUCTION

There is a well documented positive relationship between feed intake and mass of the forestomachs and intestines in cattle (20, 200, 201, 217). Increased mass generally occurs as a result of hyperplastic growth of the epithelial layer of the rumen and intestines (21, 202). However, the mechanisms by which intake and epithelial hyperplasia are linked have not been elucidated. Because the epithelial layer of the gastrointestinal tract is critical for nutrient absorption, it is important to understand how this tissue layer is regulated in response to nutrients.

In non-ruminant models, substantial evidence suggests that glucagon-like peptide-2 (GLP-2) is an important mediator of the changes in intestinal mass in response to diet amount and composition (17, 54). Secretion of GLP-2 from gastrointestinal endocrine L cells occurs in response to luminal nutrients (79-81). The most potent action of GLP-2 is increased small intestinal mass by concurrent stimulation of proliferation and inhibition of apoptosis of the intestinal epithelium (4, 6, 8). To date, no information is published describing the stimulation of GLP-2 secretion or GLP-2 action in ruminants.

The GLP-2 amino acid sequence is highly conserved among mammals; there is 89 to 97% homology between human, mouse, rat, bovine, and pig sequences, suggesting that this sequence is critical to mammalian metabolism (17). This evidence suggests that GLP-2 could be a key participant in adaptation of intestinal tissues in cattle and could potentially influence the adaptive response of ruminal epithelium to diet. Although proglucagon mRNA and GLP-2 protein have both been identified in ruminant intestine (214), the response of bovine proglucagon mRNA and GLP-2 to diet has yet to be demonstrated. This experiment was designed to determine if plasma GLP-2 and mRNA for proglucagon and the GLP-2 receptor exist in cattle and if they can be influenced by a change in dietary energy intake, which is a known stimulus for GLP-2 secretion in non-

ruminants. The hypothesis of this experiment was that increased feed intake would increase tissue proglucagon mRNA and plasma GLP-2. The objectives of this experiment were to determine if 1) cattle express proglucagon mRNA in ruminal and intestinal tissues and glucagon-like peptide-2 protein in plasma, 2) plasma concentrations of GLP-2 and gastrointestinal tissue concentrations of proglucagon mRNA change in parallel with putative ruminal and intestinal mass changes in response to intake in cattle, and 3) if changes in plasma GLP-2 concentrations coincide with changes in proglucagon mRNA in ruminal and intestinal tissues in cattle.

MATERIALS AND METHODS

Experimental design

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Four ruminally, duodenally, and ileally cannulated steers (417.2 ± 48.0 kg; mean ± SD) were utilized in this experiment. The experiment was 50 d, with 21 d for establishment of baseline variables and 29 d for collection of samples at predetermined points. For 21 d (D-21 to D-1) prior to the start of the collection period, steers were fed corn silage to meet 0.75 × net energy for maintenance (NE_M) with adequate protein and mineral supplement to meet requirements (**Low** intake). On D0, diet provided was increased to meet 1.75 × NE_M requirements (**Acute** and **Chronic** high intake). Diet ingredient and nutrient compositions are shown in Table 3.1. Steers were weighed weekly throughout the experiment (D-21, -14, -7, 0, 7, 14, 21, 29), and diet requirements were recalculated weekly on D0, 7, 14, and 21. Ruminal, duodenal, and ileal biopsies and blood samples were taken as described below at -6 and -3 d (Low), 1 and 3 d (Acute), and 7 and 29 d (Chronic) relative to the change in energy intake. Steers were housed in individual stalls in a temperature (20° C) and light-controlled (12 h light: 12 h dark) room with water available ad libitum.

Sample collection

The biopsy procedure utilized in this experiment is similar to that described in cattle for examining effects of dietary treatments on gastrointestinal endocrine parameters (206). Furthermore, a more frequent sampling protocol (-1, 0.5, 1, 2, 4, and 24 h) than the one used in this experiment (-6, -3, 1, 3, 7, and 29 d) has been demonstrated to not adversely affect animal health (218). Animals were biopsied prior to that day's feeding in a squeeze chute to minimize damage to equipment or animal. Mild sedation with xylazine (0.088 mg/kg) was utilized for animal comfort and to reduce stress. For ruminal biopsies, ruminal contents were partially or fully evacuated and 5-6 papillae sections were removed from the ventral ruminal sac using biopsy clippers. Intestinal (duodenal and ileal) biopsies were obtained by fiberoptic endoscopic biopsy by introducing an endoscope (Olympus CF, type CF-1T20L and OCV-100 camera) through the appropriate cannula. Duodenal biopsies were obtained approximately 40 to 50 cm distal to the pyloric sphincter and ileal biopsies approximately 50 to 60 cm proximal to the ileal-cecal junction. Eight 15-mg samples were taken from each site using sterilized biopsy forceps. Replicate samples were rinsed with sterile saline, frozen in liquid nitrogen, pooled into 1 sample per site, and stored at -80°C. Animals were monitored for several days following biopsy procedures for indications of infection.

Venous blood samples were obtained immediately after biopsies by jugular puncture. Aliquots of blood were placed into chilled tubes containing EDTA (10 mg/ml final concentration) with or without aprotinin (500 kallikrein inhibitory equivalents/ml), for analysis of dipeptidyl peptidase IV activity and plasma GLP-2 concentrations, respectively. Samples were centrifuged (3,000 × *g* for 10 min) to obtain plasma and were frozen at -80°C until further analysis.

Plasma GLP-2 analysis

Plasma preserved with aprotinin (300 µl) was extracted with 70 % ethanol (vol/vol, final concentration). Concentrations of GLP-2 in ethanol-extracted plasma were measured using a radioimmunoassay employing antiserum code no. 92160 and standards of human GLP-2 (proglucagon 126-158, a gift from Novo Nordisk A/S) and

monoiodinated Tyr (219) GLP-2, specific activity > 70 MBq/nmol (220). The antiserum is directed against the N-terminus of GLP-2 and therefore measures only fully processed GLP-2 of intestinal origin. Sensitivity for the assay is below 2 pmol/l, and intra-assay coefficient of variation below 6 %.

Dipeptidyl peptidase IV activity assay

Plasma dipeptidyl peptidase IV activity was determined using a colorimetric assay using *p*-nitroanaline adapted for use on a Konelab 20XT Clinical Analyzer (Thermo Scientific, Waltham, MA). Dipeptidyl peptidase IV hydrolyzes Gly-Pro-*p*-nitroanilide to Gly-Pro and *p*-nitroaniline. Briefly, 40 µl plasma was incubated with 60 µl 0.1 M TRIS buffer (0.1 M, pH 8.01; Sigma Aldrich, St. Louis, MO) and 100 µl Gly-Pro-*p*-nitroanilide (1 mM in TRIS buffer; Sigma Aldrich, St. Louis, MO). The mixture was incubated at 37°C and *p*-nitroaniline release was measured (405 nm) at 5 and 15 min. For each plasma sample, the change in *p*-nitroaniline release between the 5 and 15 minute incubations was used to determine plasma dipeptidyl peptidase activity ($nmol \cdot min^{-1} \cdot ml^{-1}$). A standard curve was generated using 0, 8, 16, 22.86, 32, and 40 nmol of *p*-nitroaniline in TRIS buffer.

Extraction of total RNA, reverse transcription, and semi-quantitative real time-PCR

Ruminal, duodenal, and ileal tissue samples were homogenized in Trizol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions to extract total RNA. The recovered RNA pellet was washed with 75% ethanol and centrifuged at 7,500 × g at 4°C for 7 minutes. The resulting pellet was resuspended in RNase/DNase-free water and stored at -80°C. Total RNA concentration was determined spectrophotometrically at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc., Waltham, MA). The quality of extracted total RNA was verified by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Total RNA (5 µg) was treated with DNase I (Amplification grade, Invitrogen, Carlsbad, CA), where 5 µg total RNA (in DNase/RNase-free water) was incubated with 1

µl 10× reaction buffer and 1 µl DNase I (1 U/µl). The mixture was incubated for 15 min at room temperature before stopping the reaction with 1 µl of 25 mM EDTA and incubation at 65°C for 10 minutes. The entire volume of DNase-treated total RNA was then used in the reverse transcription reaction using the SuperScript III First-Strand Synthesis System for RT-PCR, as suggested by the manufacturer (Invitrogen). DNase-treated total RNA was incubated with 1 µl each random hexamers (50 ng/µl) and oligo$(dT)_{20}$ (50 µM) primers for 10 minutes at 65°C before chilling on ice for 1 min. A solution containing 2 µl reaction buffer (10×), 2 µl dithiothreitol (0.1 M), 1 µl dNTP mixture (10 mM each), 4 µl $MgCl_2$ (25 mM), and 1 µl RNAse Out was added and allowed to incubate for 2 minutes at 37°C. Following the 2 minute incubation, 1 µl of Superscript III reverse transcriptase (200 U/µl) was added to the reaction mixture. The mixture was incubated at room temperature for 10 min, followed by 50 min of a 37°C incubation, and then 10 min at 65°C. The reaction was quickly chilled on ice for 1 min and was stored at -20°C until further use.

Relative abundance of mRNA for proglucagon (GCG), GLP-2 receptor (GLP2R), and for 18S rRNA were determined using semi-quantitative real-time PCR performed on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene expression assays used Custom TaqMan Primer and Probe sets that were synthesized by the Assays-by-Design service. Custom TaqMan Primer and Probe sets consists of 2 unlabeled PCR primers and TaqMan Minor Groove Binding (MGB) probe using FAM as a dye label on the 5′ end. Bovine specific nucleotide sequences were obtained from previously published sequences in Genbank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) or by performing the Basic Local Alignment Search Tool (BLAST) of the predicted bovine sequence available in Genbank with a human ortholog nucleotide sequence [GCG (Genbank NM_173916), GLP2R (Genbank XM_589370), 18S (Genbank DQ222453)]. Exon junction sites for each gene were determined with publicly available genomic data using the Ensembl Genome Browser (http://www.ensembl.org/index.html). Sequences used for primer and probe sets in this experiment are shown in Table 3.2.

Each PCR reaction consisted of 12.5 µl TaqMan Universal PCR MasterMix, No AmpErase UNG (Applied Biosystems, Foster City, CA), 1 µl cDNA template, 1.25 µl

Custom TaqMan® Assays-by-Design Primers and Probe set, and 10.25 µl DNAse/RNAse-free water. The following PCR conditions were used for the amplification and quantification: initial denaturing (95°C for 10 min), 40 cycles of a two stage amplification of denaturing (95°C for 15 s), and annealing/extension (60°C for 1 min), with a melting curve program (60 to 95°C) with a heating rate of 0.15°C/s and continuous fluorescence measurements. No-reverse transcriptase control and no-template control reactions were performed with every assay to ensure the specificity of the reaction and the absence of any contamination. Triplicate measurements of GCG, GLP2R, and 18S were made for each tissue cDNA sample.

Relative quantitation of GCG and GLP2R expression were conducted using the relative standard curve method with 18S used as an endogenous control to normalize variations in input RNA. The standard curve for each gene (GCG, GLP2R, and 18S) was generated using cDNA generated from bovine ileal tissue, which was serially diluted by 5-, 25-, 125-, 625-, 3,125-, 15,625-, and 78,125-fold. The linear range of target quantification was established to determine the appropriate amount of cDNA template to utilize in the PCR reaction. The minimal threshold (C_T) values detected by using these dilutions of cDNA were approximately 28 and 35 for the 18S and target genes, respectively. Therefore, the optimal dilutions of cDNA template generated from tissue samples used in the PCR reactions were 1-fold for GCG and GLP2R and 15,625-fold for 18S. The C_T values of 18S mRNA were statistically analyzed to ensure similarity of these values across sampling days. Semi-quantitative real-time PCR data were analyzed by normalizing GCG or GLP2R mRNA abundance to 18S abundance; 18S values used for normalization were determined in the same run as GCG or GLP2R values.

Statistical analysis

Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model included the fixed effect of day of sampling and the random effect of steer, with day included as a repeated measure with steer as the subject. Contrasts were used to determine differences among intake periods (Low, Acute high, or Chronic high) on mRNA expression and plasma variables. Additionally, a model including the fixed

effect of tissue across sampling day and steer was used to determine the difference in mRNA expression between the rumen, duodenum, and ileum. Pearson correlation coefficients were determined between steer-day observations for some parameters. Main effects of day and correlations were declared significant at $P = 0.10$ and tendencies were declared at $P = 0.15$.

RESULTS

In this experiment, measurements for plasma variables and mRNA expression were taken at -6 and -3 d for the Low intake period, at 1 and 3 d for the Acute high intake period, and at 7 and 29 d for the Chronic high intake period. Increasing energy intake from 0.75 × NE_M to 1.75 × NE_M was achieved by increasing DMI from the Low intake period to Acute high intake period (3.59 vs. 6.45 kg/d, $P < 0.0001$). Because DMI was readjusted weekly based on BW, DMI was slightly lower during the Acute high intake period than the Chronic high intake period (6.45 vs. 6.79 kg/d, $P = 0.0009$). Likewise, BW was lower during the Low intake period than the Acute high intake period (411.8 vs. 430.4 kg, $P = 0.002$), but BW was not significantly different between the Acute and Chronic high intake periods (430.4 vs. 437.2 kg, $P = 0.18$).

Plasma

Increasing energy intake from 0.75 × NE_M to 1.75 × NE_M increased plasma active GLP-2 concentrations by 37% in the Acute period ($P = 0.07$; Table 3.3), but concentrations subsequently decreased so that GLP-2 concentrations during the Chronic period were intermediate to the Acute high intake and Low intake periods and did not differ from either. Plasma DPPIV activity was not affected by an change from Low intake to Acute high intake, but plasma DPPIV activity was greater during the Chronic high intake period than the Acute high intake period ($P = 0.07$) and tended to be greater than the Low intake period ($P = 0.11$), primarily because of high DPPIV activity at D29 (Table 3.3).

Proglucagon mRNA

Proglucagon mRNA (relative to 18S rRNA) was detected in duodenal and ileal epithelial samples but not ruminal epithelial samples (Table 3.3). Duodenal and ileal proglucagon expression were differentially affected by a change in energy intake. Changing from Low intake to Acute high intake numerically decreased duodenal proglucagon mRNA expression but Chronic high intake increased duodenal proglucagon mRNA expression, resulting in higher duodenal proglucagon expression during the Chronic period of high intake compared to the Acute period of high intake ($P = 0.08$). In contrast, there was a 78% increase in ileal proglucagon mRNA expression immediately following the change from Low to Acute high intake ($P = 0.07$), but then ileal expression of proglucagon mRNA decreased slightly so that expression during the Chronic high intake period was intermediate to that in the Low intake period and the Acute high intake period and did not differ from either.

GLP-2 receptor mRNA

Expression of the GLP-2 receptor mRNA (relative to 18S rRNA) was detected in ruminal, duodenal, and ileal epithelial tissue biopsies (Table 3.3). Across all sampling days and steers, expression of GLP-2 receptor mRNA in the duodenum and ileum was approximately 39-fold greater than that in the rumen ($P < 0.0001$) but expression did not differ between the 2 intestinal sites ($P = 0.91$). Rumen GLP-2 receptor expression was variable but there was a trend for decreasing expression as animals were changed from the Low intake to Acute high level of intake and maintained at Chronic high intake; however, this effect only tended to reach significance between the Low intake and Chronic high intake periods ($P = 0.14$). Duodenal GLP-2 receptor expression was not affected by changes in energy intake level. Ileal GLP-2 receptor expression did not differ between the Low intake and Acute high intake periods, but Chronic high intake increased GLP-2 receptor expression compared to both the Acute high intake period ($P = 0.06$) and the Low intake period ($P = 0.004$).

DISCUSSION

Presence of bovine GLP-2 and mRNA for proglucagon and the GLP-2 receptor

The first objective of this experiment was to determine the existence of proglucagon and GLP-2 receptor mRNA in bovine gastrointestinal tissues and circulating plasma GLP-2. To our knowledge, this experiment is the first to report the presence of active GLP-2 in bovine plasma. The concentration of GLP-2 in bovine plasma ranged from 9 to 27 pM, which is similar to concentrations reported in rats but low compared to humans (80, 84, 221, 222). The fact that cattle have detectable active GLP-2 coupled with our observations that plasma GLP-2 concentrations change with alterations in energy intake provides evidence that GLP-2 is a nutrient-responsive hormone in ruminants.

We also extend the findings of previous research and demonstrate that mRNA for proglucagon is expressed in bovine duodenal and ileal mucosa but not ruminal tissue. Expression of proglucagon mRNA has only been previously detected in ileal tissue (214). The observation that proglucagon mRNA was not expressed in ruminal tissue agrees with the general consensus that the rumen is not a secretory organ (223). Our observed localization of proglucagon mRNA to the duodenum and ileum is consistent with data from non-ruminants (38, 57, 63). Further support of our results is that in both ruminants and non-ruminants, the L cells are found in high numbers in ileum and colon (33, 35), and L cells have also been identified in the duodenum (35, 36, 56, 57, 205, 224).

Expression of GLP-2 receptor mRNA has previously only been detected in ruminant ileal tissue (214), but our results demonstrate that mRNA for the GLP-2 receptor is additionally expressed in ruminal and duodenal tissue. The real-time PCR methodology used in this experiment is extremely sensitive (225, 226), and may be more powerful in detecting genes with low expression such as proglucagon and the GLP-2 receptor. However, it is important to note that although the GLP-2 receptor was detected in ruminal tissue, mRNA expression was substantially lower than that in the intestines and suggests that GLP-2 may not be an important mediator of ruminal growth. Our observation that the GLP-2 receptor is expressed in duodenal and ileal tissue is consistent with data from non-ruminants (5, 148, 153). Of all the segments of the small intestine, jejunum is reported to have the greatest expression of the GLP-2 receptor (148, 153);

however, this site could not be reached using the biopsy technique utilized in this experiment.

The precise cellular localization of the GLP-2 receptor remains unclear. The current consensus is that the GLP-2 receptor is not localized to the intestinal columnar absorptive cell, but instead is found in intestinal enteroendocrine cells (14, 153, 156), subepithelial myofibroblasts (5), and enteric neurons (14, 156, 157). Because we detected mRNA for the GLP-2 receptor in all of our biopsy tissues, this would most likely suggest that the biopsy samples we obtained contained subepithelial myofibroblasts, which exist in ruminal papillae (227, 228) and ruminant intestinal mucosa, or enteroendocrine cells.

Nutrient responsiveness of bovine GLP-2 and mRNA for proglucagon and the GLP-2 receptor

The second objective of this experiment was to determine if expression of proglucagon and GLP-2R mRNA and plasma GLP-2 concentrations was responsive to changes in energy intake using three periods: a Low level of energy intake (-6 and -3 d), after an Acute increase in energy intake (1 and 3 d), and after a Chronic increase in energy intake (7 and 29 d). Most experiments have investigated effects of fasting and refeeding on proglucagon mRNA expression or plasma concentrations of the glucagon-like peptides (1 and 2) rather than differences in feeding level per se. Proglucagon mRNA expression generally decreases with fasting and increases with refeeding; these effects have been observed in the jejunum (79) and ileum (53, 54, 84). This pattern is also observed with plasma concentrations of GLP-2 (84) and GLP-1 (79). In cattle, although changes in ileal proglucagon mRNA expression were not observed during or after a 48-h fast, plasma GLP-1 concentrations decreased with fasting and were restored to pre-fast levels by 8 h after refeeding (206). While the fasting/refeeding model may be expected to induce more substantial changes than the model used in the current experiment in which there is continuous digesta flow, our observations that ileal proglucagon mRNA expression and plasma concentrations of GLP-2 respond positively to increased energy intake agrees with previously reported literature. Furthermore, the changes in ileal proglucagon expression slightly precede the temporal pattern of plasma

active GLP-2 concentration and may reflect transcription of proglucagon with subsequent secretion of GLP-2 from ileal L-cells. These results suggest that ileal L cells play an important role in the early response to dietary changes whereas duodenal L cells that are consistently exposed to luminal nutrients are less responsive.

It is unclear if changes in proglucagon mRNA expression and plasma GLP-2 concentrations result from a change in total energy intake or changes in a specific nutrient flow. It is generally well accepted that GLP-2 secretion in non-ruminants is induced by mixed diets as well as carbohydrates and fats in particular, but peptides have also demonstrated a stimulatory effect (80, 229). Additionally, fermentable fiber and resistant starch increases intestinal proglucagon mRNA expression and secretion of the glucagon-like peptides in non-ruminants (53, 57, 58, 98, 230, 231), which could have important implications for the ruminant in which the diet contains of a high proportion of fiber. The effects of fermentable substrates on proglucagon mRNA and glucagon-like peptides are likely the result of SCFA production. Butyrate dose-dependently stimulated proglucagon mRNA expression in cell culture (57, 58), and similar results were observed with propionate and acetate (58). Even more interesting, the increase in proglucagon mRNA in response to SCFA was greater at lower pH (6.0 versus 7.5) (58), suggesting that greater SCFA accumulation in the intestinal tract will more strongly stimulate proglucagon transcription. Butyrate and propionate also stimulate secretion of GLP-1 from the isolated vascularly perfused rat ileum (103) at concentrations (20 mM) that are comparable to those observed in the small intestine of the fed ruminant (232). This may suggest that the high SCFA concentrations present in the ruminant intestinal tract could play an important role in stimulating the mRNA expression and subsequent release of glucagon-like peptides.

There is a relative paucity of information about nutrient-induced secretion of GLP-1 or -2 from the L cell in the ruminant. Plasma concentrations of GLP-1 in cattle are increased by abomasal infusion of lipid (209-211), although this effect was only observed at 7 d of continuous infusion and not at 1 d of infusion (212). In contrast, casein infusion increased plasma GLP-1 in cattle after 1 d but not 7 d of infusion (212). In stark contrast to non-ruminant data, plasma GLP-1 concentrations in sheep were not increased within 6 h after a concentrate meal (233) and starch infusion decreased plasma GLP-1

concentrations (212), suggesting that unlike non-ruminants, ruminant glucagon-like peptide secretion may not be very responsive to starch. Because of forestomach fermentation of dietary nutrients, proportionally less dietary starch reaches the ruminant intestine than in a non-ruminant. Experiments with ruminants have demonstrated a different response to increasing dietary starch than non-ruminants; for example, in rats dietary carbohydrate increased pancreatic α-amylase secretion (Snook, 1971), but in cattle abomasal infusion of starch hydrolysate reduced pancreatic α-amylase secretion (234). Therefore, the stimulatory effect of starch on glucagon-like peptide secretion observed in non-ruminants may be attenuated or even absent in ruminants.

There is a definitive lack of data regarding the changes in GLP-2 receptor expression in response to changes in nutrient intake in all species. In the few experiments that have examined GLP-2 receptor expression in relation to dietary intake, there is little consensus. In mice, jejunal GLP-2 receptor expression was not affected by 24-h fast nor a 24-h fast followed by a 24-h refeeding period (54). In pigs, replacement of dietary methionine with 2-hydroxy-4-methylthiobutyrate, a SCFA that can act as a methionine precursor, increased stomach expression of GLP-2 receptor but did not affect expression in the proximal or distal small intestine (235). In contrast, introduction of enteral food reduced GLP-2 receptor mRNA expression at 6 d in term and preterm piglets (236); however, the authors suggested that the rapid increase in mucosal growth that occurred with the intake of enteral nutrients was associated with a degree of unspecific dilution of intestinal proteins such as the GLP-2 receptor. Some of the difficulty in eliciting the effects of nutrient intake per se on GLP-2 receptor expression is that these possible effects cannot be separated from effects of nutrient intake on GLP-2 secretion and subsequent GLP-2 receptor expression changes in response to changes in plasma GLP-2. It is interesting to note that we observed increased expression of ileal GLP-2 receptor mRNA despite greater plasma GLP-2 concentrations, similar to observations in rats given exogenous GLP-2 (63). Therefore, if high concentrations of GLP-2 cause desensitization of the GLP-2 receptor as observed in vitro (145, 149, 170), this does not appear to affect in vivo GLP-2 receptor mRNA expression.

Dipeptidyl peptidase IV activity

Biological activity of GLP-2 is related to the amount secreted and the proportion of active GLP-2 in the blood, which is dictated by the activity of dipeptidyl peptidase IV (DPPIV), the putative enzyme responsible for deactivation of active GLP-2 (1-33) to its inactive form, GLP-2 (3-33). Because plasma DPPIV activity does not appear to be regulated by diet (237, 238), the increased plasma DPPIV activity we observed during the chronic period of high energy intake could suggest that DPPIV activity might be regulated as a second control mechanism to carefully modulate the amount of active GLP-2 available to induce gastrointestinal growth. It has been suggested that the regulation of active GLP-1 is dictated more by the activity of the membrane-bound DPPIV lining the capillaries draining the gastrointestinal tract than plasma DPPIV activity (238). However, because GLP-2 has a longer half-life than GLP-1 (81), plasma DPPIV activity may play a greater role in regulating GLP-2 biological activity.

In conclusion, we have identified that GLP-2 and its receptor exist in cattle and overall appear to function similar to other non-ruminant species in response to nutrient intake. Although the GLP-2 receptor is expressed in the rumen and thus forestomach epithelial growth could be regulated to some extent by GLP-2, expression levels were low and suggest that other factors are likely more important. Ileal tissue appears to respond to an increase in energy intake by increasing proglucagon mRNA expression and likely secretion of GLP-2, thus contributing toward greater plasma GLP-2 levels, whereas duodenal proglucagon mRNA is less responsive to changes in dietary energy intake. These data provide evidence that GLP-2 changes occur prior to intestinal mass changes observed in ruminants with increasing energy intake, and could suggest the possibility for a role for GLP-2 in at least partially mediating intestinal growth in response to increasing energy intake in cattle.

Table 3.1. Ingredient and nutrient composition of diet used in experiment.

	% of DM
Ingredient composition of diet	
Corn silage	87.7
Supplement	12.3
Ingredient composition of supplement	
Ground corn	74.0
Dicalcium phosphate	10.2
Limestone	9.50
Choice white grease	3.00
Trace mineral mix[1]	3.00
Vitamin ADE mix[2]	0.30
Nutrient Composition of Diet[3]	
DM	46.3
CP	12.7
NDF	34.7
ADF	20.8
NE_M, Mcal/kg	1.70

[1] Trace mineral mix contained 96.0-98.5% NaCl, 3500 mg/kg Zn, 2,000 mg/kg Fe, 1,800 mg/kg Mn, 370 mg/kg Mg, 350 mg/kg Cu, 100 mg/kg I, 90 mg/kg Se, and 60 mg/kg Co.

[2] Vitamin ADE mix contained 1.8 million IU/kg vitamin A, 3.6 million IU/kg vitamin D, and 227 IU/kg vitamin E.

[3] Nutrient compositions of diet were determined by analysis of composited ingredient samples of corn silage (n = 6) and supplement (n = 3).

Table 3.2. Primer and probe sets used for real-time PCR analyses of GCG, GLP2R, and 18S RNA.

Primer and Probe	Location on template (bp)	Sequence[1]	Amplicon size (bp)
GCG (Genbank NM_173916)			
Forward	457-475	5′-CTGGTGAAAGGCCGAGGAA-3′	
Probe (rev.)	478-493	5′-FAM-CTTCTGGGAAATCTCG-3′	64
Reverse	500-520	5′-GGCGGAGTTCTTCAACGATGT-3′	
GLP2R (Genbank XM_589370)			
Forward	1409-1431	5′-CCTCGCAGTATTGCTTTGCTAAT-3′	
Probe (rev.)	1432-1448	5′-FAM-TCAGCCTTCACCTCTCC-3′	61
Reverse	1452-1469	5′-CGGGCCCACTGTTTTCG-3′	
18S (Genbank DQ222453)			
Forward	548-572	5′-CCCTGTAATTGGAATGAGTCCACTT-3′	
Probe (rev.)	593-611	5′-FAM-CCAGACTTGCCCTCCAATG-3′	100
Reverse	625-647	5′-ACGCTATTGGAGCTGGAATTACC-3′	

[1] FAM = six-carboxy-fluorescein used as a reporter dye

Table 3.3. Means of plasma variables and relative abundance of proglucagon and GLP2R mRNA in steers switched from a low (d -6 and -3) to high (d 1, 3, 7, and 29) level of energy intake.

	Day							*P* =		
	Low		Acute		Chronic		SEM [1]	Low vs. Acute	Low vs. Chronic	Acute vs. Chronic
	-6	**-3**	**1**	**3**	**7**	**29**				
Plasma										
Active GLP-2, pM	14.0	10.25	15.5	17.75	12.5	16.5	2.250	0.07	0.31	0.36
DPPIV activity, nmol ml^{-1} min^{-1}	17.9	19.1	19.1	17.1	16.3	27.7	5.98	0.85	0.11	0.07
Proglucagon / 18S										
Rumen [2]	ND	ND	ND	ND	ND	ND				
Duodenum	1.80	0.84	0.65	0.82	2.09	1.48	0.710	0.31	0.42	0.08
Ileum	0.89	0.92	1.67	1.55	0.96	1.35	0.359	0.07	0.50	0.22
GLP-2 receptor / 18S										
Rumen	0.051	0.031	0.029	0.037	0.009	0.015	0.0252	0.62	0.14	0.28
Duodenum	0.47	1.17	1.01	1.48	1.34	1.62	0.650	0.49	0.28	0.70
Ileum	0.61	0.84	1.27	1.04	1.28	2.24	0.421	0.17	0.004	0.06

[1] n = 4

[2] ND= not detected

CHAPTER 4: INFLUENCE OF SITE OF SUPPLEMENTAL ENERGY ON GASTROINTESTINAL MASS AND EXPRESSION OF PROGLUCAGON AND GLP-2 RECEPTOR MRNA IN THE RUMINANT GASTROINTESTINAL TRACT

INTRODUCTION

It has been well established that in ruminants, gastrointestinal tract size is positively related to energy intake (20-22). Because the gastrointestinal tract utilizes up to 25% of the animal's daily energy requirement (1), understanding factors that control gastrointestinal tract size is important to maximizing animal productivity. There are a number of regulatory factors that are produced by the gastrointestinal tract, including hormones, peptide growth factors, and cytokines (239).

One potential mechanism linking gastrointestinal growth to increased energy intake is glucagon-like peptide-2 (GLP-2). Glucagon-like peptide-2 is a 33-amino acid hormone secreted by the enteroendocrine L-cell of the gastrointestinal tract. Expression of mRNA for proglucagon, the GLP-2 precursor, is stimulated by the presence of nutrients in the intestinal lumen (54, 55, 57, 99), and secretion of GLP-2 from the L-cell is also stimulated by luminal nutrients (50, 80, 98, 240). In non-ruminants, GLP-2 increases the growth of the mucosal epithelium of the small intestine by reducing villus apoptosis and increasing crypt proliferation, thus increasing the total mass of the small intestine (2, 6-10, 161). However, there is a paucity of data regarding GLP-2 in ruminants. Expression of mRNA for proglucagon and the GLP-2 receptor has been identified in ruminant ileum previously (214). Our laboratory has recently demonstrated expression of proglucagon mRNA in bovine duodenum and ileum and expression of GLP-2 receptor mRNA in bovine rumen, duodenum, and ileum (241). However, those results were obtained using a biopsy technique and thus had access to a limited number of gastrointestinal tissues.

Therefore, the objectives of this experiment were to 1) determine if starch infusion into the rumen or abomasum would affect gastrointestinal mass and expression of

proglucagon and GLP-2 receptor mRNA and 2) further describe the expression of proglucagon and the GLP-2 receptor mRNA in the ruminant gastrointestinal tract. Our hypotheses were that starch infusion would increase gastrointestinal mass in the region it was infused, expression of proglucagon mRNA would be greater in the distal intestinal tract compared to the proximal intestinal tract, and increased energy, provided either ruminally or abomasally, would increase proglucagon mRNA expression.

MATERIALS AND METHODS

Experimental design

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Eighteen Angus steers (260 ± 17 kg) were surgically fitted with ruminal and abomasal infusion cannulae (242) and duodenal T-cannulas (243). The experiment was a repeated random complete block design with 3 periods repeated over time, with 2 blocks of 3 steers each per period. Steers were blocked by weight and treatments were assigned randomly to steer within block so that each block had each of the three treatments represented. The experiment consisted of 3 periods of 21 d each, with 7 d for infusion adaptation.

Treatments were **Control** (water) or infusion of starch hydrolysate (**SH**; to provide an additional 20% of ME intake) into either the rumen (**R-SH**) or abomasum (**A-SH**). Water was infused into the infusion cannula site that did not receive carbohydrate infusion. During the first 7 d of the adaptation period, amount of starch infused was incrementally increased each day so that by d 7 the full starch infusion amount was attained. The full amount of starch treatment was infused for the next 12 to 14 d. Starch hydrolysate stock solution (25% DM) was prepared by heating cornstarch and tap water with heat-stable α-amylase (Spezyme Delta-AA Genecor, Rochester, NY) and 25 g $CaCl_2$ to partially hydrolyze the starch (234, 244). Starch hydrolysate stock solution was mixed with tap water to provide each steer with an additional 20% of daily ME intake as starch in a total infusate quantity of 5,500 g/d. The solution was continuously stirred while being infused at a rate of 229 mL/h over 22.5 h out of each experimental day.

Beginning at least 21 d prior to the experimental period and throughout the experimental period, steers were fed alfalfa cubes (17.8% crude protein and 1.31 Mcal of NE_M/kg, DM basis) at 1.33 × NE_M. Additional dietary nutrient analysis has been previously reported (Liao et al., 2008). Feed was offered in 12 meals daily (2 h intervals) using automatic feeders (Ankom Co., Fairport, NJ). Once daily, steers were fed a supplement of 40 g/d trace mineralized salt (245) and 20 g of poloxolene (Phibro Animal Health, Ridgefield Park, NJ) daily to minimize incidence of bloat. Steers were individually housed in metabolism tie stalls (1.2 × 2.4 m^2) in a temperature-controlled room (20° C) with water available ad libitum.

Sample collection

Steers were slaughtered in the University of Kentucky UDSA-approved slaughter facility on either d 19 or 21. Steers were stunned and exsanguinated, and immediately eviscerated. Forestomachs (reticulorumen, omasum, and abomasum), intestines, and liver were obtained. Forestomachs were separated from intestines and were stripped of all connective and adipose tissues. Reticulorumen, omasum, and abomasum were separated and digestive contents were emptied. Tissue samples from the rumen, omasum, and abomasum were obtained from the cranial ventral sac, the large order I and II omasal plies, and the antral region, respectively. All forestomach samples were rinsed extensively with ice-cold saline, and epithelial tissue was harvested by scraping with a glass slide (abomasal tissue) or stainless steel scraper (ruminal and omasal tissue) over an ice-cold tray. Epithelial tissue was weighed, partitioned into tubes for RNA extraction, and homogenized before snap freezing. Two g of epithelial tissue were homogenized in 20 ml of ice-cold Tri-Reagent (Molecular Research Center, Cincinnati, OH) for RNA extraction. Remaining whole tissue pieces were snap frozen. All tissue samples were stored at -80°C.

Small and large intestines and cecum were separated from the mesentery, divided, and lengths were determined by looping the intestine across a wet stationary board, fitted with pegs at 2-m increments, without tension to minimize stretching. Based on the total measured length, the small intestine was divided at the midpoint into proximal and distal

sections. One-meter intestinal sections were then excised from the duodenum (0.5 – 1.5 m distal to the pyloric sphincter), jejunum (0.5 m to either side of the midpoint of the proximal small intestine), and ileum (0.5 m to either side of the midpoint of the distal small intestine). A one meter section of colon (0.5 m to 1.5 m distal of the ileocecal junction) was excised from the large intestine. Excised sections were immediately cut into two 0.5-m segments, gently stripped of digesta, everted, rinsed extensively with ice-cold physiological saline, blotted dry, and weighed. One section was snap frozen and epithelial tissue from other 0.5-m segment was harvested with a glass slide and preserved for RNA and protein isolation as described previously. After removal and weighing of remaining epithelial tissue and fat, the 0.5-m segment was reweighed to obtain a weight for non-epithelial tissue (primarily muscle).

All sampled tissues were weighed, processed, and frozen within approximately 45 minutes of exsanguination. After sections were removed for sampling, remaining forestomachs (reticulorumen, omasum, and abomasum), intestines (small and large), cecum, and liver were rinsed with warm tap water to remove any digesta or debris, allowed to drip dry, and were weighed. To obtain whole organ weights, weights of sampled sections and remaining organ were added together. Weights of all other components (adipose, connective tissue, hide, remaining organs, and carcass) were obtained to calculate whole body weight.

Extraction of total RNA, reverse transcription, and semi-quantitative real time-PCR

Total RNA was extracted from homogenized epithelial tissue samples according to manufacturer's instructions (Tri-Reagent; Molecular Research Center, Cincinnati, OH). The recovered RNA pellet was washed with 75% ethanol and centrifuged at 7,500 × g at 4°C for 7 minutes. The resulting pellet was resuspended in RNase/DNase-free water and stored at -80°C. Total RNA was purified by a column-based kit (RNeasy Mini Kit, Qiagen, Valencia, CA). Resulting purified total RNA was eluted with 60 µl of DNase/RNase-free water. Total RNA concentration was determined spectrophotometrically at 260 nm using a Nanodrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc., Waltham, MA). The quality of extracted total RNA was

verified by analysis with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Total RNA (5 µg) was treated with DNase I (Amplification grade, Invitrogen, Carlsbad, CA), where 5 µg total RNA (in DNase/RNase-free water) was incubated with 1 µl 10× reaction buffer and 1 µl DNase I (1 U/µl). The mixture was incubated for 15 min at room temperature before stopping the reaction with 1 µl of 25 mM EDTA and incubation at 65°C for 10 minutes. The entire volume of DNase-treated total RNA was then used in the reverse transcription reaction using the SuperScript III First-Strand Synthesis System for RT-PCR, as suggested by the manufacturer (Invitrogen). DNase-treated total RNA was incubated with 1 µl each random hexamers (50 ng/µl) and oligo$(dT)_{20}$ (50 µM) primers for 10 minutes at 65°C before chilling on ice for 1 min. A solution containing 2 µl reaction buffer (10×), 2 µl dithiothreitol (0.1 M), 1 µl dNTP mixture (10 mM each), 4 µl $MgCl_2$ (25 mM), and 1 µl RNAse Out was added and allowed to incubate for 2 minutes at 37°C. Following the 2 minute incubation, 1 µl of Superscript III reverse transcriptase (200 U/µl) was added to the reaction mixture. The mixture was incubated at room temperature for 10 min, followed by 50 min of a 37°C incubation, and then 10 min at 65°C. The reaction was quickly chilled on ice for 1 min and was stored at -20°C until further use.

Relative abundance of mRNA for proglucagon (GCG), GLP-2 receptor (GLP2R), and for 18S rRNA were determined using semi-quantitative real-time PCR performed on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene expression assays used Custom TaqMan Primer and Probe sets that were synthesized by the Assays-by-Design service. Custom TaqMan Primer and Probe sets consists of 2 unlabeled PCR primers and TaqMan Minor Groove Binding (MGB) probe using FAM as a dye label on the 5′ end. Bovine specific nucleotide sequences were obtained from previously published sequences in Genbank (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD) or by performing the Basic Local Alignment Search Tool (BLAST) of the predicted bovine sequence available in Genbank with a human ortholog nucleotide sequence [GCG (Genbank NM_173916), GLP2R (Genbank XM_589370), 18S (Genbank DQ222453)]. Exon junction sites for each gene were determined with publicly available genomic data using the Ensembl

Genome Browser (http://www.ensembl.org/index.html). Sequences used for primer and probe sets in this experiment are shown in Table 4.1.

Each PCR reaction consisted of 12.5 µl TaqMan Universal PCR MasterMix, No AmpErase UNG (Applied Biosystems, Foster City, CA), 1 µl cDNA template, 1.25 µl Custom TaqMan® Assays-by-Design Primers and Probe set, and 10.25 µl DNAse/RNAse-free water. The following PCR conditions were used for the amplification and quantification: initial denaturing (95^{o}C for 10 min), 40 cycles of a two stage amplification of denaturing (95^{o}C for 15 s), and annealing/extension (60^{o}C for 1 min), with a melting curve program (60 to 95^{o}C) with a heating rate of 0.15^{o}C/s and continuous fluorescence measurements. No-reverse transcriptase control and no-template control reactions were performed with every assay to ensure the specificity of the reaction and the absence of any contamination. Triplicate measurements of GCG, GLP2R, and 18S were made for each tissue cDNA sample.

Relative quantitation of GCG and GLP2R expression were conducted using the relative standard curve method with 18S used as an endogenous control to normalize variations in input RNA. The standard curve for each gene (GCG, GLP2R, and 18S) was generated using a pooled cDNA sample generated from a composite of 1 µl each of duodenal, jejunal, and ileal cDNA from all animals in the experiment, which was serially diluted by 5-, 25-, 125-, 625-, 3,125-, 15,625-, and 78,125-fold. The linear range of target quantification was established to determine the appropriate amount of cDNA template to utilize in the PCR reaction. The minimal threshold (C_T) values detected by using these dilutions of cDNA were approximately 28 and 32 to 35 for the 18S and target genes, respectively. Therefore, the optimal dilutions of cDNA template generated from tissue samples used in the PCR reactions were 1-fold for GCG and GLP2R and 15,625-fold for 18S. The C_T values of 18S mRNA were statistically analyzed to ensure similarity of these values across treatment. Semi-quantitative real-time PCR data were analyzed by normalizing GCG or GLP2R mRNA abundance to 18S abundance; 18S values used for normalization were determined in the same run as GCG or GLP2R values.

Products from PCR were gel purified according to manufacturer's instructions (PureLink Quick Gel Extraction Kit, Invitrogen) and submitted for DNA sequencing.

Approximately 250 µl of pooled PCR reaction mixture was eletrophoresed on a 1.5% agarose slab gel. Under UV light, a single cDNA band of the correct size was excised and placed into a sterile 1.5-ml microcentrifuge tube. The gel was dissolved with gel solubilization buffer and the cDNA was extracted with an extraction column and washed with washing buffer. The column-bound cDNA was then eluted using 50 µl of DNase/RNase-free water. The purified real-time PCR products were sequenced by the University of Florida DNA Sequencing Core Laboratory using the appropriate forward and reverse primers. Resulting sequences were then compared to the expected sequence to validate the real-time RT-PCR method.

Statistical analysis

Whole animal measures were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC.). The model included treatment as a fixed effect and period, block(period), and treatment*block(period) as random effects. Tissue measures were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC.). The model included treatment, tissue, and their interaction as fixed effects and period and block(period) as random effects. Forestomach and intestinal tissues were analyzed separately using this model. Repeated measures were done on tissues with the subject as treatment*block(period). When a significant tissue effect was detected for mRNA expression, means were compared using the Tukey-Kramer multiple comparison test. To determine treatment effects, orthogonal single degree of freedom contrasts were used to determine the effects of Control versus SH (average of R-SH and A-SH) and the effects of R-SH versus A-SH. Because there were no treatment × tissue interactions, treatment and tissue means are presented separately. A model including tissue across treatment and steer was used to determine the difference in mRNA expression between the forestomach and intestinal sampling sites. Pearson correlation coefficients were determined between observations within a tissue site for some parameters. Main effects of treatment (model for whole animal measures) or treatment and tissue (model for tissue measures) and correlations were declared significant at $P = 0.05$ and tendencies were declared at $P = 0.10$.

One animal from the first block was excluded from all analyses due to removal from the experiment because of poor adaptation to the ruminal infusion treatment. Therefore, $n = 6$ for control and abomasal treatments but $n = 5$ for ruminal treatment. For measures of intestinal section weight and proportions of epithelial and non-epithelial tissue, data from the first block were excluded because fat-free tissue weights were not obtained; therefore reported data are obtained from 4 animals on each treatment across 2 blocks.

RESULTS

Treatment effects on visceral tissue mass

Infusion of SH, either ruminally or abomasally, tended to increase live BW and empty BW ($P = 0.11$ and $P = 0.15$, respectively; Table 4.2) compared to Control. Wet tissue mass of the reticulorumen was not affected by treatment. SH did not increase wet reticulorumen mass versus Control, but A-SH numerically increased reticulorumen mass (Table 4.2, $P = 0.16$) compared to R-SH. Infusion of SH (versus Control) did not affect omasal mass, but A-SH increased omasal mass compared to R-SH ($P = 0.03$). Infusion of SH tended to increase abomasal mass ($P = 0.12$). Small intestinal mass was increased by SH ($P = 0.003$) compared to Control, primarily because A-SH increased small intestinal mass compared to R-SH ($P = 0.0009$). Wet tissue mass of the large intestine and liver were unaffected by treatment. Although SH did not increase small intestinal length versus Control, A-SH tended to increase small intestinal length versus R-SH ($P = 0.13$). Large intestinal length was not affected by treatment.

On an empty BW (EBW) basis, SH did not increase reticulorumen or omasal mass versus Control, but A-SH increased reticulorumen and omasal mass (Table 4.3, $P = 0.08$ and $P = 0.01$, respectively) compared to R-SH. Abomasal mass was not affected by treatment. Infusion of SH increased small intestinal mass ($P = 0.09$; Table 4.3), primarily because A-SH increased small intestinal mass by 18% compared to R-SH ($P = 0.002$). Large intestinal mass was not affected by treatment. Liver weight was reduced by SH compared to Control ($P = 0.04$, Table 4.3). Infusion of A-SH increased small intestinal length (cm/kg EBW) compared to R-SH ($P = 0.09$, Table 4.3), but large intestinal length was not affected by treatment.

Infusion of SH increased small intestinal mass:length (g/m) versus Control ($P = 0.08$; Table 4.4), but large intestinal mass:length was not affected by treatment. Increased overall small intestinal mass:length was likely the result of 2 different effects of starch infusion. Infusion of A-SH (versus R-SH) increased proximal intestine fat-free section weights (duodenum and jejunum) by 27-30%, although this was only statistically significant in the jejunum ($P = 0.06$). Increased proximal intestine fat-free section weights with A-SH were a result of a 25-30% increase in duodenal and jejunal epithelium weight ($P = 0.07$ and $P = 0.14$, respectively) with lesser increases in non-epithelial weight. A different effect was seen in the ileum, where SH (compared to Control) increased fat-free section weight of the ileum by 34% ($P = 0.03$) by increasing the weights of non-epithelial tissue by 34% ($P = 0.03$) and epithelial tissue by 26% ($P = 0.14$) compared to control steers. In the colon, although fat-free section mass and epithelial tissue mass were unaffected by treatment, SH tended to increase non-epithelial mass ($P = 0.08$; Table 4.4) compared to Control.

Gastrointestinal distribution of proglucagon and GLP-2 receptor mRNA

Proglucagon mRNA was detected in all 7 tissues analyzed (Figure 4.1A and B). However, intestinal tissue (duodenum, jejunum, ileum, and colon) expression of GCG mRNA (relative to 18S rRNA) was approximately 5000-fold greater ($P < 0.0001$) than forestomach expression (rumen, omasum, and abomasum). Within the forestomachs, abomasal expression of GCG mRNA was greater than ruminal and omasal expression ($P = 0.004$; Figure 4.1A). Within intestines, duodenal GCG mRNA expression was less than that in the jejunum, ileum, and colon ($P = 0.0005$; Figure 4.1B) but expression in distal intestinal segments did not differ from each other.

Expression of GLP2R mRNA was detected in all 7 tissues analyzed (Figure 4.1C and D). However, intestinal tissue (duodenum, jejunum, ileum, and colon) expression of GLP2R mRNA (relative to 18S rRNA) was approximately 49-fold greater ($P < 0.0001$) than forestomach expression (rumen, omasum, and abomasum). Within the forestomachs, abomasal expression of GLP2R mRNA was greater than ruminal and omasal expression ($P < 0.0001$; Figure 4.1C). Within intestines, expression of GLP2R

mRNA was greatest in the ileum and lowest in the colon ($P = 0.01$; Figure 4.1D), and jejunal and ileal expression of GLP2R mRNA did not differ from either ileum or colon.

Expression of GCG and GLP2R mRNA were positively correlated in the jejunum ($r^2 = 0.59$, $P = 0.0003$), ileum ($r^2 = 0.28$, $P = 0.02$), and colon ($r^2 = 0.51$, $P = 0.001$), but not in duodenum or any of the forestomachs (rumen, omasum, abomasum).

Treatment effects on proglucagon and GLP-2 receptor mRNA

Proglucagon mRNA expression in the rumen or omasum was not affected by treatment (Table 4.5). Although SH (versus Control) did not affect GCG mRNA expression in the abomasum, A-SH tended to decrease abomasal GCG mRNA expression ($P = 0.09$; Table 4.5) compared to R-SH. Ruminal GLP2R mRNA expression was not affected by treatment. Omasal GLP2R mRNA expression tended to be greater with A-SH than R-SH ($P = 0.08$; Table 4.5). Abomasal GLP2R mRNA expression was not affected by treatment.

Duodenal GCG mRNA expression tended to be lower with SH ($P = 0.06$; Table 4.5) compared to Control. Treatment did not affect GCG mRNA expression in jejunum, ileum, or colon. Duodenal GLP2R mRNA expression was greater with R-SH than A-SH ($P = 0.0003$, Table 4.5), but GLP2R mRNA expression in jejunum, ileum, and colon was not affected by treatment.

DISCUSSION

Treatment effects on visceral tissue mass

It has been well established that gastrointestinal mass is positively related to energy intake (20). In this experiment, we found that A-SH increased mass (EBW-basis) of the forestomachs compared to R-SH. Despite greater energy infusion, steers infused with R-SH had the lowest reticulorumen and omasal masses (EBW-basis) of all three treatment groups. In contrast to our results, McLeod et al. (246) found that masses of the forestomach organs were increased by R-SH compared to A-SH. It is plausible that in the current experiment, infusion of starch into the rumen adversely affected the ruminal

environment to reduce fiber digestibility (247, 248). Indeed, one animal was removed from the experiment because of poor adaptation to R-SH treatment. The conflicting results of the current experiment and that of McLeod et al. (246) may also be related to the experimental differences in length of infusion periods; the 35-d infusion period used in the experiment of McLeod et al. (246) could have allowed for a greater time for the ruminal environment to adapt to the increased starch load and any effects of digestibility depression on forestomach mass may have been overcome by the time that mass measurements were taken. Finally, differences in diet may have also contributed to these differing results.

Infusion of A-SH increased small intestinal mass compared to R-SH, but both A-SH and R-SH starch infusion increased intestinal mass:length (g/m). Infusion of SH, regardless of site, increased mass of the ileum by parallel increases in epithelial and non-epithelial mass. In contrast, A-SH increased mass of the proximal intestine (duodenum and jejunum) by disproportionately increasing epithelial mass compared to non-epithelial mass. Similar increases in jejunal and ileal section masses were observed in response to starch infusion in another experiment (246). Several researchers have concluded that the small intestinal growth observed with increasing energy intake is primarily the result of hyperplastic growth of the mucosal compartment (21, 202). Additionally, increased dietary energy in the form of starch increased villus height of the proximal (duodenum and jejunum) intestinal mucosa in cattle and goats (249, 250). A variety of hormonal and trophic factors, including GLP-2, increase intestinal epithelial proliferation in response to luminal nutrients (239, 251-253). Because the hallmark of GLP-2 action is increased small intestinal mass resulting from small intestinal mucosal growth, we thought that GLP-2 could potentially be an important mediator of ruminal intestinal growth in response to luminal nutrients. However, the gastrointestinal distribution of GLP-2 and its receptor had not yet been fully described in ruminants.

Gastrointestinal distribution of proglucagon and GLP-2 receptor mRNA

To our knowledge, this is the first experiment to fully describe the distribution of GCG and GLP2R mRNA across the ruminant gastrointestinal tract. Proglucagon mRNA

and protein has been previously detected in ruminant ileum, but not other tissues (214). Because the real-time RT-PCR technique used in this experiment is highly sensitive and can detect very low expression of mRNA (225, 226), we were able to identify expression of both GCG and GLP2R mRNA in all 7 measured tissues.

Expression of GCG mRNA in the rumen and omasum is low enough to be considered negligible, and abomasal expression was greater but still very low. Glucagon-immunoreactive cells have been observed in the abomasum of ruminants (205, 224), but were not detected in the rumen or omasum (224). It is important to note that although mRNA for proglucagon was detected in rumen and omasum, expression levels are extremely low and it is unlikely that these tissues would contribute toward circulating glucagon-like peptides. The majority of circulating glucagon-like peptides are presumably secreted from L-cells of the intestinal tract.

Intestinal expression of GCG mRNA was substantially greater than forestomach expression, and within the intestinal segments expression was lower in the duodenum compared to jejunum, ileum, and colon. This distribution in the intestinal tract agrees with data describing the expression patterns of GCG mRNA and glucagon-like positive cells in non-ruminants (33, 35, 36, 56, 57) and also agrees with data describing the distribution of glucagon-like positive cells in ruminants (205, 224).

Expression of GLP2R mRNA is extremely low in the rumen and omasum, and may suggest that GLP-2 does not significantly affect growth or function of the ruminant forestomachs. Expression of GLP2R mRNA in the abomasum is greater than that in the rumen and omasum, but still substantially lower than intestinal expression. However, GLP2R mRNA expression is also low in the stomach of non-ruminants (148, 153), yet GLP-2 has been demonstrated to inhibit antral motility and gastric acid secretion in the stomach (254, 255). Thus, it is possible that despite low GLP2R mRNA expression, GLP-2 may have a physiological role in the abomasum of cattle.

In this experiment, GLP2R mRNA expression was highest in the ileum and lowest in the colon, with the duodenum and jejunum intermediate to the two. This agrees with data in rodents in that GLP2R expression is generally lowest in the stomach, intermediate in the duodenum, and highest in the jejunum, with ileum and colon expression similar to either duodenal or jejunal expression (148, 153). The distribution of the receptor for

GLP-2 corresponds to the tissues that exhibit the greatest growth response to exogenous GLP-2 administration (4, 10).

Treatment effects on proglucagon and GLP-2 receptor mRNA

Because GLP-2 secretion is increased by luminal nutrients in non-ruminants (80, 111), we hypothesized that infusion of additional energy as starch hydrolysate would increase proglucagon mRNA expression in ruminants. Furthermore, we hypothesized that A-SH may be more stimulatory than R-SH, which might be expected to increase microbial protein flow to the intestine, because fat and carbohydrate has been reported to be more effective in stimulating GLP-2 secretion than protein (80, 111). However, in contrast to our hypothesis, A-SH reduced GCG mRNA expression in the abomasum and duodenum and did not affect GCG mRNA expression in other gastrointestinal tissues.

Regulation of GLP2R mRNA expression is not well-described in the literature. Although A-SH tended to increase omasal GLP2R expression compared to R-SH, the expression of GLP2R is so low in this tissue that the biological significance of this result is questionable. In this experiment, A-SH decreased duodenal GLP2R expression compared to R-SH, whereas expression in other tissues was unchanged by infusion of starch. This coincides with the apparent inhibitory effect of A-SH on duodenal GCG mRNA expression. Additionally, we did not observe any significant correlations between GCG or GLP2R mRNA expression and fat-free weight or epithelial weight of intestinal sections.

We had anticipated greater changes in proglucagon and GLP-2 receptor mRNA in response to treatment, but tissues were sampled approximately 20 d after initiation of infusion treatments, a time when a transition in regulatory signals may have passed. However, our aim was to also measure changes in tissue mass thus an extended infusion period was required. Previous research in our laboratory has demonstrated that ileal GCG mRNA expression and plasma active GLP-2 concentrations increase within 1 and 3 d of increased energy intake, but these effects are not apparent after 7 or 29 d (241). Additionally, response and subsequent action of GLP-2 is relatively rapid, as small intestinal mass changes are evident by 6 d (4, 12), and increases in intestinal protein mass

can be detected as early as 4 h after exogenous GLP-2 administration (13). Furthermore, although proglucagon mRNA is increased by starch in non-ruminants (80, 111), ruminants seem to exhibit a different response to dietary starch. In contrast to non-ruminants, concentrations of plasma GLP-1 (the hormone co-secreted with GLP-2) are either unchanged or decreased after a high concentrate meal or starch infusion (212, 233), and lipid or casein infusion is more stimulatory to GLP-1 secretion in cattle (209, 210, 212).

In conclusion, infusion of additional energy increased small intestinal mass:length and ileal section weight by increasing both the epithelial and non-epithelial tissue fractions. Infusion of starch into the abomasum increased total small intestinal mass, in part due to an increase in jejunal mass as a result of epithelial tissue growth. In ruminants, forestomach expression of both GCG and GLP2R mRNA is detectable but is substantially lower than intestinal expression. Distribution of GCG mRNA increased from the duodenum to the mid-intestine but did not differ among jejunum, ileum, and colon, whereas distribution of GLP2R mRNA was greatest in ileum and least in colon with duodenum and jejunum intermediate to the two. Data from this experiment are important to describe the distribution of proglucagon and GLP-2 receptor mRNA in order to establish the possible role of GLP-2 in the ruminant.

Table 4.1. Primer and probe sets used for real-time PCR analyses of GCG, GLP2R, and 18S RNA.

Primer and Probe	Location on template (bp)	Sequence[1]	Amplicon size (bp)
GCG (Genbank NM_173916)			
Forward	457-475	5′-CTGGTGAAAGGCCGAGGAA-3′	
Probe (rev.)	478-493	5′-FAM-CTTCTGGGAAATCTCG-3′	64
Reverse	500-520	5′-GGCGGAGTTCTTCAACGATGT-3′	
GLP2R (Genbank XM_589370)			
Forward	1409-1431	5′-CCTCGCAGTATTGCTTTGCTAAT-3′	
Probe (rev.)	1432-1448	5′-FAM-TCAGCCTTCACCTCTCC-3′	61
Reverse	1452-1469	5′-CGGGCCCACTGTTTTCG-3′	
18S (Genbank DQ222453)			
Forward	548-572	5′-CCCTGTAATTGGAATGAGTCCACTT-3′	
Probe (rev.)	593-611	5′-FAM-CCAGACTTGCCCTCCAATG-3′	100
Reverse	625-647	5′-ACGCTATTGGAGCTGGAATTACC-3′	

[1] FAM = six-carboxy-fluorescein used as a reporter dye

Table 4.2. The effect of additional starch (0 or 20% of ME) and site of starch infusion (ruminal or abomasal) on visceral organ mass and intestinal length of growing beef steers.

Starch	0%	20% ME (SH)			*P* =	
Item	Control	R-SH	A-SH	SEM[1]	Control versus SH	R-SH versus A-SH
Weight, kg						
Live BW	266	279	274	13.7	0.11	0.50
Empty BW	214	223	221	13.9	0.15	0.73
Rumen/ Reticulum	6.97	6.98	7.68	0.346	0.37	0.16
Omasum	2.59	2.23	2.75	0.251	0.58	0.03
Abomasum	1.12	1.24	1.34	0.094	0.12	0.41
Small Intestine	3.05	3.15	3.66	0.155	0.003	0.0009
Large Intestine	1.95	2.03	2.34	0.327	0.30	0.27
Liver	3.63	3.51	3.46	0.198	0.26	0.77
Length, m						
Small Intestine	28.0	26.1	29.4	1.52	0.92	0.13
Large Intestine	5.64	5.10	5.40	0.283	0.28	0.47

[1] $n = 6$ except for R-SH treatment where $n = 5$

Table 4.3. The effect of additional starch (0 or 20% of ME) and site of starch infusion (ruminal or abomasal) on visceral organ mass and intestinal length as a % of empty body weight of growing beef steers.

Starch	0%	20% ME (SH)			*P* =	
Item	Control	R-SH	A-SH	SEM[1]	Control versus SH	R-SH versus A-SH
Organ, % EBW						
Rumen/ Reticulum	3.26	3.04	3.53	0.205	0.92	0.08
Omasum	1.22	1.01	1.27	0.166	0.23	0.01
Abomasum	0.53	0.55	0.62	0.062	0.15	0.18
Small Intestine	1.43	1.41	1.67	0.051	0.09	0.002
Large Intestine	0.93	0.93	1.11	0.222	0.47	0.28
Liver	1.71	1.56	1.57	0.065	0.04	0.91
Length: EBW, cm / kg						
Small Intestine	13.2	11.5	13.4	0.83	0.42	0.09
Large Intestine	2.57	2.24	2.37	0.153	0.18	0.55

[1] n = 6 except for R-SH infusion treatment where n = 5

Table 4.4. The effect of additional starch (0 or 20% of ME) and site of starch infusion (ruminal or abomasal) on mass of intestinal sections of growing beef steers.

	0 % ME	20 % ME (SH)		SEM[1]	*P* =	
	Control	R-SH	A-SH		Control versus SH	R-SH versus A-SH
Mass: Length, g/ m						
Small Intestine	110	124	126	6.7	0.08	0.88
Large Intestine	352	393	397	50.6	0.33	0.93
Duodenum						
Fat-free section mass, g	50.2	54.0	68.8	4.77	0.30	0.24
Epithelial mass, g	30.8	31.2	40.5	2.05	0.24	0.07
Non-epithelial mass, g	22.9	24.9	30.5	1.95	0.27	0.26
Jejunum						
Fat-free section mass, g	38.4	37.4	48.8	2.12	0.32	0.06
Epithelial mass, g	26.2	24.9	31.2	1.79	0.60	0.14
Non-epithelial mass, g	16.5	17.3	19.2	1.24	0.49	0.51
Ileum						
Fat-free section mass, g	39.6	55.5	50.5	2.45	0.03	0.43
Epithelial mass, g	26.2	33.6	32.3	2.09	0.14	0.79
Non-epithelial mass, g	17.7	26.3	21.1	1.12	0.03	0.07
Colon						
Fat-free section mass, g	90.4	104.8	130.1	12.15	0.32	0.42
Epithelial mass, g	33.7	39.7	50.0	4.08	0.22	0.32
Non-epithelial mass, g	41.3	62.7	81.0	7.37	0.08	0.34

[1] For mass:length measures, $n = 6$ except for R-SH treatment where $n = 5$. For mass measures of individual intestinal sections, $n = 4$ for all treatments.

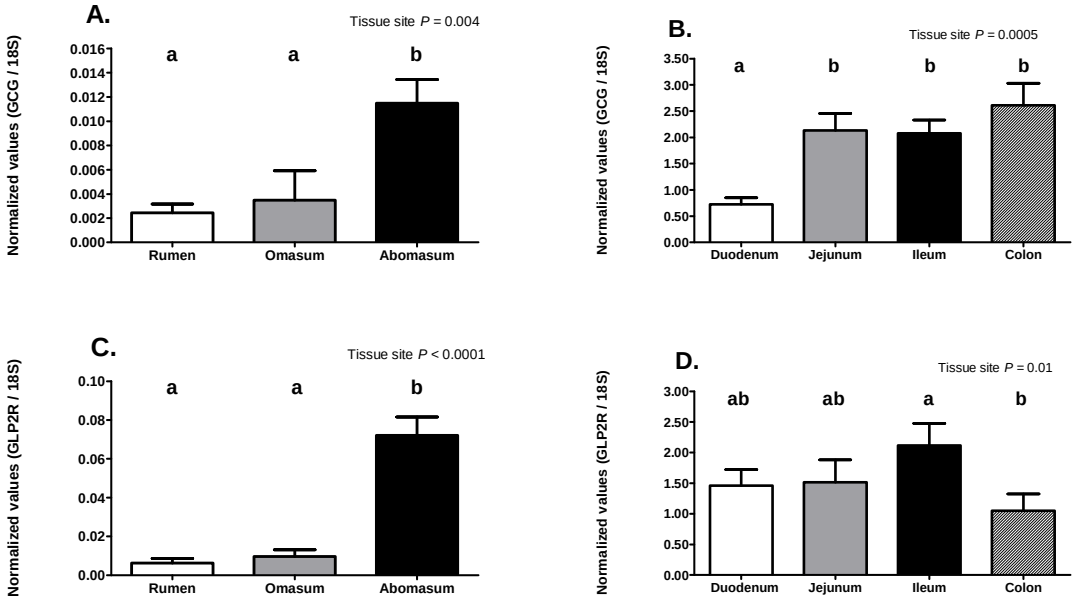
Table 4.5. The effect of additional starch (0 or 20% of ME) and site of starch infusion (ruminal or abomasal) on expression of GCG and GLP2R mRNA in forestomach and intestinal tissues of growing beef steers.

	0 % ME	20 % ME (SH)		SEM[1]	*P* =	
	Control	R-SH	A-SH		Control versus SH	R-SH versus A-SH
Forestomach Tissue						
GCG/18S[2]						
Rumen	3.60	1.97	1.75	0.717	0.31	0.90
Omasum	2.11	1.16	7.17	2.440	0.71	0.32
Abomasum	11.01	16.22	7.19	1.967	0.86	0.09
GLP2R/18S[2]						
Rumen	5.52	5.55	7.60	2.387	0.55	0.30
Omasum	7.33	4.33	17.25	3.435	0.55	0.08
Abomasum	91.51	74.96	49.96	9.434	0.15	0.30
Intestinal tissue						
GCG/18S						
Duodenum	1.0739	0.5644	0.5380	0.1248	0.06	0.93
Jejunum	2.0900	2.0911	2.2142	0.3234	0.93	0.88
Ileum	1.9094	1.7092	2.6113	0.2523	0.63	0.18
Colon	2.8428	2.8419	2.1439	0.4190	0.69	0.52
GLP2R/18S						
Duodenum	1.3885	1.9587	1.0359	0.2600	0.49	0.0003
Jejunum	1.6981	1.2737	1.5723	0.3660	0.62	0.69
Ileum	1.7595	2.4685	2.1225	0.3605	0.33	0.61
Colon	1.1506	1.0251	0.9760	0.2727	0.52	0.87

[1] $n = 6$ except for R-SH treatment where $n = 5$

[2] Values presented in table are expressed as 1000 × value obtained in mRNA analysis.

Figure 4.1. Relative expression of proglucagon (GCG) mRNA in forestomach (A) and intestinal tissues (B) and relative expression of GLP-2 receptor (GLP2R) mRNA in forestomach (C) and intestinal tissues (D) of growing beef steers. Results are expressed as means ± SEM (n = 17). Means without a common superscript letter differ (Tukey-Kramer test, $P < 0.05$).



CHAPTER 5: EFFECTS OF GLUCAGON-LIKE PEPTIDE-2 ON GASTROINTESTINAL GROWTH, SPLANCHNIC BLOOD FLOW, AND SPLANCHNIC NUTRIENT FLUX IN RUMINATING CALVES

INTRODUCTION

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid hormone secreted from the gastrointestinal tract in response to luminal nutrients (50, 80, 98, 240). The hallmark of GLP-2 action is an increase in small intestinal mass (2, 5, 6, 9, 10, 12, 61). This increase in small intestinal mass is a result of increased growth of the intestinal mucosa as evidenced by greater villus height (7, 144, 151, 175, 176) and crypt depth (7, 144, 175). Treatment with GLP-2 results in greater proliferation of crypt cells (4, 6, 8) and reduced villus cell apoptosis (6-8, 161).

A second established action of GLP-2 is a rapid increase in blood flow, specifically of vessels supplying and draining the small intestine such as the superior mesenteric artery and portal vein (13-16, 185). Increased blood flow is evident within 10 minutes of GLP-2 infusion and quickly returns to baseline with cessation of GLP-2 infusion (13, 15, 16). The action of GLP-2 is nitric-oxide dependent, as the nitric oxide synthase (NOS) inhibitor L-NAME drastically attenuates GLP-2-mediated increases in blood flow and GLP-2 increases NOS activity (13, 14, 16). However, the effect of GLP-2 on blood flow has only been investigated in experiments utilizing short-term infusions (< 4 h) of GLP-2; no experiments have been conducted evaluating the blood flow response to GLP-2 after extended GLP-2 administration.

The vast majority of published research investigating the effects of GLP-2 on intestinal growth and blood flow has been conducted in non-ruminant animals (rodents, pigs, humans), and the effects of GLP-2 in the ruminant are unknown. However, some data is available in ruminants. Cattle express mRNA for proglucagon (the GLP-2 precursor) and the GLP-2 receptor across the gastrointestinal tract but the greatest expression is found in the small intestine (256). Moreover, increasing dietary energy intake increased ileal proglucagon mRNA expression and plasma concentrations of active

GLP-2 (241). Because GLP-1 and GLP-2 are co-secreted from the L-cell (73), changes in GLP-2 secretion can be inferred from changes in GLP-1 secretion. In cattle, plasma GLP-1 concentrations increase in parallel with food intake (206, 207), and with infusions of oil or casein into the abomasum (209-212). These changes in secretion patterns are similar to those observed in non-ruminants (56, 80, 90), and suggest that the secretion of glucagon-like peptides is similar between ruminants and non-ruminants. The digestive system of the ruminant has a more intricate stomach complex that alters foodstuffs prior to their entry into the intestines which could potentially influence regulatory signals. However, like non-ruminants, ruminants exhibit increases in intestinal mass in response to greater nutrient intake (20, 200, 201, 217) primarily by expansion of the intestinal mucosal layer (21). Thus, GLP-2 may provide a mechanism to mediate small intestinal mucosa growth in response to increased nutrient intake in the ruminant.

Therefore, the objectives of this experiment were to examine the effects of exogenously-administered GLP-2 on splanchnic blood flow and gastrointestinal growth in the ruminant. Ruminant calves were given GLP-2 by subcutaneous injection for 10 d to evaluate effects on gastrointestinal mass and morphology. We hypothesized that GLP-2 would increase small intestinal mass by increasing the villus height and crypt depth of the proximal intestine compared with Control calves. Additionally, the blood flow response to GLP-2 was investigated at the beginning and end of the experiment to evaluate the response of splanchnic blood flow to a short (1 h) continuous GLP-2 infusion before and after 10 d of GLP-2 administration. We hypothesized that GLP-2 infusion would increase blood flow of the superior mesenteric artery (SMA), portal vein, and hepatic vein in calves acutely exposed to GLP-2, but after 10 d of GLP-2 administration this effect would be diminished. Furthermore, we evaluated the uptake and release of several key nutrients across the portal-drained viscera and liver.

MATERIALS AND METHODS

Animals and surgical procedures

All experimental procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee. Eight Holstein calves (41 ± 3 d old) were

obtained for use in this experiment. Calves were fed milk replacer twice daily with water and calf starter (Table 5.1) available ad libitum before weaning (50 ± 3 d of age). After weaning the diet was gradually adjusted to a 50:50 (by weight) mixture of alfalfa cubes and calf starter. Calves were fed this diet at 2.75% of BW before and throughout the experiment; their daily allotment was fed in two equally sized meals at 0730 and 1730 h.

When calves were 97 ± 7 d of age they were surgically prepared with a 4-mm ultrasonic flow probe (4R; Transonic Systems, Ithaca, NY) around the superior mesenteric artery along the distal duodenum. The criteria for probe placement included a repeatable location and an area with predominant arterial supply to the small intestine. The arterial supply to the proximal small intestine was located and the probe was placed as proximal as possible to any arterial branches to represent as much of the arterial supply as possible. Additionally, chronic indwelling catheters were inserted in a carotid artery, mesenteric vein, the hepatic portal vein, and the hepatic vein by procedures adapted from Katz and Bergman (1969) and Huntington et al. (257) as described by McLeod et al. (258). Catheters were prepared as described by Huntington et al. (257). Catheters and flow probe cable were tunneled subcutaneously and exteriorized along the spine at approximately the 5th thoracic vertebrae. Before surgery, feed and water were withheld for 24 and 12 h, respectively. On the day of surgery, calves were induced with xylazine (0.09 mg/kg) and ketamine (1.8 mg/kg), intubated, and maintained with isoflurane-oxygen (2-5% isoflurane) for the duration of the surgery. Prior to surgery calves were injected with the long-acting antibiotic ceftiofur (Excede, 6.6 mg/kg BW, Pfizer Animal Health, New York) and were administered flunixin meglumine (1.1 mg/kg BW), with additional flunixin meglumine administered following surgery as needed for analgesia. Catheter exteriorization sites and suture sites were treated daily with antimicrobial ointment. Catheter patency was maintained by biweekly flushing and filling the catheters with a solution of gentamicin sulfate (20 mg/ml) and chymotrypsin (225 U/ml). Experimental procedures began approximately 16 d after surgery (113 ± 8 d of age), once animals had maintained feed intake for a minimum of 5 d.

Experimental design

Calves were paired by age before being assigned randomly to treatment, Control (n = 4) or GLP-2 (n = 4). Only two calves (one per treatment) began experimental periods at any one time. Experimental periods were 11 d in length. On d 1 (**Acute**), a blood flow experiment was performed as described below. After the blood flow measurements, calves were given their first dose of treatment via subcutaneous injection; injections were either vehicle (0.5% bovine serum albumin in saline, **Control**) or GLP-2 (50 μg/kg BW GLP-2 in vehicle, **GLP-2**). The GLP-2 used for both daily injections and the blood flow experiment was synthesized based on the native bovine GLP-2 sequence (California Peptide Research, Inc., Napa, CA). Treatments were administered by subcutaneous injection twice daily (every 12 h) for 10 days. On d 10 (**Chronic**) a second blood flow experiment was performed as described below. Calves were slaughtered on d 11 approximately 2 h after an intravenous injection of 5-bromo-2′-deoxyuridine (BrdU), as described below.

Blood flow measurements

On d 1 and 10 of the experiment, blood flow was monitored for 2.5 h using the chronically implanted probe to measure superior mesenteric artery blood flow and using *p*-aminohippuric acid (*p*AH) to measure portal vein, hepatic vein, and hepatic artery plasma flow. Blood flow measurements were conducted after withholding the morning feeding to minimize prandial blood flow changes. Beginning 1 h before the blood flow experiment and continuing throughout the sampling period, *p*AH (2.0% wt/vol, pH 7.4) was continuously infused into the mesenteric vein catheter (0.98 ml/min) with a peristaltic pump (Watson Marlow 205U, Wilmington, MA). The blood flow experiment consisted of 3 periods; 1) baseline infusion (B_1 or B_{10} for baseline on d 1 or 10, respectively) to establish baseline blood flow and nutrient flux during a 30-min infusion of physiological saline, 2) treatment challenge infusion (C_1 or C_{10}) in which calves were infused with their assigned treatment, either Control or GLP-2 (1000 $pmol \cdot kg^{-1} \cdot h^{-1}$) for 60 min, and 3) saline infusion (S_1 or S_{10}) in which calves were infused with physiological saline for 60 min to observe the recovery of blood flow and net nutrient flux after

treatment challenge infusion. On d 10, the blood flow experiment was started 3 to 7 h after the subcutaneous injection of treatment for that morning. Solutions were infused into the mesenteric vein catheter at a rate of 0.70 ml/min with a syringe pump (Harvard Apparatus model 22, Holliston, MA).

During all three infusion periods, real-time transit blood flow of the intestinal mesenteric artery was recorded using a bloodflow meter (T106, Transonic Systems, Ithaca, NY) and data acquisition package (Windaq 194 datalogger and Data Acquisition System, Dataq Instruments, Akron, OH). Portal and hepatic venous and arterial blood samples (5 ml each) were taken from their respective catheter every 15 min during the entire 2.5-h recording period. This sampling frequency was chosen to ensure that several points would be obtained for any infusion period (B, C, or S). Blood samples were drawn into a chilled syringe containing sodium EDTA (final concentration 10 mg EDTA/ml blood). An aliquot of blood was transferred to a chilled tube containing aprotinin (to obtain a final concentration of 500 kallikrein inhibitory equivalents /ml blood), centrifuged at 10,000 × g for 10 min, and plasma stored at -20ºC for analysis of plasma GLP-2. Blood was immediately analyzed for hematocrit using a microhematocrit centrifuge (Autocrit Ultra 3, Becton Dickinson, Franklin Lakes, NJ) at 13,700 × g for 3 min. Remaining whole blood was centrifuged (3,000 × g for 10 min) to obtain plasma and was stored at -20°C.

Plasma metabolite and hormone analysis

Plasma concentrations of *p*AH and urea were determined by automated analysis (AutoAnalyzer II, Technicon Industrial Systems, Tarrytown, NY) according to the methods described by Harvey and Brothers (259) and Marsh et al. (260), respectively. The standard curve for *p*AH assay was developed from the *p*AH infusion solution used that day. Plasma was analyzed by membrane-immobilized enzymes (YSI Inc., Yellow Springs, OH) for L-lactate (coupled to L-lactate oxidase), D-glucose (coupled to glucose oxidase), L-glutamate (coupled to L-glutamate oxidase) and L-glutamine (coupled to glutaminase and L-glutamate oxidase). Plasma concentrations of β-hydroxybutyrate were determined using a commercially available assay (Stanbio Laboratory, Boerne, TX)

using procedures adapted for use on a Konelab 20XTi Analyzer (Thermo Electron Corporation, Waltham, MA).

Plasma preserved with aprotinin (300 µl) was extracted with 70 % ethanol (vol/vol, final concentration). Concentrations of GLP-2 in ethanol-extracted plasma were measured using a radioimmunoassay employing antiserum code no. 92160 and standards of human GLP-2 (proglucagon 126-158, a gift from Novo Nordisk A/S) and monoiodinated Tyr GLP-2 (219), specific activity > 70 MBq/nmol (220). The antiserum is directed against the N-terminus of GLP-2 and therefore measures only fully processed GLP-2 of intestinal origin. Sensitivity for the assay was below 2 pmol/l and intra-assay coefficient of variation was below 6 %.

Tissue harvest

Calves were fasted for approximately 12 h before tissue harvest. On d 11, approximately 2 h after an intravenous injection of 10 mg/kg BW BrdU (5-bromo-2′-deoxyuridine; Sigma Aldrich, St. Louis, MO), calves were euthanized using an overdose of barbituate and immediately eviscerated to obtain forestomachs (reticulorumen, omasum, and abomasum) and intestines (duodenum, jejunum, ileum, and colon). Forestomachs were separated from intestines and were stripped of all connective and adipose tissues.

Reticulorumen, omasum, and abomasum were separated and emptied of digestive contents. Tissue samples from the rumen, omasum, and abomasum were obtained from the cranial ventral sac, the large order I and II omasal plies, and the antral region, respectively. All forestomach samples were rinsed extensively with ice-cold saline before preserving for morphometric and immunohistochemical analyses by placing representative samples (2.5×2.0 cm^2) in cassettes and immersing in 10% phosphate-buffered formalin (Fisher Scientific SF 100-4) for 48 h before transferring to 70% ethanol. Epithelial tissue was dissected away from tissue samples using scissors or scraping with a glass slide, and aliquots of epithelial tissue (500 mg) were snap frozen in foil packs in liquid N before storage at -80°C for DNA and protein analysis.

Small and large intestines and cecum were separated from the mesentery, divided, and lengths determined by looping the intestine across a wet stationary board, fitted with pegs at 2-m increments, without tension to minimize stretching. Based on the total measured length, the small intestine was divided at the midpoint into proximal and distal sections. One-m intestinal sections were excised from the duodenum (0.5 – 1.5 m distal to the pyloric sphincter), jejunum (0.5 m to either side of the midpoint of the proximal small intestine), and ileum (0.5 m to either side of the midpoint of the distal small intestine). A 1-m section of colon (0.5 m to 1.5 m distal of the ileocecal junction) was excised from the large intestine. Excised sections were immediately cut into two 0.5-m segments, gently stripped of digesta, cut longitudinally, and rinsed extensively with ice-cold physiological saline. Representative samples (2.5×2.0 cm^2) were removed from one 0.5-m section and placed in 10% phosphate-buffered formalin for morphometric and immunohistochemical analyses as described for forestomach tissue samples. Epithelial tissue was harvested from the other 0.5-m section by scraping with a glass slide over an ice-cold tray. All epithelial tissue and fat was removed from the 0.5-m section and components (epithelial, fat, and non-epithelial tissue) were weighed to determine the proportions of epithelial and non-epithelial tissue. Aliquots of epithelial tissue (500 mg) were snap frozen in foil packs in liquid N before storage at -80°C for DNA and protein analysis.

After sample removal, remaining forestomachs (reticulorumen, omasum, and abomasum), intestines (small and large), cecum, and liver were rinsed with warm tap water to remove any digesta or debris, allowed to drip dry, and weighed. To obtain whole organ weights, weights of sampled sections and remaining organ were added together. Weights of all other components (adipose, connective tissue, hide, remaining organs, and carcass) were obtained to calculate whole body weight.

Morphometry

Formalin-fixed tissue pieces (2.5×2.0 cm^2) were dehydrated and embedded in paraffin. Blocks were sectioned (5 μm thick), mounted with each slide containing 2 to 3 sections, and stained with hematoxylin and eosin. Mean villus height and crypt depth

were evaluated in at least 15 well-oriented crypt-villus units using an Axiophot microscope (Carl Zeiss Inc, Werk Göttingen, Germany) and Scion Image software (Scion Corporation, Frederick, MD).

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections (5 μm thick) were incubated at 70°C for 10 min, rehydrated with distilled deionized water, rinsed with phosphate-buffered saline (PBS), and incubated in 1× target unmasking fluid (Invitrogen, Carlsbad, CA) at 90°C for 10 min. The slides were incubated with 10% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) at 40°C for 15 min, followed by a 45 min incubation of the slides with BrdU antibody/nuclease reagent (mouse monoclonal antibody clone # BU-1, RPN202, GE Healthcare, Piscataway, NJ) at 40ºC. Slides were rinsed with PBS to remove excess primary antibody and incubated with PBS containing 0.3% H_2O_2 and 0.1% NaN_3 for 10 min at room temperature followed by a 40 min incubation of the slides with biotinylated universal second antibody (goat anti-mouse IgG_{2a}, sc-2073, Santa Cruz Biotechnology, Santa Cruz, CA) at 40°C. Cells were incubated in avidin-biotin horseradish peroxidase complex (Vectastain ABC Kit, PK-4000, Vector Laboratories, Inc., Burlingame, CA) and were visualized using 3,3'-diaminobenzidine (DAB Substrate Kit for peroxidase, SK-4100, Vector Laboratories, Inc., Burlingame, CA). Slides were counter-stained with 0.1% hematoxylin solution for 35 s and dehydrated through an increasing series of ethanol and xylene solutions. The proportion of proliferating crypt cells was quantified by counting the number of BrdU-labeled nuclei in 20 vertically well-oriented crypts and expressing this as a percentage of total nuclei per crypt.

DNA and protein analysis of epithelial tissue

Epithelial tissue (approximately 200 mg) was homogenized in 4 ml water for 1 min and aliquots of homogenate were used for DNA or protein analysis. Analysis of DNA was performed using bis-benzimide (261). Protein analysis was performed using the

bicinchoninic acid protein assay (Pierce, Rockford, IL) and a standard curve generated from bovine serum albumin.

Calculations and statistical analysis

Means were calculated for *p*AH and metabolite concentrations in arterial, portal venous, and hepatic venous blood samples and were used to determine blood flow and nutrient flux. Therefore, each measurement had 6 means per calf; 3 for the B, C, and S periods during the Acute period (B_1, C_1, and S_1, respectively) and 3 for the B, C, and S periods during the Chronic period (B_{10}, C_{10}, and S_{10}, respectively).

The following equations were used to calculate blood flow and net nutrient flux:

(1) Plasma flow = *p*AH infusion rate / ($C_V - C_A$) where C_V is the *p*AH concentration in portal or hepatic plasma and C_A is the *p*AH concentration in arterial plasma

(2) Hepatic arterial flow = hepatic plasma flow – portal plasma flow

(3) Net portal drained viscera (PDV) flux = portal plasma flow × ($C_P - C_A$) where C_P and C_A are metabolite concentrations in portal and arterial plasma, respectively

(4) Net total splanchnic (TS) flux = hepatic plasma flow × ($C_H - C_A$) where C_H and C_A are metabolite concentrations in hepatic and arterial plasma, respectively

(5) Net hepatic flux = net TS flux – net PDV flux

A positive net flux indicates net release or production by the PDV or liver and a negative net flux indicates net extraction or uptake by the PDV or liver.

(6) Net PDV extraction ratio (PER) = 1 – (PDV output / PDV input) × 100, where PDV output was PDV flux and PDV input was (portal plasma flow × arterial nutrient concentration).

(7) Hepatic extraction ratio (HER) = 1 – (hepatic output/hepatic input) × 100, where hepatic output was net hepatic flux and hepatic input was [(portal plasma flow × portal nutrient concentration) + (arterial plasma flow × arterial nutrient concentration)]

The statistical model for analyzing gastrointestinal organ mass, morphometric measurements, and BrdU proliferative index included treatment as a fixed effect and

block and block by treatment as random effects. The statistical model for analyzing blood flow, plasma GLP-2 and nutrient concentrations, and net nutrient flux included infusion (B_1, C_1, S_1, B_{10}, C_{10}, and S_{10}), treatment (Control or GLP-2), and their interaction as fixed effects and block as a random effect. Repeated measures were conducted on infusion with calf(treatment) as the subject. Multiple t-tests were used to compare the effect of treatment within each infusion and to compare the differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions within the GLP-2 treatment. A Bonferroni correction was used to correct *P*-values for multiple comparisons. Pearson correlation coefficients were determined between calf observations for some parameters. Data the blood flow probe for 1 calf in the GLP-2 treatment group were not measured because of flow probe failure; therefore for SMA blood flow data n = 4 for the Control treatment group but n = 3 for the GLP-2 treatment group. Results are expressed as least square means and their respective SE, and significance for treatment effects and correlations was declared at $P < 0.05$.

RESULTS

Gastrointestinal organ mass and intestinal morphology

After 10 d of treatment, neither live BW nor empty BW was affected by treatment (Table 5.2). Total gastrointestinal tract mass was not affected by treatment, and mass of all gastrointestinal organs except small intestine did not differ between Control and GLP-2 treatments. Treatment with GLP-2 for 10 d increased small intestinal mass by 17% ($P = 0.03$) whereas lengths of the small and large intestines were not affected by treatment. Similar results were observed on an empty BW basis. Total gastrointestinal weight and weight of reticulorumen and omasum were not affected by GLP-2 treatment. Abomasal mass tended to be reduced by GLP-2 treatment ($P = 0.09$, Table 5.3). Similar to the observed effects on wet small intestinal mass, GLP-2 increased small intestinal mass on an empty BW basis by 24% ($P = 0.04$, Table 5.3). Large intestinal mass and liver mass were not affected by treatment. Length of the small and large intestines on an empty BW basis were not affected by treatment (Table 5.3). In contrast, GLP-2 increased

mass:length (g/m) of the small intestine (P = 0.02, Table 5.3) but large intestinal mass:length was unaffected by treatment.

In the duodenum there was no effect of treatment on total section mass or masses of the epithelium and smooth muscle (Table 5.4). Treatment with GLP-2 tended to increase DNA content of 0.5-m duodenal sections (P = 0.10, Table 5.4) and did increase protein content of 0.5-m duodenal sections (P = 0.03, Table 5.4). Duodenal DNA concentration did not change (P = 0.91, data not shown) and protein concentration tended to increase (121 vs. 107 mg/g mucosa, P = 0.10) with GLP-2 treatment. In the jejunum, GLP-2 increased total section mass (P = 0.009, Table 5.4) by increasing epithelium mass (P = 0.008, Table 5.4) and not affecting smooth muscle mass. Treatment with GLP-2 increased DNA content of 0.5-m jejunal sections (P = 0.01, Table 5.4) but did not affect protein content of jejunal sections (Table 5.4) nor DNA or protein concentrations in jejunal mucosa (P = 0.43 and P = 0.32, respectively, data not shown). Similar to effects observed in the jejunum, GLP-2 increased ileal total section mass (P = 0.06, Table 5.4) by increasing epithelium mass (P = 0.04, Table 5.4) and not affecting smooth muscle mass. Mucosal DNA and protein content in 0.5-m ileal sections were not affected by treatment (Table 5.4), nor were DNA or protein concentrations in ileal mucosa (P = 0.68 and P = 0.80, respectively, data not shown). Tissue mass and DNA and protein content in 0.5-m colon sections were not affected by treatment (Table 5.4), nor were DNA or protein concentrations in colonic mucosa (P = 0.53 and P = 0.97, respectively, data not shown).

To evaluate the effect of GLP-2 on intestinal morphology, villus height and crypt depth were measured and the number of BrdU-positive cells were counted as an index of crypt cell proliferation (Table 5.5 and Figure 5.8). In the duodenum, GLP-2 increased villus height and crypt cell proliferation (P = 0.03 and P = 0.02, respectively, Table 5.5) and tended to increase duodenal crypt depth (P = 0.06, Table 5.5). In the jejunum, GLP-2 increased crypt depth and crypt cell proliferation in the jejunum (P = 0.02 and P = 0.01, respectively, Table 5.5) and tended to increase jejunal villus height (P = 0.06, Table 5.5). Treatment with GLP-2 tended to increase ileal villus height (P = 0.09, Table 5.5). Although ileal crypt depth was not affected by treatment, crypt cell proliferation in the

ileum was increased with GLP-2 (P = 0.05, Table 5.5). Colon crypt depth and crypt cell proliferation were not affected by treatment.

Overall, growth increases were greatest in the jejunum, and several measures of growth were positively correlated. Jejunal epithelial mass in the 0.5-m section was positively correlated with jejunal villus height (r^2 = 0.68; P = 0.007), jejunal crypt depth (r^2 = 0.67; P = 0.008), and jejunal crypt cell proliferation (r^2 = 0.50; P = 0.03). Additionally, mucosal protein content was positively related with epithelial mass of duodenal (r^2 = 0.83; P = 0.001) and jejunal (r^2 = 0.25; P = 0.12) sections. Furthermore, jejunal epithelium mass was positively correlated with PDV extraction ratios of glutamate (r^2 = 0.61; P = 0.04) and glutamine (r^2 = 0.49; P = 0.07), and a similar correlation was observed between ileal epithelium mass and PDV extraction ratio of glutamine (r^2 = 0.53; P = 0.06).

Blood flow

The effects of treatment infusion on plasma active GLP-2 concentrations are shown in Figure 5.1. As expected, GLP-2-treated calves had greater arterial concentrations of active GLP-2 during the treatment infusion periods than those treated with Control; in addition, Chronic treatment with GLP-2 increased arterial GLP-2 concentrations during the baseline infusion period compared to Control-treated calves (T × I P < 0.0001, Figure 5.1).

Infusion of GLP-2 increased SMA blood flow to 175% of baseline in the Acute period but only to 137% of baseline in the Chronic period, whereas Control infusion did not affect SMA blood flow (T × I P = 0.0002, Figure 5.2A). Blood flow of the SMA returned to baseline values during the saline infusion following GLP-2 infusion. Portal and hepatic plasma flow (% of baseline) demonstrated similar trends to SMA blood flow (Figure 5.2 B and C, respectively). Blood flow (ml/min) of the SMA during the baseline period was not different between Acute and Chronic periods, but did tend to be greater for calves assigned to the GLP-2 treatment group (774 ± 126.3 vs. 509 ± 105.7 ml/min; P = 0.09). Hepatic arterial flow (% of baseline) did not differ between treatments or among infusion periods (data not shown). Portal plasma flow (L/h) tended to be greater for

GLP-2-treated calves during the treatment challenge infusion in the Acute period (288 ± 56.8 vs. 203 ± 46.5 L/h), but did not differ from Control-treated calves during the baseline or saline infusions in the Acute period or any infusion during the Chronic period (T × I P = 0.07). Hepatic plasma flow did not differ between treatment for any infusion during the Acute period (267 L/h), but tended to be less for GLP-2-treated calves (T × I P = 0.12) during the Chronic period across the baseline (269 ± 11.8 vs. 389 ± 39.1 L/h, GLP-2 vs. CON, respectively) and treatment challenge infusions (271 ± 28.4 vs. 402 ± 32.4 L/h, GLP-2 vs. CON, respectively) but not the saline infusion period (208 ± 29.5 vs. 286 ± 33.1 L/h, GLP-2 vs. CON, respectively). Likewise, hepatic arterial plasma flow tended to be lower for GLP-2-treated calves (48.8 ± 4.61 vs. 68.0 ± 5.61 L/h), particularly during the Chronic period (T P = 0.07, data not shown). When blood flow was expressed on a BW basis ($L \cdot kg\ BW^{-1} \cdot h^{-1}$) there were no differences in portal and hepatic plasma flow (T × I P = 0.16 and P = 0.30, respectively, data not shown). Arterial hematocrit did not differ between treatments (30.3 ± 0.63 vs. 29.3 ± 0.61 % for CON and GLP-2, respectively).

Nutrient flux

Infusion of GLP-2 affected the arterial concentrations of several key metabolites. Calves in the GLP-2 treatment group had greater plasma glucose concentrations than those in the Control group (T P = 0.006, Figure 5.3A). Baseline arterial lactate concentrations were greater for GLP-2- vs. Control-treated calves in the Acute period but were not different from Control-treated calves in the Chronic period (T × I P = 0.0008, Figure 5.3B); there were no differences between treatments during the other infusion periods. Arterial glutamate concentrations were similar between treatments during the Acute period but in the Chronic period GLP-2 reduced arterial glutamate concentrations during the saline infusion period (T × I P = 0.006, Figure 5.3C). Likewise, arterial glutamine concentrations did not differ between treatments during the Acute period, but in the Chronic period GLP-2 reduced arterial glutamate concentrations during all three infusion periods compared with Control, as well as compared with the corresponding infusion during the Acute period in GLP-2-treated calves (T × I P = 0.0001, Figure

5.3D). Arterial β-hydroxybutyrate concentrations were lower for calves in the GLP-2 treatment group than the Control treatment group (T P = 0.05, Figure 5.3E), although this is due primarily to differences in the Acute period. Arterial urea concentrations tended to be lower in GLP-2-treated calves than Control-treated calves in the Acute period, but in the Chronic period GLP-2-treated calves tended to have greater arterial urea concentrations than Control-treated calves (T × I P = 0.08, Figure 5.3F).

Net PDV flux of glucose was not affected by treatment (Figure 5.4A). Net PDV lactate flux in calves Acutely treated with GLP-2 was not different or was greater than calves Acutely treated with Control, but calves Chronically treated with GLP-2 had lower net PDV lactate flux than those Chronically treated with Control (T × I P = 0.05, Figure 5.4B); however, when expressed on a BW basis ($mmol \cdot h^{-1} \cdot kg\ BW^{-1}$), there were no differences among treatment (data not shown). Net flux of glutamate, glutamine, β-hydroxybutyrate, and urea by the PDV did not differ between treatments (Figure 5.4C, D, E, and F, respectively).

Treatment with GLP-2 tended to increase the net PDV extraction ratio of glucose (T P = 0.09, Figure 5.5A). The net PDV extraction ratio of glutamate did not differ between treatments. However, Chronic GLP-2 treatment increased the net PDV extraction ratio of glutamine, especially in the baseline period, compared to Chronic Control treatment, whereas there was no difference between treatment in Acutely-treated calves (T × I P = 0.004, Figure 5.5C).

Net hepatic glucose flux tended to be reduced by GLP-2 administration (T P = 0.09, Figure 5.6A), because net hepatic glucose flux in chronically-exposed GLP-2 calves was lower than Control calves during the treatment challenge and saline infusion periods on D10; this effect was also observed when net hepatic glucose flux was expressed on a BW basis (T x I P = 0.006, data not shown). There were no treatment differences observed for net hepatic flux of lactate, glutamate, glutamine, β-hydroxybutyrate, or urea (Figure 5.6B, C, D, E, and F, respectively). The hepatic extraction ratio of lactate did not differ between treatments in the Acute period, but GLP-2 increased the lactate hepatic extraction ratio compared with Control during the baseline infusion in the Chronic period (Figure 5.7, T x I P = 0.006).

Because treatment did not affect glucose flux by the PDV but did influence net hepatic glucose flux, net total splanchnic glucose flux followed a similar pattern to hepatic glucose flux when expressed in mmol/h (T × I P = 0.06, data not shown) and as mmol·h^{-1}·kg BW^{-1} (T × I P = 0.06, data not shown). There were no differences observed for net total splanchnic flux of lactate, glutamate, glutamine, β-hydroxybutyrate or urea.

DISCUSSION

Gastrointestinal organ mass and intestinal morphology

The importance of GLP-2 in inducing gastrointestinal mucosal growth of the small intestine has been well documented in non-ruminant animals (2, 5, 6, 9, 10, 12, 61). The research reported here extends these findings by demonstrating that GLP-2 also increases gastrointestinal growth of the ruminant. As observed in non-ruminants (6, 7), GLP-2 preferentially increased mass of the small intestine without affecting total body mass or mass of other gastrointestinal organs. This growth increase was due to an increase in the mucosal compartment of the small intestine, especially in the jejunum. Increases in jejunal epithelial mass, BrdU labeling, villus height, and crypt depth were observed with high correlations between these parameters. Increased villus height and BrdU labeling was observed in all three segments of the small intestine, whereas increased crypt depth was observed in duodenum and jejunum and increased mucosal mass was observed in jejunum and ileum. The results from the current experiment agree with others (6, 7, 12, 144) that jejunal growth is most affected by GLP-2. The localization of mucosal growth observed in this experiment agrees with the localization of the GLP-2 receptor. Expression of the GLP-2 receptor is throughout small intestine in rat, mouse, and man, but the greatest staining is observed in the proximal small intestine in all species (5).

This experiment also investigated the effects of GLP-2 on splanchnic blood flow and nutrient flux after Acute or Chronic exposure to GLP-2. The purpose of this design was to determine if long-term administration (10 d) would attenuate the responsiveness of the gastrointestinal vessels to a short (1 h) continuous infusion of GLP-2. It should be noted that both the short-term infusion and long term subcutaneous injections were able

to achieve pharmacological plasma GLP-2 concentrations (Figure 5.1). Furthermore, it would be expected that the arterial GLP-2 concentrations in GLP-2-treated calves during the baseline period of 10 are primarily reflective of the blood concentrations achieved between 3 to 7 h after subcutaneous injection of exogenous GLP-2 as endogenous GLP-2 concentrations would be expected to be low in calves fasted for 12 h. Indeed, calves in the Control treatment group had arterial GLP-2 concentrations of 16 pM during the same time period whereas calves in the GLP-2 treatment group had arterial GLP-2 concentrations of 512 pM.

An important finding of this experiment was that a short continuous infusion of GLP-2 increased SMA blood flow to ~175% of baseline flow in Acute calves but only increased flow to ~ 135% of baseline flow in Chronic calves that had received GLP-2 for 10 d. Similar results were observed in the portal and hepatic veins. However, SMA blood flow during the baseline period (before treatment infusion) did not differ between the two treatment groups, demonstrating that GLP-2 did not affect the basal blood flow of the SMA but rather the responsiveness of the SMA to GLP-2 infusion. The lack of treatment effect on hepatic arterial plasma flow (as calculated by the difference between hepatic and portal plasma flows) is consistent with other research observing an effect of GLP-2 only on blood flow to "intestinal vessels" (15, 16). The blood flow response to GLP-2 has only been documented in response to short-term GLP-2 administration (4 h or less), and the magnitude of the blood flow response to GLP-2 in Acute calves is similar to that reported in the literature (13, 14, 16). Our observation that chronic administration of GLP-2 reduced the blood flow response to a short continuous infusion of GLP-2 extends the findings of previous researchers and suggests that in vivo desensitization to exogenous GLP-2 may have occurred in response to chronic GLP-2 administration.

Although homologous desensitization of the GLP-2 receptor has been observed in vitro (145, 170), no definitive evidence of GLP-2R desensitization has been observed in vivo. The putative mechanism of blood flow increases as a result of GLP-2 administration is an increase in the activity and expression of eNOS, which presumably increases the amount of NO generated and thus acts as a vasodilator of local vessels; however, because the GLP2R is also co-expressed with VIP and 5-HT, these two substances may also be involved in the vasodilatory response to GLP-2 (13, 14, 16).

Whether long-term GLP-2 administration affects its own receptor or responsiveness of secondary or tertiary messenger generation is unknown, but the current data demonstrate a clear downregulation of the blood flow response to GLP-2 in calves. This finding could have important implications for both the understanding of GLP-2 action and the potential of GLP-2 use for clinical applications.

PDV nutrient uptake and release

In Acute calves, short-term GLP-2 infusion did not appear to significantly alter net uptake of energy substrates. Notably, net PDV glucose uptake was not affected by GLP-2 infusion in Acute calves. This is in contrast to results observed in TPN-fed piglets, in which acute GLP-2 infusion increased both PDV glucose uptake and extraction % (13). Furthermore, in Acute calves we did not observe changes in glutamine uptake by the PDV during GLP-2 infusion, in contrast to the results of Guan et al. (13). Differences between experiments may be the result of several factors. The difference in species and physiological status of the animals may have caused differences between these two experiments. The experiment by Guan et al. (13) utilized neonatal piglets that had been fed by exclusively by TPN, whereas our experiment utilized enterally-fed calves that, although fasted for 12 h before the infusion protocol, were not truly post-absorptive because of the long retention time of feedstuffs in the rumen. Because the TPN model is associated with intestinal atrophy (262), nutrient uptake and utilization could be substantially different than in a healthy animal. Additionally, the length of GLP-2 infusion in this experiment was 1 h, whereas in the experiment by Guan et al. (13) a 4 h infusion was utilized. Perhaps a greater period of time (or greater total amount of GLP-2 infused) is necessary to observe alterations in PDV nutrient uptake.

More marked changes in net nutrient flux appeared after chronic exposure to treatment. Arterial concentrations of glutamine were lower in calves chronically exposed to GLP-2, similar to observations in piglets treated with GLP-2 (263). Although net PDV glucose, glutamate, and glutamine uptake were unchanged, Chronic GLP-2 exposure increased the PDV extraction ratio of glutamine and tended to have a similar effect on PDV extraction ratios of glutamate and glucose, particularly during the baseline infusion

period. Typically, the PDV extracts 15-33% of arterial glutamine and of this half to two-thirds is oxidized (summarized in Bertolo and Burrin (264)), whereas 10-15% of the glutamate C is recovered in protein or other acid-insoluble materials and the remainder is exported from the PDV as amino or organic acids (265). Additionally, the PDV actively extracts and oxidizes arterial glutamate to CO_2, but a corresponding reduction in net flux is not observed because of the high conversion of arterially extracted glutamine to glutamate, which is then exported from the PDV (266). Thus, the lack of treatment effects on net glutamate and glutamine flux in this experiment may not demonstrate a lack of treatment effect on PDV uptake and subsequent release; however, it is impossible to ascertain the true fate of these metabolites from this experiment using only net flux measurements, especially because of this substantial interchange between glutamate and glutamine.

Glutamate and glutamine are important amino acids for not only gut energy metabolism, but also growth and intestinal function (239, 265), including synthesis of other amino acids such as arginine, proline, ornithine, and citrulline (263, 264). Our results suggest that GLP-2 increased the net extraction of arterial glutamine and glutamate by the PDV, likely in support of the GLP-2-induced increases in small intestinal mass. Indeed, PDV net extraction ratio of glutamate was positively correlated with jejunal epithelial mass and PDV net extraction ratio of glutamine was positively correlated with epithelial mass of the jejunum and ileum. Thus the epithelial growth stimulated by GLP-2 increased net arterial extraction of glutamate and glutamine across the PDV; however, because there were presumably a greater number of cells present, there is no indication that GLP-2 upregulated arterial nutrient extraction per se. Additionally, the reduced net PDV lactate release observed in calves chronically treated with GLP-2 would suggest that if the uptake of substrates by the PDV was increased, more C was being retained in the PDV or was oxidized rather than being exported as lactate. Alternatively, export of C and N could be achieved by altering alanine, proline, citrulline, ornithine, or arginine flux (267), which were not measured in this experiment.

Hepatic nutrient utilization and production

Net hepatic nutrient uptake and release also was not substantially affected by short-term GLP-2 infusion in Acute calves. In contrast, Chronic exposure to GLP-2 may have altered glucose and lactate metabolism across the liver. Despite a greater lactate hepatic extraction ratio in GLP-2-exposed calves, infusion of GLP-2 into calves Chronically exposed to GLP-2 had lower net hepatic glucose release compared with Control calves. This may have contributed to the lower arterial glucose concentrations observed in calves chronically exposed to GLP-2. Hepatic glucose release is primarily a result of gluconeogenesis and glycogenolysis. One major factor influencing the rate of gluconeogenesis is the availability of gluconeogenic substrates such as propionate, lactate, glycerol, and amino acids, primarily alanine and glutamine (268). Because calves were fasted, the propionate contribution would be much less than that of a fed animal. In cows fasted for 1 d, the contribution of propionate to hepatic glucose output decreased from 46 to 15% but the contribution of lactate increased from 16 to 42% (269). Calves chronically exposed to GLP-2 tended to have lower absolute portal and hepatic plasma flows than their Control-exposed counterparts as well as a lower net PDV lactate release, which may have resulted in decreased lactate flow to the liver that would be available for gluconeogenesis. This reduction in lactate uptake could explain a lower net hepatic output of glucose. In support, net hepatic glucose output was positively correlated with net hepatic lactate uptake ($R^2 = 0.18$, $P = 0.02$). Although changes in hepatic glucogenic amino acids could have a similar effect, no changes were observed in net PDV or hepatic glutamate or glutamine flux, and other glucogenic amino acids such as alanine were not measured in this experiment.

Although alterations in hepatic nutrient flux can most likely be explained by changes in glucogenic precursor availability as a result of blood flow, there are important endocrine controls of gluconeogenesis that could potentially be affected by GLP-2 administration. The GLP-2 receptor colocalizes with proglucagon in the pancreatic islets in rats and humans, and GLP-2 infusion increases secretion of glucagon from the perfused rat pancreas but does not affect insulin secretion (154). Because glucagon promotes gluconeogenesis and glycogenolysis to increase hepatic glucose output, alterations in glucagon secretion could have occurred after chronic GLP-2 exposure.

Perhaps similar to the potential desensitization of blood flow to chronic GLP-2 administration, long-term GLP-2 exposure could reduce the pancreatic sensitivity to a subsequent GLP-2 exposure which may result in decreased glucagon release. However, because glucagon concentrations were not measured in this experiment, this is purely speculative.

In summary, this experiment demonstrates that ruminants respond to GLP-2 administration in a similar manner to non-ruminants. Treatment with GLP-2 increased small intestinal mass by increases in crypt cell proliferation in the small intestine, contributing to increases in villus height, crypt depth, and mucosal mass. Furthermore, we show that GLP-2 infusion increases blood flow of the superior mesenteric artery, portal vein, and hepatic vein in calves not previously exposed to exogenous GLP-2. However, we have found for the first time that long-term administration of GLP-2 attenuates this blood flow response substantially. These results extend our understanding of the actions of GLP-2 and may have significant implications for the use of GLP-2 in clinical care settings.

Table 5.1. Ingredient and nutrient compositions of the experimental diet.

	% of DM
Ingredient composition of diet	
Alfalfa cubes	50.0
Calf starter	50.0
Ingredient composition of calf starter	
Crimped oats	35.12
Cracked corn	30.13
Soybean meal	23.90
Molasses	7.60
Limestone	1.00
Sodium chloride	0.75
Dicalcium phosphate	0.75
Rumensin premix[1]	0.50
Trace mineral premix[2]	0.15
Selenium premix[3]	0.10
Nutrient Composition of Diet[4]	%
DM	89.1
CP	19.9
NDF	28.6
ADF	19.9
NE_M, Mcal/kg	1.635

[1] Rumensin premix contained 6.6 g/kg Rumensin.

[2] Trace mineral premix contained 338 mg/kg zinc sulfate, 281 mg/kg manganese sulfate, 159 mg/kg copper sulfate, 6 mg/kg iodine EDDI, 23 mg/kg bentonite, and 180 mg/kg mineral oil.

[3] Selenium premix contained 90 mg/kg Se.

[4] Nutrient compositions of diet were determined by analysis of ingredient samples of alfalfa cubes and calf starter composited by block (n = 4).

Table 5.2. The effect of subcutaneous injection of GLP-2 (100 μg·kg $BW^{-1}·d^{-1}$) for 10 d on visceral organ mass and intestinal length of Holstein calves.

	Control	GLP-2	SEM[1]	*P* =
Weight, kg				
Live BW	137	128	8.6	0.48
Empty BW	122	115	7.5	0.55
Total GIT	9.61	9.82	0.480	0.76
Rumen/Reticulum	3.49	3.17	0.228	0.36
Omasum	0.84	0.87	0.092	0.83
Abomasum	0.74	0.62	0.056	0.19
Small Intestine	3.27	3.84	0.116	0.03
Large Intestine	1.07	1.08	0.095	0.93
Liver	2.79	2.45	0.237	0.34
Length, m				
Small Intestine	30.7	29.8	1.54	0.72
Large Intestine	5.11	5.10	0.401	0.98

[1] n = 4.

Table 5.3. The effect of subcutaneous injection of GLP-2 (100 μg·kg $BW^{-1}·d^{-1}$) for 10 d on visceral organ mass and intestinal length as a percentage of empty body weight of Holstein calves.

	Control	GLP-2	SEM[1]	*P* =
Organ, % EBW				
Total GIT	7.96	8.54	0.272	0.18
Rumen/Reticulum	2.90	2.75	0.136	0.48
Omasum	0.69	0.75	0.040	0.30
Abomasum	0.61	0.54	0.040	0.09
Small Intestine	2.72	3.36	0.168	0.04
Large Intestine	0.89	0.94	0.065	0.44
Liver	2.29	2.13	0.085	0.23
Length:EBW, cm/kg				
Small Intestine	25.6	26.0	1.50	0.86
Large Intestine	4.30	4.41	0.394	0.85
Mass:Length, g/m				
Small Intestine	106.9	129.4	4.22	0.02
Large Intestine	211.3	213.0	15.28	0.94

[1] n = 4.

Table 5.4. The effect of subcutaneous injection of GLP-2 (100 μg·kg $BW^{-1}·d^{-1}$) for 10 d on mass and DNA and protein content in small intestinal sections (0.5-m) of Holstein calves.

	Control	GLP-2	SEM[1]	*P* =
Duodenum				
Total section weight, g	52.05	58.39	8.188	0.60
Epithelium weight, g	24.70	30.94	2.888	0.22
Smooth muscle weight, g	24.00	25.58	5.795	0.86
DNA, mg	228	282	28.6	0.10
Protein, mg	989	1100	79.4	0.03
Jejunum				
Total section weight, g	47.44	62.88	3.388	0.009
Epithelium weight, g	27.44	44.50	3.771	0.008
Smooth muscle weight, g	18.63	17.88	1.521	0.74
DNA, mg	265	412	35.3	0.01
Protein, mg	1066	1105	97.8	0.66
Ileum				
Total section weight, g	42.76	55.60	3.919	0.06
Epithelium weight, g	22.73	36.37	3.650	0.04
Smooth muscle weight, g	16.38	17.95	1.516	0.49
DNA, mg	245	373	52.4	0.18
Protein, mg	1216	1158	105.7	0.71
Colon				
Total section weight, g	102.5	78.94	15.693	0.33
Epithelium weight, g	15.02	24.24	4.521	0.21
Smooth muscle weight, g	46.35	37.25	6.705	0.37
DNA, mg	123	183	46.5	0.42
Protein, mg	705	710	104.7	0.97

[1] n = 4.

Table 5.5. The effect of subcutaneous injection of GLP-2 (100 μg·kg $BW^{-1}·d^{-1}$) for 10 d on small intestinal morphology of Holstein calves.

	Control	GLP-2	SEM[1]	*P* =
Duodenum				
Villus height, μm	308.1	367.0	13.650	0.03
Crypt depth, μm	166.7	208.6	12.452	0.06
$BrdU^+$cells, % of total cells	15.17	21.11	1.288	0.02
Jejunum				
Villus height, μm	294.4	373.1	24.098	0.06
Crypt depth, μm	188.2	240.1	11.824	0.02
$BrdU^+$cells, % of total cells	16.77	23.09	0.859	0.01
Ileum				
Villus height, μm	227.0	270.6	15.024	0.09
Crypt depth, μm	167.8	161.6	8.760	0.57
$BrdU^+$cells, % of total cells	16.90	19.99	1.602	0.05
Colon				
Crypt depth, μm	317.1	329.1	20.111	0.52
$BrdU^+$cells, % of total cells	7.74	8.14	1.124	0.71

[1] n = 4.

Figure 5.1. Arterial concentrations of active glucagon-like peptide-2 (GLP-2) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}·h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}·d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as mM (means ± SE). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

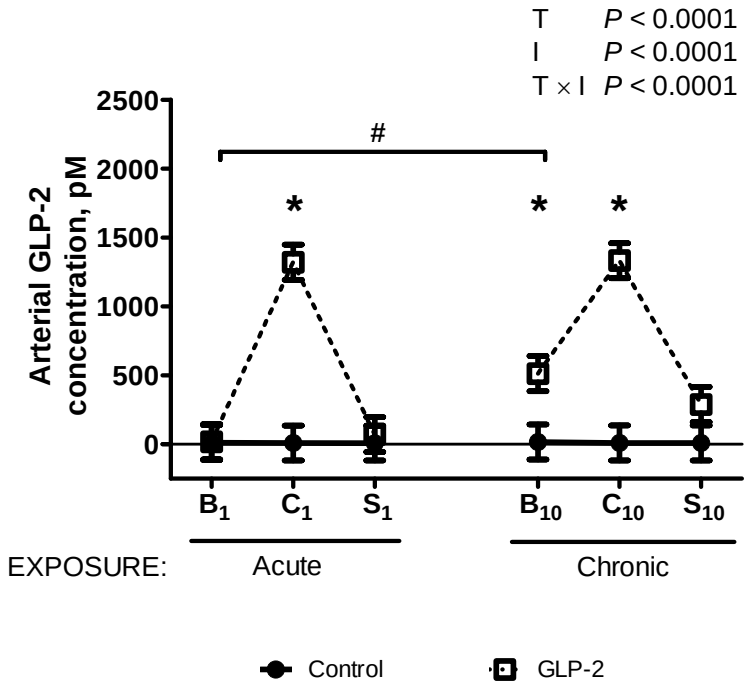


Figure 5.2. Blood flow of the superior mesenteric artery (A) and plasma flow of the portal vein (B) and hepatic vein (C) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 3 for super mesenteric artery blood flow but n = 4 for portal and hepatic vein plasma flow). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}·h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}·d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as percent of the blood or plasma flow during the baseline infusion period (means ± SE). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

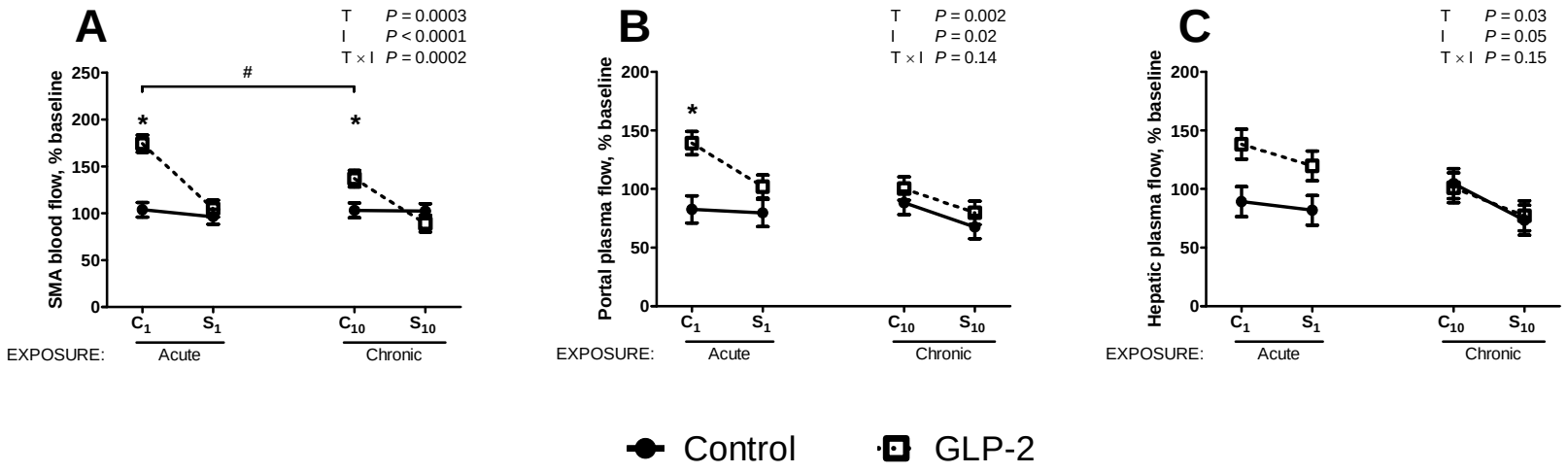


Figure 5.3. Arterial concentrations of glucose (A), lactate (B), glutamate (C), glutamine (D), β-hydroxybutyrate (E), and urea (F) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}\cdot h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}\cdot d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as mM (means ± SE). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

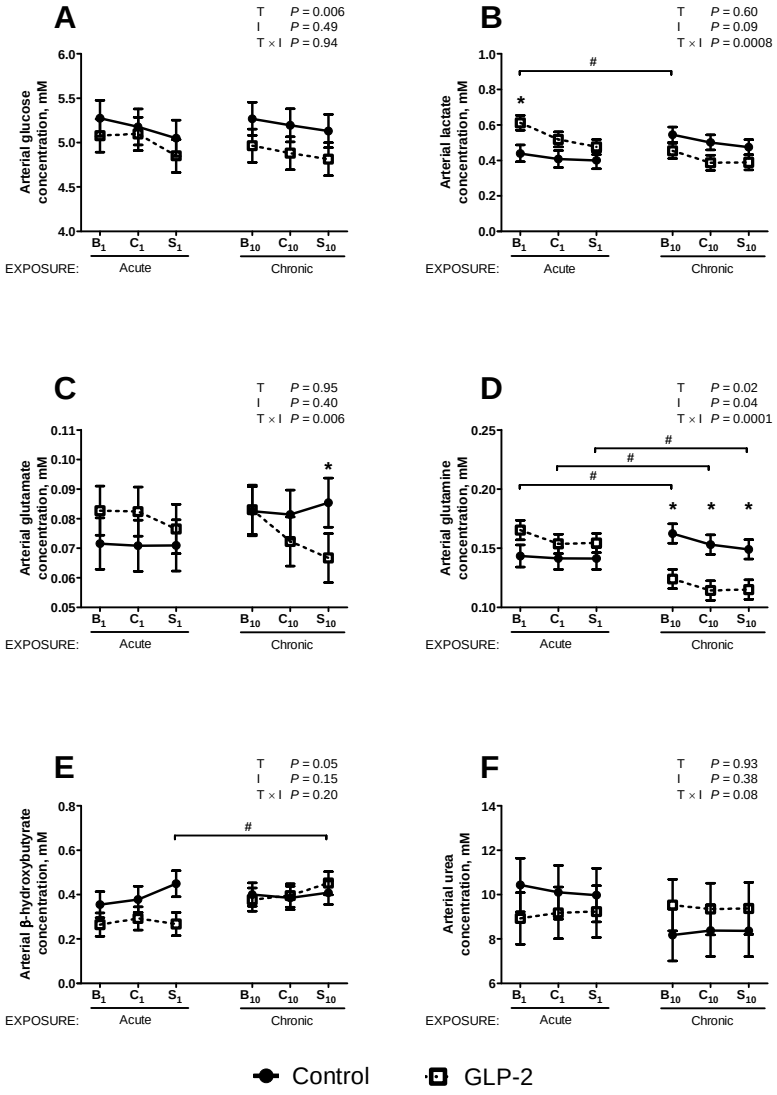


Figure 5.4. Net PDV flux of glucose (A), lactate (B), glutamate (C), glutamine (D), β-hydroxybutyrate (E), and urea (F) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}·h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}·d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as mmol/h (means ± SE). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

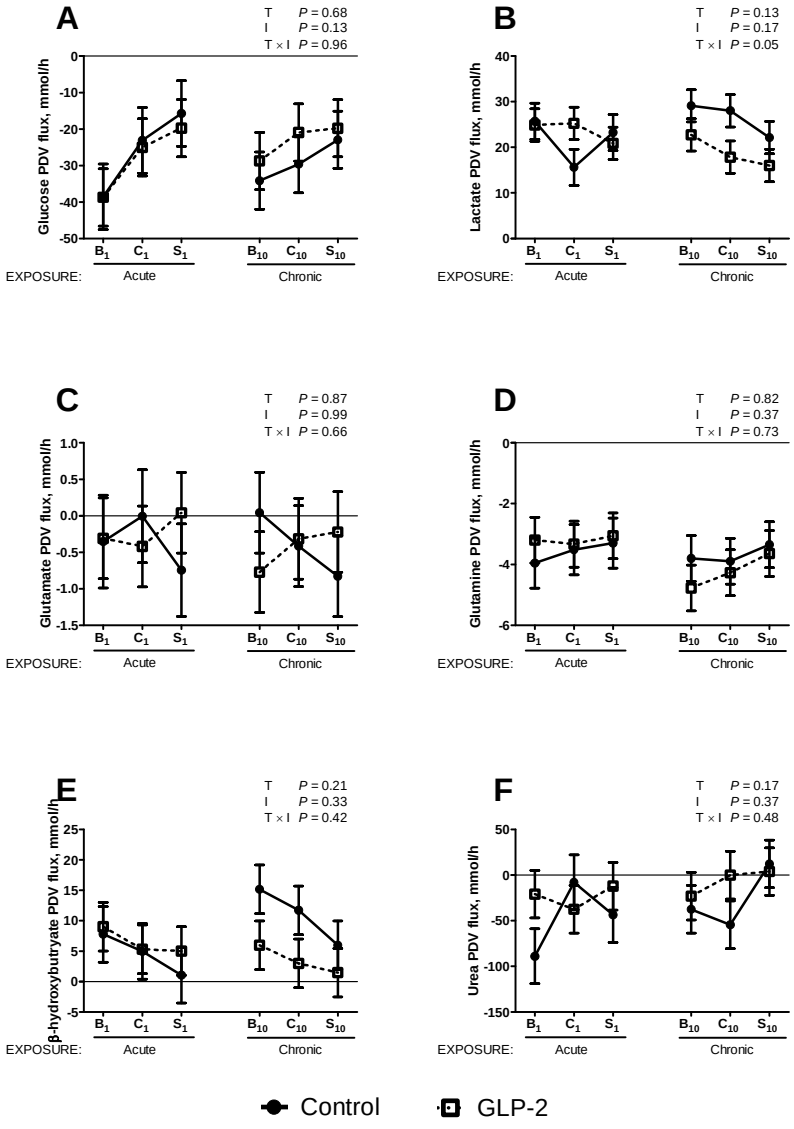


Figure 5.5. Net PDV extraction ratio of glucose (A), glutamate (B), and glutamine (C) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg BW^{-1}·h^{-1} GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg BW^{-1}·d^{-1} GLP-2 as subcutaneous injection for 10 d. Values are expressed as % (means ± SE). Net PDV extraction ratio (PER) = 1 – (PDV output / PDV input) × 100, where PDV output was PDV flux and PDV input was (portal plasma flow × arterial nutrient concentration). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

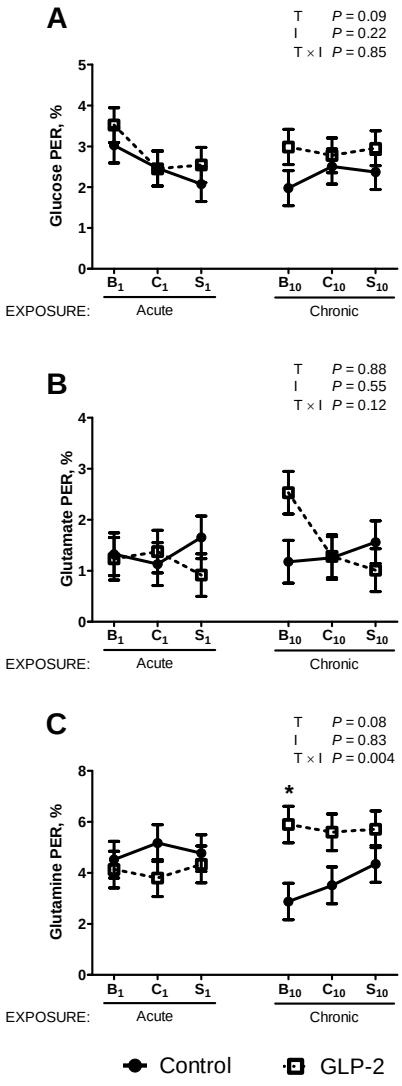


Figure 5.6. Net hepatic flux of glucose (A), lactate (B), glutamate (C), glutamine (D), β-hydroxybutyrate (E), and urea (F) in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}·h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}·d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as mmol/h (means ± SE). Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

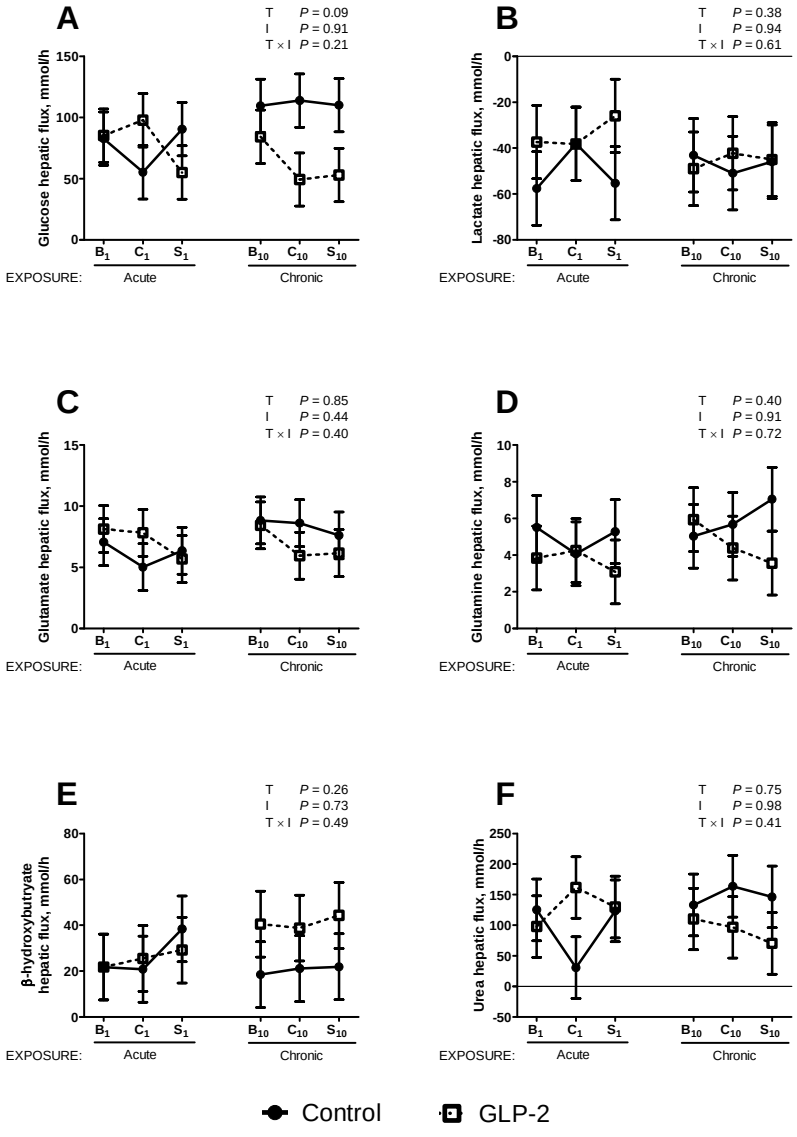


Figure 5.7. Net hepatic extraction ratio (HER) of lactate in calves treated with Control (•; n = 4) or GLP-2 (□, n = 4). Response to baseline, treatment challenge, or saline infusion in calves not previously exposed to treatment (Acute; B_1, C_1 and S_1) or after 10 d of treatment exposure (Chronic; B_{10}, C_{10} and S_{10}) was evaluated. Calves in the Control group were given vehicle (BSA) during both the treatment challenge infusion period (C_1 and C_{10}) and as subcutaneous injection for 10 d. Calves in the GLP-2 treatment group were given 1000 pmol·kg $BW^{-1}·h^{-1}$ GLP-2 during the treatment challenge infusion period (C_1 and C_{10}) and 100 µg·kg $BW^{-1}·d^{-1}$ GLP-2 as subcutaneous injection for 10 d. Values are expressed as % (means ± SE). Hepatic extraction ratio (HER) = 1 – (hepatic output/hepatic input) × 100, where hepatic output was net hepatic flux and hepatic input was [(portal plasma flow × portal nutrient concentration) + (arterial plasma flow × arterial nutrient concentration)]. Fixed effect *p*-values for Treatment (T; Control or GLP-2), Infusion period (I; B_1, C_1, S_1, B_{10}, C_{10}, or S_{10}), and their interaction are shown for each figure. Significant differences between T (Control vs. GLP-2) within I are denoted by * ($\alpha < 0.05$). Within GLP-2 treatment, significant differences between baseline (B_1 vs. B_{10}), treatment challenge (C_1 vs. C_{10}) or saline (S_1 vs. S_{10}) infusions are denoted by # ($\alpha < 0.05$).

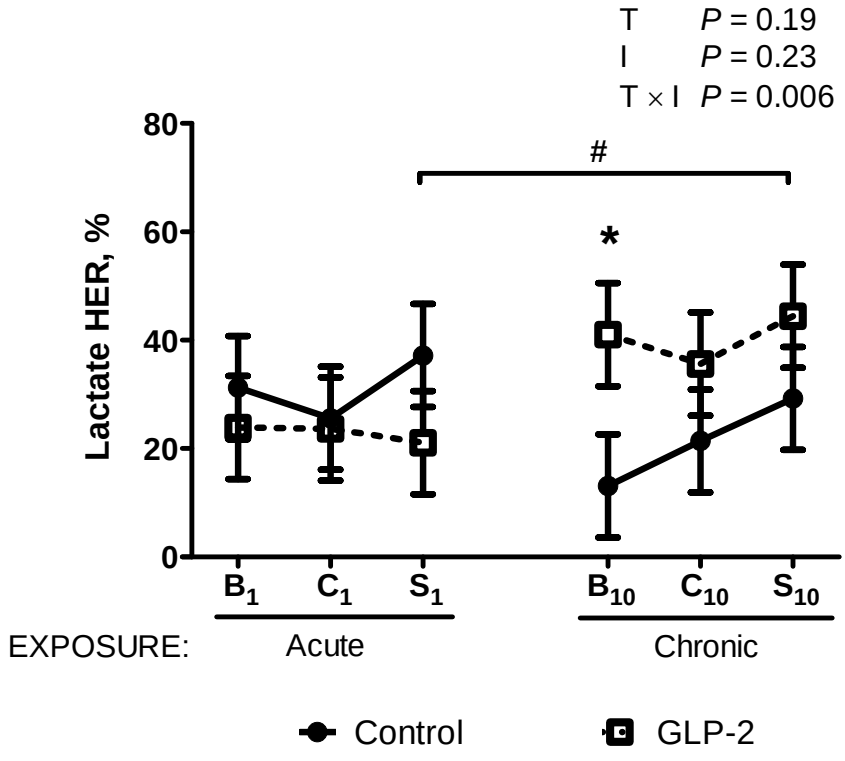
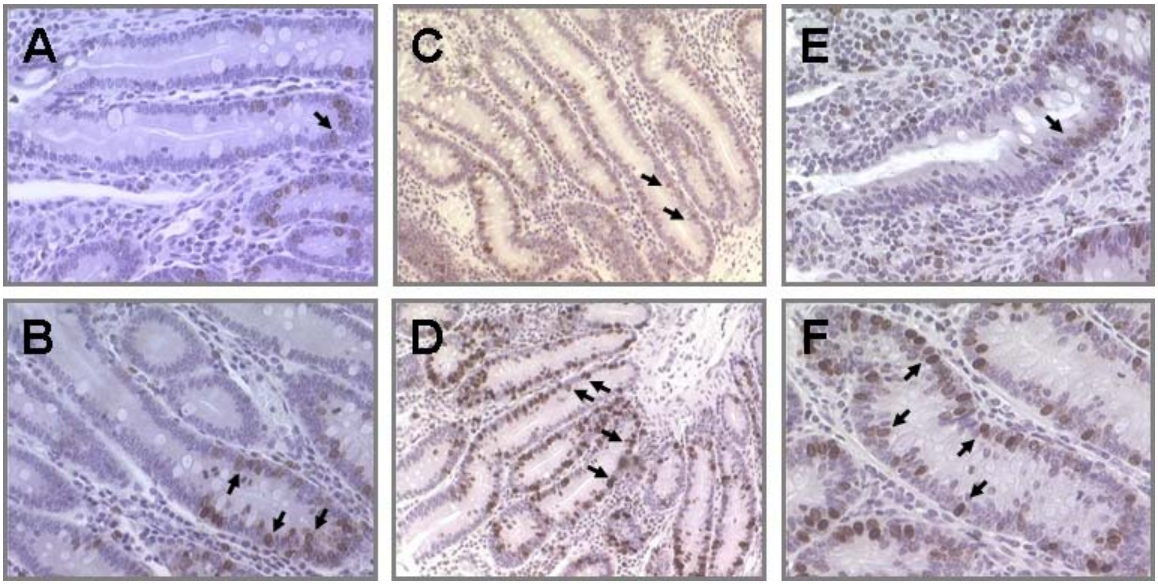


Figure 5.8. Representative light micrographs of duodenal, jejunal, and ileal tissue stained for BrdU in calves treated for 10 d with Control (n = 4; A, C, and E, respectively) or 100 μg·kg $BW^{-1}·d^{-1}$ GLP-2 (n = 4; B, D, and F, respectively). Images of duodenum and ileum (A, B, E, and F) were obtained at ×200 magnification and images of jejunum (C and D) were obtained at ×125 magnification. Crypt cells staining positive for BrdU are labeled dark brown (black arrows) and are counterstained with hematoyxlin.



CHAPTER 6: CONCLUSIONS AND IMPLICATIONS

The overall hypotheses of this dissertation are that GLP-2 and its receptor exist in ruminants, respond to physiological stimuli, and GLP-2 can induce gastrointestinal growth and intestinal blood flow. The aim of this research was to systematically characterize and evaluate the role of GLP-2 in the control of gastrointestinal growth and splanchnic blood flow in ruminants. Prior to this, only indirect evidence was available to suggest the existence of GLP-2 in ruminants.

Our first experiment established the existence of plasma GLP-2 in cattle and the expression of mRNA for proglucagon (the GLP-2 precursor) and the GLP-2 receptor in bovine intestinal tract tissue. Moreover, increasing dietary energy intake increased ileal proglucagon mRNA expression and concentrations of active GLP-2 in plasma. These changes demonstrate that GLP-2 is responsive to physiologic changes in nutrient intake and can thus be relevant to "real-world" feeding practices. These results prompted a second experiment to further define the gastrointestinal expression patterns of proglucagon and GLP-2 receptor mRNA. For both proglucagon and GLP-2 receptor mRNA, expression is substantially greater in the intestinal tissues than in the ruminant forestomachs, suggesting that the greatest impact of GLP-2 would be observed in the intestines.

To assess the action of GLP-2 in the ruminant, our third experiment tested the effect of exogenous bovine GLP-2 on splanchnic blood flow, splanchnic nutrient flux, and gastrointestinal growth in a ruminating calf model. In agreement with data collected in non-ruminants, GLP-2 treatment increased small intestinal weight by increasing small intestinal crypt cell proliferation, villus height, crypt depth, and mucosal mass. The fact that forestomach mass was not affected by GLP-2 treatment further strengthens our conclusions from the first two experiments that GLP-2 would have few effects in the ruminant forestomachs associated with the extremely low GLP-2 receptor mRNA expression. We suggest, based on our own data and that from non-ruminants, that the growth-stimulating effect of GLP-2 is restricted primarily to the small intestine. We also used this experiment to test the effect of GLP-2 on splanchnic blood flow and the

potential changes in responsiveness of blood flow after extended GLP-2 treatment. We found that GLP-2 increases blood flow of the superior mesenteric artery, portal vein, and hepatic vein in calves not previously exposed to exogenous GLP-2. However, the blood-flow response to GLP-2 was markedly reduced after 10-d administration of exogenous bovine GLP-2. To our knowledge, this apparent down-regulation in the blood flow response to GLP-2 has never been reported in any species prior to this dissertation. The data presented in this dissertation shows that ruminants possess a functional GLP-2 signaling system that is responsive to nutrient ingestion. Administration of exogenous GLP-2 increases small intestinal epithelial growth and intestinal blood flow.

This research sets the foundation for several directions of future research. Our research demonstrating the increases in small intestinal growth and blood flow with GLP-2 suggests that this hormone could substantially modify the capacity of the gastrointestinal tract for nutrient absorption by affecting the total surface area of the absorptive compartment (growth) and the potential concentration gradient for nutrient absorption (blood flow). Other research has shown that GLP-2 increases the number and activity of specific nutrient transporters on both sides of the intestinal absorptive cell, thus specifically increasing absorption of certain nutrients, especially glucose (191, 193), but whether this occurs in ruminants is unknown. Therefore, GLP-2 could substantially modify the capacity of the gastrointestinal tract to absorb nutrients and may possibly change the amount and profile of nutrients available for productive purposes.

If nutritionists can understand how the gastrointestinal tract adapts to the nutrient profile presented to it, a more complete understanding of the temporal profile of nutrient absorption and presentation to productive tissues can be achieved. Furthermore, this knowledge will allow improvements in nutrient utilization and feed efficiency and could allow for targeted nutrient feeding protocols to reduce overfeeding and minimize excretion of excess nutrients into the environment. Additionally, GLP-2 may give the industry a tool to modify the timing of gastrointestinal tract growth to better suit the upcoming needs of the animal. For example, growth of the gastrointestinal tract of early lactation dairy cows occurs in concert with the negative energy balance caused by low feed intake and high milk production (19). It may be possible to use GLP-2 to modify the timing of this gastrointestinal growth to the pre-parturient period when nutrients are

relatively abundant and demands are much less. This may reduce the energy demands of the cow during the post-parturient period and thus lessen the incidence and severity of negative energy balance disorders. As negative energy balance in early lactation directly and indirectly costs the dairy industry millions of dollars annually in lost milk yield and culling, this type of tool could be extremely powerful.

In addition to research that impacts ruminant animal productivity, significant advances could be achieved in basic biomedical research. It has long been known that glucagon-like peptide-1 (GLP-1, which is co-secreted with GLP-2) stimulates insulin secretion, but it has recently been reported that GLP-2 induces glucagon secretion (154). Therefore, these gastrointestinal hormones impact not only the profile of nutrients absorbed from the gastrointestinal tract but may also indirectly affect their utilization in peripheral tissues. This will be an important area of research in the future in that it may allow a more complete understanding of the interactions between diet and potential downstream metabolic effects via insulin and other metabolic hormones.

Finally, our research discovered an important aspect of GLP-2 that has not previously been reported in any species. The attenuated blood flow response to GLP-2 after chronic GLP-2 administration suggests a downregulation of the in vivo response, although the precise mechanism is unknown. Further investigation is needed using in vivo models to better understand this response to extended GLP-2 administration. This research also has significant implications for the prolonged use of GLP-2 in treatment regimens for human disease such as short-bowel syndrome.

This dissertation research systematically characterized and evaluated the potential role of GLP-2 in the control of gastrointestinal growth and splanchnic blood flow in ruminants. While it extends the knowledge of hormonal control of the gastrointestinal tract in ruminants, it also adds crucial information to the larger body of work investigating the actions of GLP-2. This dissertation research has contributed to the groundwork necessary to enable the use of glucagon-like peptide-2 in improving the health and productivity of a diverse group of mammalian species.

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VITA

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EDUCATION

2004-current **Ruminant Nutritional Physiology**
Department of Animal and Food Sciences, University of Kentucky
- GPA: 4.0/4.0
- Dissertation: "Identification and characterization of the glucagon-like peptide-2 hormonal system in ruminants."
- Advisor: Dr. David Harmon

M.S. 2003 **Ruminant Nutrition and Metabolism**
Department of Animal Science, Michigan State University
- GPA: 3.94/4.0
- Thesis: "Interactions of endosperm type of corn grain and brown midrib 3 mutation of corn silage on production and metabolism of lactating dairy cows."
- Advisor: Dr. Michael Allen

B.S. 2001 **Animal Science, Pre-Veterinary Medicine**
Degree with Distinction, Magna Cum Laude
University of Delaware
- GPA: 3.69/4.0
- Senior Thesis: "The effect of *Lactobacillus buchneri* on the fermentation and aerobic stability of high moisture corn."
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RESEARCH

Experience

2004-present Research Assistant, Dr. David Harmon, University of Kentucky

2001-2003 Research Assistant, Dr. Michael Allen, Michigan State University

1999-2001 Research Assistant, Dr. Limin Kung Jr., University of Delaware

Grants

Preliminary Assessment of Interactions of Intake and Glucagon-Like Peptide-2 in Angus Steers

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TEACHING

2006-2007	Teaching Assistant, Dr. David Harmon, University of Kentucky
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2005	Teaching Assistant, Dr. William Silvia, University of Kentucky
2005	Teaching Assistant, Dr. Eric Vanzant, University of Kentucky
2002	Teaching Assistant, Dr. Michael VandeHaar, Michigan State University

HONORS AND AWARDS

2008	University of Kentucky Dissertation Enhancement Award
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2008	University of Kentucky Dissertation Completion Fellowship
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2004	Outstanding Master's Student, Dept of Animal Science, Michigan State University
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2001	Wilbur R. Hesseltine Scholarship
2001	Alpha Zeta Outstanding Senior Award
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1997-2001	State of Delaware Caravel Agricultural Scholar Award
1997	University of Delaware Honors Program Scholarship

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