THE EFFECT OF NUTRIENTS ON THE RATE OF PROTEIN DEGRADATION IN ISOLATED SMALL INTESTINAL ENTEROCYTES FROM *SPRAGUE-DAWLEY* RATS

by

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ABSTRACT

Nutrients affect small intestinal protein mass and metabolism. Studies on the effect of nutrients on small intestinal protein degradation are limited due to a lack of a proper method. The objectives of this study were to establish a method to directly estimate protein degradation in isolated enterocytes from rats, and to test the effect of energy substrates and amino acids on protein degradation. Male Sprague-Dawley rats (150-200 g, n ≥8 per treatment) were used. Cell viability, tyrosine release as an indicator of protein degradation, and the effect of osmolarity were measured to establish the method. The effects of energy substrates (50 mM glucose, 20 mM β-hydroxybutyrate, 4.7 mM butyrate, and 30 mM glutamine) and amino acid solutions (30 mM amino acids mix, 30 mM glutamate, 1% ammonium, and 30 mM amino acids mix plus 50 mM glucose) on protein degradation were measured. Average viability at time 30 min was 85.8% (range 81-94%). Tyrosine release was linear over the course of experiments, indicating constant protein degradation (R^2= 0.9943) ($p<0.05$). Osmolarity, glucose, and glutamine had no effect on protein degradation. β-hydroxybutyrate significantly decreased it (-16%; $p<0.05$), whereas butyrate slightly increased it (+ 5%; $p<0.05$). The mixture of amino acids and glutamate increased ($p<0.05$) protein degradation (+ 10%) compared to control. Ammonium had no effect. The mixture of amino acids plus glucose decreased (-13%; $p<0.05$) protein degradation compared to the mix of amino acids only. High viability and constant rate of protein degradation indicate successful establishment of a method to estimate protein degradation in isolated small intestinal enterocytes from rats. The mixture of amino acids or glutamate may have acted as an energy source for the highly energy dependent enterocyte. Addition of glucose to the mixture of amino acids may have decreased the concentration of amino acids in tissue and abolished their

effect. The large effect of β-hydroxybutyrate suggests a potential positive role for ketone bodies to limit the loss of small intestinal protein mass by decreasing protein degradation. These findings might be particularly important in catabolic conditions such as starvation or total parenteral nutrition, where the loss of small intestinal protein mass is substantial.

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DEDICATION

To my precious little "Darya" who is a "sea" of joy and hope in my life, and to Parastoo, without whom starting into this path would have been impossible.

CHAPTER ONE: Introduction and literature review

1.1 GENERAL INTRODUCTION:

Specific nutrients affect small intestinal protein mass and metabolism. Studies have shown that some nutrients such as glutamine, glucose, short-chain fatty acids and ketone bodies influence the small intestinal protein mass (Baracos et al. 2000). The small intestine is an important organ in the body. It is one of the most metabolically active organs and has one of the highest rates of protein synthesis in the body. The small intestine plays a vital role in maintaining the nutritional status of the entire animal through digestion and absorption of nutrients. It also provides an effective barrier between the body and the external environment to prevent the passage of bacteria and toxins into the systemic circulation (McBurney 1994). The protein mass of this important and vital organ is easily affected by diet and stressors. Loss of small intestinal mass will not only lead to change and loss of small intestinal function, but will also affect the health and well being of the entire animal. To understand how nutrients affect small intestinal protein mass, several studies have investigated the effect of nutrients on the rate of small intestinal protein synthesis. In these studies the change in the rate of small intestinal protein synthesis was not totally clear and did not explain the changes in the small intestinal mass. It seems possible that a process other than protein synthesis might be involved to contribute to and account for the loss of small intestinal mass. On the other hand, studies on the effect of nutrients on the rate of small intestinal protein degradation are very limited because as yet there is no satisfactory direct method to measure the rate of small intestinal protein degradation. Developing a method to directly measure the rate of small intestinal protein degradation is necessary and very important. Also, it is of

crucial importance to develop a method to look at the direct effect of nutrients or nutritional status on protein degradation under different conditions.

1.2 SMALL INTESTINE:

In order to understand how the small intestine functions, it is important to have an understanding of the small intestine structure and cell types. The small intestine extends from the gastric pylorus to the ileocecal valve and is divided into three regions; the duodenum, the jejunum, and the ileum. Its wall contains four distinctive layers: the mucosa, sub mucosa, muscularis propria, and serosa or adventitia. The mucosa is composed of three distinct layers: the epithelium, lamina propria, and muscularis mucosae. The epithelial layer forms a barrier between the lumen and the underlying tissues (Junqueira et al.1995). The epithelial lining of the small intestine is made up of several different cell types including stem cells, Paneth's cells, absorptive cells, and goblet cells. Absorptive cells (enterocytes) are the most common cells lining the villi. The second most prominent cell type found in the epithelium are goblet cells. They secrete mucus that forms a protective lubrication lining the lumen of the small intestine. Paneth's cells function in immune response (Potten 1995). Stem cells are situated at the base of the crypts and provide a source of cells for the crypt and villus (Yoshida et al. 1992).

1.2.1 Importance and functions of small intestine

The small intestine performs a vital role in maintaining the nutritional status of the entire animal. It is responsible for digestion and absorption of nutrients. It also provides an effective barrier between the external environment and the body, to prevent the passage of bacteria and toxins into the systemic circulation of the small intestine (McBurney 1994). It has an important role in providing immune function. The small intestine is one of the most

metabolically active organs and has one of the highest rates of protein synthesis in the body (McBurney 1994).

1.2.2 Protein mass of the small intestine

The protein mass of the small intestine is easily altered by diet and stressors. Loss of protein mass may result in loss of small intestinal function. For example, if the intestinal barrier is weakened as a result of loss in protein mass, bacterial translocation may occur, leading to infection (Bouteloup-Demange et al. 2000). Conditions such as starvation, protein deficiency, total parenteral nutrition (TPN), and cancer cachexia could lead to loss of small intestinal protein mass. Starvation greatly decreases the protein mass of the small intestine (Emery et al. 1986, Samuels et al. 1996), and causes much greater losses from the small intestine than from the rest of the body (Emery et al. 1986). A protein deficient diet fed for 1-8 weeks led to an overall reduction in small intestine mass in pigs (Wykes et al. 1996). TPN compared to enteral feeding, leads to a great loss of the small intestinal mass in rats (O'Dwyer et al. 1989). Cancer cachexia has been shown to reduce protein mass of the small intestine in mice (Samuels et al. 2000). The effect of cancer cachexia was also shown to be independent of food intake. Infection is associated with a decrease in small intestinal protein mass (Samuels and Baracos 1995), an effect that was also independent of food intake.

1.2.3 Protein turnover

To understand how nutrients and nutritional status affect tissue protein mass, it is necessary to have an understanding of the contributing processes in protein turnover. Protein in the body is continuously being degraded and reconstructed. This process is called protein turnover. Protein turnover in most tissues is comprised of two different processes, protein synthesis and protein degradation. The difference in rates of these two processes determines

protein mass of the body (Sugden and Fuller 1991). To maintain body protein at a constant level, the rates of protein synthesis and degradation should be in balance with each other. If the rate of synthesis is greater than degradation, the result is growth. If the rate of protein degradation exceeds the rate of synthesis, wasting of body protein or atrophy will happen. The difference between the rates of synthesis and degradation will affect protein mass of the tissue, regardless of whether the difference resulted from a change in the rate of synthesis, degradation, or a combination of the two rates. Changes in protein mass of most tissues in the body will result from changes in the net balance between the rates of synthesis and degradation, but will not reflect how and to what extent protein synthesis and degradation are affected by the changes. Only by measuring the rate of protein synthesis and the rate of protein degradation, can the effect of each process on protein mass be determined (Sugden and Fuller 1991).

1.2.3.1 Protein synthesis

1.2.3.1.1 Description

Protein synthesis is one of the processes involved in protein turnover. In the process of protein synthesis, amino acids are incorporated into proteins. First, the information encoded within DNA is transferred to RNA. This process of transcription produces ribosomal RNA (rRNA), transfer RNA (tRNA), and messenger RNA (mRNA) (Sugden and Fuller 1991). The mRNA then serves as a template from which cytosolic ribosomes can use aminoacyl tRNAs as building blocks to synthesize protein (Lehninger et al. 1993).

1.2.3.1.2 Methodology

There are different methods available to measure the rate of protein synthesis in a tissue, and all are based on the same general principle (Sugden and Fuller 1991). The rate of protein synthesis can be measured by monitoring the incorporation of an isotopically labeled amino acid into protein, over a specific period of time. The most common methods for determining the incorporation of tracer amino acids into tissue protein are the flooding dose and the constant infusion methods. The flooding dose method involves the injection of a large dose of a radio labeled amino acid, followed by measuring the amount of radio labeled amino acid incorporated into the protein of a tissue of interest. This method estimates the rate of tissue protein synthesis over a short period of time; usually 10-30 min. Short labeling time is beneficial when measuring protein synthesis in tissues with high secretory activity and high rate of protein turnover such as the small intestine. The flooding dose method is the most reasonable way to measure the rate of tissue protein synthesis in small animals, because it involves a single rapid injection of tracer, instead of a prolonged infusion period (Davis and Reeds 2001). The constant infusion method involves the infusion of a trace amount of a labeled amino acid at a constant rate for a longer period of time, usually 4-8 h, until steady state labeling of the free amino acid pool is obtained. This method is suitable to measure protein synthesis in tissues with slow protein turnover rate such as muscle. In tissues with rapid protein turnover rate such as the small intestine, the rate of protein synthesis might be affected by the amount of tracer that has been recycled. The constant infusion method also might underestimate the rate of protein synthesis in tissues such as small intestine, in which a significant amount of synthesized protein is exported and escapes measurement (Davis and Reeds 2001).

1.2.3.2 Protein degradation

1.2.3.2.1 Description

Protein degradation is the second process that is essential to protein turnover. There are several pathways for the degradation of proteins. Three known pathways are the lysosomal, Ca2+ activated, and ubiquitin-proteasome systems. Lysosomal proteases, such as cathepsins, degrade extracellular, intracellular, membrane and secretory proteins (Attaix et al. 1994). Lysosomes are largely found in the liver and kidneys (Attaix et al. 1994). Calcium dependent proteases are also known as calpains. Their specific functions are not clear and it seems that they do not perform a major role in protein turnover. However, calpains may be involved in regulating the degradation of short-lived proteins (Attaix et al. 1994).

The ubiquitin-proteasome system is a major pathway for protein degradation in most cells. It involves the ubiquitin and the proteasome complex (Attaix et al. 1994). This pathway is generally believed to be the major pathway for the degradation of abnormal proteins or short-lived regulatory components. The ubiquitin-proteasome system has been reported to be the key pathway in the breakdown of skeletal muscle proteins in animal models of cancer cachexia, sepsis, starvation, and in some human diseases (Attaix et al. 1994, Attaix et al. 1998). In the ubiquitin-proteasome pathway, ubiquitin is first activated in the presence of ATP to a high-energy mediator by the ubiquitin-activating enzyme (E1) to form polyubiquitin chains. Then the polyubiquitin chains are covalently attached to the target protein with the help of ubiquitin conjugating enzyme (E2). Attachment of polyubiquitin chains to target proteins is recognized as a degradation signal by the 26s proteasome complex. The next step is the degradation of polyubiquitylated proteins by 26s proteasome complex. The 20s proteasome is the core of the proteolytic machinery. The 20s core of the

26s proteasome contains many subunits that have protease activity. The breakdown of ubiquitylated proteins results in peptides and free and reusable ubiquitin (Attaix et al. 1998).

1.2.3.2.2 Methodology

Different methods are available to measure the rate of protein degradation (Sugden and Fuller 1991, Attaix et al. 1998). Indirect measurement estimates the rate of protein degradation in vivo as the difference between protein synthesis and growth. Protein degradation could also be estimated directly in vitro by measurement of the release of an amino acid such as tyrosine (Sugden and Fuller 1991). However, this method can only measure protein degradation in very small or thin tissues, as it is necessary for all the cells to be in close contact with incubation media (Fulks et al. 1975).

1.2.4 Small intestinal protein turnover

Small intestinal protein turnover is dependent not only on the rate of protein synthesis and protein degradation, but also upon several other factors. In the small intestine, cells are continuously sloughing off into the lumen (McBurney 1994), significantly reducing the protein content of the small intestine. In addition, a large proportion of the synthesized protein in the small intestine is secretory protein. These proteins are secreted both into the lumen and systemically and therefore, reduce the protein content of the small intestine (Reeds et al. 1993).

1.2.4.1 Small intestinal protein synthesis

1.2.4.1.1 Description and methodology

One of the factors contributing to protein turnover in small intestine is protein synthesis. The small intestine has one of the highest rates of protein synthesis in the body. In

young growing mammals, it accounts for 9-16% of whole body protein synthesis, even with the fact that its protein mass only accounts for a small percentage of total body protein (Attaix et al. 1988). The fractional rate of protein synthesis in the small intestine in rats has been estimated to be as high as 100%, indicating that over a period of one day, the whole protein mass in the small intestine can be reconstructed (Samuels et al. 1996). The flooding dose method is the method of choice to measure protein synthesis in small intestine. This method avoids problems of high protein turnover, high secretory contribution of the small intestine, and high recycling rate of infused amino acids.

1.2.4.2 Small intestinal protein degradation

1.2.4.2.1 Description

Small intestine protein mass is also affected by protein degradation. The contribution of protein degradation on small intestinal protein balance has not been widely investigated. However, studies on small intestinal protein metabolism have concluded that protein degradation may be an important determinant of intestinal protein balance. This was concluded because although during a number of different experimental situations, protein mass decreased, protein synthesis either slightly increased or remained unchanged (Samuels et al. 1996, Burrin et al. 2000, Vann et al. 2000). Also results from a study on overnight fasted piglets, to test the effects of nutrients on intestinal protein metabolism, suggested that small intestinal catabolism contributes to the small intestinal protein balance (Adegoke et al. 2003). To understand the meaning of the changes in the small intestinal mass where protein synthesis is relatively unchanged, it is necessary to determine protein degradation itself.

1.2.4.2.2 Methodology

Measuring the rate of protein degradation in the small intestine is difficult. The rate of protein degradation cannot be simply measured by the difference between the growth rate and the rate of protein synthesis, as indirect in vivo measurements. In the subtracting method, the assumption is that the difference between the growth rate and the rate of protein synthesis is the rate of protein degradation. This assumption is not true in the small intestine, as small intestinal protein content is reduced by factors other than protein degradation. In the small intestine, cells are continuously sloughing off into the lumen (McBurney 1994). Also, a large proportion of the synthesized protein in the small intestine is secreted both into the lumen and systemically (Reeds et al. 1993). These two factors significantly reduce the protein content of the small intestine by mechanisms other than protein degradation.

The rate of protein degradation can be directly estimated in vitro by measuring the rate of release of an amino acid such as tyrosine (Sugden and Fuller 1991). Tyrosine is used as an indicator of protein degradation because this amino acid is not metabolized nor degraded in the small intestine (Lin and Knox 1958, Gillam et al. 1974, Rodwell 1988, Fitzpatrick 2003). This property of tyrosine shows that all the released tyrosine is the product of protein degradation, not the result of tyrosine metabolism from other sources. However, to my knowledge, there are no published data on measuring the rate of protein degradation in the intestine using in vitro techniques. Another way to indirectly determine the rate of protein degradation is to measure the mRNA levels for the various proteolytic pathways. In skeletal muscle, increased mRNA levels of components of proteolytic systems have been shown to be related to increase excretion of urinary 3-methylhistidine. Increase in excretion of urinary 3-methylhistidine is an indicator of skeletal muscle protein degradation in vivo (Samuels et al.

1996, Attaix et al. 1998). Although measuring mRNA levels do not indicate changes in proteolysis, they can show the possible molecular mechanisms that are responsible for protein wasting. There are as yet no satisfactory direct methods for estimating protein degradation in the small intestine.

1.3 NUTRITION AND SMALL INTESTINAL PROTEIN TURNOVER

Nutritional status plays a role in regulating small intestinal protein metabolism and mass. The effect of nutritional status on the regulation of intestinal protein degradation is much less well known compared to that of protein synthesis (Adegoke et al. 2003). Different nutritional conditions affect protein synthesis, protein degradation, or both, but it is not clear how these conditions regulate intestinal protein mass and turnover (Baracos et al. 2000).

1.3.1 Starvation

Starvation has a significant negative impact on protein mass of the small intestine. For example, protein content of the small intestine in the adult rat decreased about 26% after 1 day of starvation. After 5 days of starvation, the protein content of the small intestine decreased about 47% (Samuels et al. 1996). Most studies have shown that the rate of protein synthesis in the small intestine of starved rats either slightly decreased or did not change (McNurlan et al. 1979, Emery et al. 1986, Burrin et al. 1991a). For example, Samuels and colleagues (1996) showed that small intestinal protein synthesis rate did not change after 1 day of starvation. Concluding from these studies, it is not clear how small intestinal mass is regulated during starvation. To my knowledge, the effect of starvation on the rate of small intestinal protein degradation has not yet been directly studied.

1.3.2 Feeding

Feeding increases small intestinal protein mass. Re-feeding stimulates the rate of small intestinal protein synthesis in 7-and 26-day-old pigs (Davis et al. 1996). Protein deficiency on the other hand, decreases small intestinal protein mass. Wykes and colleagues (1996) showed that the rate of jejunal mucosal protein synthesis was lower in young pigs fed a protein deficient diet for 8 weeks. Different studies have investigated the effect of nutritional status on small intestinal protein turnover. These studies have shown that protein synthesis might play an important role in regulating small intestinal protein turnover. However, the effect of nutritional status on small intestinal protein degradation has not yet been investigated.

1.3.3 Total parenteral nutrition (TPN)

Total parenteral nutrition, introduced to clinical medicine about 40 years ago, is usually considered a significant advance in the nutritional support of a large proportion of patients. TPN has become a standard nutritional therapy for patients who are temporarily or sometimes permanently not able to have oral feeding, are unable to absorb enterally provided nutrients, or cannot tolerate enteral feeding (Buchman et al. 1995). It is commonly used as an alternative nutritional support in different conditions in clinical settings (Dudley et al. 1998). In seriously ill patients who cannot be fed orally or those with intestinal disorders such as short bowel, TPN is the choice of feeding and becomes vital to their survival (Thakur et al. 2002). If TPN is not given to patients with extensive intestinal resection, the result is severe malnutrition that leads to increased morbidity and mortality (Jeejeebhoy 2001).

1.3.3.1 TPN and small intestinal protein mass and turnover

TPN feeding may have a large and significant adverse effect on small intestinal mass. TPN feeding is associated with intestinal atrophy, decrease in protein and DNA content, decrease in villus height (Burrin et al. 1994, Bertolo et al. 1999), and impaired intestinal barrier to bacterial translocation (Li et al. 1994). It may reduce intestinal barrier function and immunological competence in trauma patients (Kudsk et al. 1992). Its atrophic effect on small intestinal morphology and function has been shown in human subjects (Pironi et al. 1994, Buchman et al. 1995). TPN feeding significantly decreased villus height and crypt depth in two patients after 2 months compared to five healthy controls on oral feeding (Pironi et al. 1994). TPN resulted in decreased total mucusa thickness by decreasing villus cell count and height (Buchman et al. 1995). In this study however, the crypt depth was not affected. The loss of mucosal structure may result in increased intestinal permeability, the clinical significance of which remains to be defined. Total parenteral nutrition compared to oral feeding is also associated with a significant decrease in the rate of intestinal protein synthesis in rats (Stein et al. 1994) and in piglets (Dudley et al. 1998). Animal studies have shown significant intestinal villus atrophy after a few days (Miura et al. 1992), but although in human subjects intestinal morphologic and functional changes occur when TPN is the sole nutritional source, the findings are substantially less significant than those observed in animal models. Small intestinal atrophy has not been seen even after one month of sole TPN feeding in humans and there is little evidence that intestinal atrophy and increased bacterial translocation occur in humans while on TPN feeding (Jeejeebhoy 2001). The effect of TPN on small intestinal protein degradation has not been studied yet.

1.4 OSMOLARITY

Cellular hydration and consequently cell volume is affected by the exposure of cells to different and unequal osmotic pressures. Hypo-osmotic pressure results in cell swelling and increased cell volume. Conversely, when cells are exposed to hyper-osmotic pressure, the result is cell shrinkage and decreased cell volume. However, there are mechanisms to regulate cell volume. These mechanisms act to prevent excessive cell volume changes and restore regular cell volume (Häussinger 1996). The mechanisms responsible for regulating cell volume after exposure of cells to different osmotic pressures, involve the activation of ion transport systems in the plasma membrane, and in some cell types, the release or accumulation of organic osmolytes. Cellular hydration states play an important role in cellular metabolic function to such an extent that it is believed that hormones and nutrients exert their effects on metabolism partially by modifying cell volume (Häussinger 1996).

1.4.1 Cellular hydration and protein turnover

Cellular hydration state plays a major role in the control of proteolysis in liver (Häussinger et al. 1991). Studies have shown a relationship between proteolytic activity and hepatic cellular hydration, indicating that cell swelling inhibits protein degradation, whereas cell shrinkage stimulates protein degradation (Häussinger et al. 1990, Halbrucker et al. 1991, Häussinger et al. 1991, Von Dahl et al. 1991). However, it remains unclear how cellular hydration exerts its control on protein degradation. The study by Meijer and colleagues (1993) adds to this uncertainty. They showed that in isolated hepatocytes (in the absence of amino acids) compared to iso-osmotic conditions, hypo-osmotic pressure not only did not inhibit protein degradation, but also slightly stimulated it. However, with addition of amino acids to the perfusion medium, cell volume increased and proteolysis was inhibited. The

difference in results of Häussinger et al (1991) and Meijer et al (1993) is due to the presence or absence of amino acids. Häussinger concludes that cell swelling following hypo-osmotic pressure (in the presence of amino acids) inhibits protein degradation and claims that it is the hypo-osmotic pressure that inhibits protein degradation, whereas Meijer claims that hypo-osmotic pressure increases the sensitivity of the inhibitory effect of amino acids on protein degradation and shows that in the absence of amino acids, hypo-osmotic pressure does not inhibit protein degradation. When amino acids are added, hypo-osmotic pressure inhibits protein degradation.

1.4.2 Cellular hydration and small intestinal protein metabolism

Small intestinal mucosa is exposed to unequal osmotic pressure and possesses variable states of cellular hydration (Ferraris et al. 1990). There is limited knowledge about the effect of aniso-osmotic pressure on small intestinal protein metabolism. Using their in-situ system, Adegoke and colleagues (1999a) showed that osmolarity did not affect small intestinal mucosal protein synthesis in piglets. In contrast with this, Marsman and McBurney (1996) showed that a hyper-osmotic NaCl solution compared to control, suppressed protein synthesis in isolated rat colonocytes. These two studies are different in species and cell or tissue types. To my knowledge the direct effect of osmolarity on the rate of small intestinal protein degradation has not been studied yet.

1.5 NUTRIENTS AND SMALL INTESTINAL PROTEIN TURNOVER

A number of specific nutrients influence the regulation of intestinal protein metabolism and mass. Studies have shown that glutamine, glucose, short-chain fatty acids, and ketone bodies affect small intestinal mass and metabolism (Baracos et al. 2000). The exact nature of this influence is not known and the results are contradictory. Structure of the

small intestine is an important factor to consider when investigating the regulation of intestinal protein metabolism. The unique aspect of the small intestine is the exposure of luminal and systemic membranes of the mucosa to two different environments; therefore, feeding through either side could affect and regulate small intestinal protein metabolism. Studies on intestinal protein turnover during TPN, indicate that luminal nutrients *per se* might be needed to conserve small intestinal protein mass. Luminal nutrients might exert their effect by increasing protein synthesis, decreasing protein degradation, or a combination of both processes (Baracos et al. 2000). When studying the effect of nutrients on small intestinal mass and metabolism, it is necessary to consider different factors. These factors include 1: differences between the routes of nutrient delivery (luminal versus intravenous feeding), 2: the direct or indirect effect of nutrients, 3: if treatments are short-term or long-term, and 4: if animals or subjects are healthy and non-stressed or sick and under stress (Baracos et al. 2000). To study direct effects of nutrients on the rate of small intestinal protein synthesis, Adegoke and colleagues (1999a) developed an in situ experimental system. In this system, nutrients are exposed to the luminal side of the mucosa while the systemic effect is avoided; therefore, the direct effect of nutrients on small intestine mass is investigated. This is important because the sole effect of any given nutrient is studied, in the absence of a systemic effect. Also this system can be used to study the effect of both luminal and systemic routes.

1.5.1 Amino acids

1.5.1.1 Amino acids and small intestinal mass and growth

Glutamine is the major fuel for enterocytes (Windmueller 1982, Souba et al. 1985). It promotes growth, metabolism, structure, and function of the intestinal mucosa (Windmueller

1982). The small intestine is the main organ for glutamine utilization in post-absorptive rats (Windmueller and Spaeth 1980) and pigs (Rerat et al. 1992). In vitro studies have shown that glutamine stimulates intestinal cell proliferation (Scheppach et al. 1994, Rhoads et al. 1997). It has been shown that intravenous glutamine delivery during recovery from prolonged starvation, increases small intestinal mass (Inoue et al. 1993). In this study, starved animals re-fed by glutamine-supplemented (1.5% glutamine in a standard mix of amino acids) TPN solution showed a significant recovery in their intestinal mucosal weight, mucosal thickness, and villous height after 7 days compared to animals re-fed with standard solution lacking glutamine. Also, O'Dwyer and colleagues (1989) showed increased jejunal mucosal weight, DNA content, and villus height in healthy rats receiving intravenous standard parenteral solution supplemented with glutamine after 7 days. Addition of glutamine to parenteral nutrition feeding has been shown to promote villus height and to prevent TPN induced increase in intestinal permeability (Van der Hulst et al. 1993, Kaibara et al. 1994, and Li et al. 1994). Addition of glutamine (0.20-0.26 g/kg bodyweight) to a standard TPN solution fed to patients for 10-14 days prevented intestinal permeability and improved mucosal structure (Van Der Hulst et al. 1993). However, intravenous glutamine failed to influence small intestinal mass in healthy animals. Burrin and colleagues (1991b) showed that addition of glutamine (5% of total amino acid content) to TPN solution had no affect on small intestinal mass in healthy young piglets after 7 days of exclusive TPN feeding. The effect of long-term intravenous glutamine was also investigated in stressed animals. Garcia-Arumi and co-workers (1995) showed that addition of glutamine to a standard TPN solution did not significantly increase jejunal mucosal thickness, DNA content, or protein content in rats with non-hypercatabolic surgical stress after 8 days.

Glutamine has also been shown to enhance protein content in rat jejunum when given orally to rats with enterocolitis (Fox et al. 1988). In this study, oral delivery of glutamine (2% glutamine) to rats with methotrexate- induced enterocolitis resulted in increased mucosal weight, protein, and DNA content of the jejunum compared to control rats receiving glycine (2% glycine).

The effect of glutamate on small intestinal mass and growth has also been studied. Glutamate has metabolic characteristics similar to those of glutamine in enterocytes under normal physiologic conditions (Reeds et al. 1997). Glutamate also plays a significant role in biosynthesis of glutathione (a three peptide that can be synthesized in mammals from glutamate, glycine, and cysteine). Glutathione is an important peptide because of its role in the protection of the mucosa from peroxidative damage and dietary toxins (Aw and Williams 1992) and that its rate of synthesis in the mucosa is very high (Jahoor et al. 1996). Addition of glutamic acid to TPN solution at physiological doses (5% of total amino acid content) fed to healthy young piglets did not affect small intestinal protein or DNA content after 7 days (Burrin et al. 1996). In this study, plasma glutamic acid concentration did not change.

1.5.1.2 Amino acids and small intestinal protein synthesis

The effects of glutamine supplementation on small intestinal protein synthesis have been investigated in animal models using in vivo and in vitro methods. Results of these studies do not agree. This may be due to the differences in length of study, whether the nutrients were supplied via oral or TPN, whether subjects were healthy or sick, and whether the nutrient had direct or indirect effects.

In vitro studies have shown that glutamine increases enterocyte protein synthesis. Higashiguchi and co-workers (1993) showed that different glutamine concentrations

stimulated protein synthesis in isolated enterocytes from rat jejunum. Incubation of enterocytes in a medium with a glutamine concentration of 0.67 mmol/L, stimulated protein synthesis compared to a medium lacking glutamine (this amount reflects the normal plasma glutamine concentration). Addition of glutamine at five times its plasma concentration to the medium did not significantly stimulate the rate of protein synthesis compared to 0.67 mmol/L. Further studies indicated that the stimulatory effect might not be a specific effect of glutamine because energy substrates increased the rate of protein synthesis to the same degree as glutamine (Higashiguchi et al. 1993).

The effect of long-term intravenous glutamine on small intestinal protein synthesis in healthy animals has been investigated. Addition of glutamine (11.1 g/L of glycyl glutamine) to a standard TPN solution in healthy rats for 5 days, stimulated small intestinal protein synthesis (Stein et al. 1994). Short (4 h) intravenous infusion of glutamine to healthy, well-nourished, and growing dogs after 24 h fasting, failed to stimulate the rate of duodenal protein synthesis compared to either intravenous saline or iso-nitrogenous glycine infusion (Marchini et al.1999). Intravenous glutamine delivery to septic rats was investigated by Yoshida and colleagues (1992). They showed that addition of glutamine to a TPN solution increased the rate of protein synthesis in the intestinal mucosa of septic rats. However, addition of glutamine to a standard TPN solution failed to increase the rate of small intestinal protein synthesis in rats with non-hypercatabolic surgical stress after 8 days (Garcia-Arumi et al. 1995).

The effect of luminal glutamine on the rate of small intestinal protein synthesis has also been investigated. Enteral glutamine delivery (5 g/kg body weight/day) to human subjects with hypercatabolic state induced by treatment with glucocorticoids failed to

significantly increase the rate of mucosal protein synthesis in the post-absorptive state and during feeding (Bouteloup-Demange et al. 2000).

Using an in situ method that allows exposure of the mucosa to nutrients on the luminal side without having systemic effects, Adegoke and co-workers (1999b) showed that short-term luminal glutamine (1.8 mmol/L) delivery to healthy overnight fasted piglets had no effect on the rate of protein synthesis compared to animals receiving saline. This amount of glutamine represents the amount of glutamine in rat's digesta in the fed state (Adibi and Mercer 1973). Luminal perfusion of 30 mmol/L of glutamine decreased the rate of mucosal protein synthesis by 20 to 25% compared to saline (Adegoke et al. 1999a). This suggests that glutamine does not directly or acutely increase protein synthesis. If glutamine affects the rate of small intestinal protein synthesis, it might be indirect.

The effect of amino acid mixtures on small intestinal protein synthesis has been investigated in different studies. Luminal delivery of a 30 mM amino acid mixture (including glutamine) to over-night fasted piglets, decreased mucosal protein synthesis by 20 to 25% compared to saline (Adegoke et al. 1999b). This concentration of amino acids reflects that found in digesta after a meal (Adibi and Mercer 1973).

A mixture of amino acids (containing glutamine) at plasma concentration, stimulated protein synthesis in isolated enterocytes in rats compared to an amino acid mixture lacking glutamine (Higashiguchi et al. 1993). The stimulatory effect of an amino acid mixture (with glutamine) was also seen in a mixture containing a concentration five times plasma concentration. Amino acid mixtures at plasma concentration and at five times plasma concentration had no effect on protein synthesis in isolated enterocytes when glutamine was absent in the mixtures (Higashiguchi et al. 1993). Results from this study indicate that an

amino acid mixture (with no glutamine) does not affect protein synthesis in isolated enterocytes from rats. The effect of the amino acid mixture was due to the presence of glutamine. Again, the effect of glutamine may be non-specific because energy substrates increased the rate of protein synthesis to the same degree as glutamine.

The effect of glutamate on small intestinal protein synthesis has also been studied. Glutamate has metabolic characteristics similar to those of glutamine for the enterocytes in normal physiologic conditions (Reeds et al. 1997). Addition of glutamate to enteral nutrition may increase mucosal protein synthesis (Hasebe et al. 1999). In this study, an enteral diet containing 30% glutamate (of total amino acids) was fed to rats with severe burns for 64 h. Results of this study indicate that glutamate may enhance mucusal protein synthesis in burned rats (Hasebe et al. 1999). In conclusion, the results from the studies mentioned above do not give a clear answer to the direct and the degree of effect of amino acids on small intestinal protein synthesis. This indicates the importance and value of investigating the effect of amino acids on small intestinal protein degradation.

1.5.1.3 Amino acids and small intestinal protein degradation

The effect of amino acids on small intestinal protein degradation has not been directly studied, since there are as yet no satisfactory direct methods for estimating the rate of this process. However, Adegoke et al. (2003) measured the mRNA levels for components of the ubiquitin-proteasome system to indirectly examine the effect of amino acids on small intestinal protein degradation. Using their in-situ system, Adegoke et al. (2003) measured mRNA levels for components of proteolytic systems in response to a 30 mM amino acid mixture or 30 mM glutamine. The amino acid levels in the luminal perfusate were formulated based on analysis of jejunal digesta after feeding (Adibi and Mercer 1973). Thirty

mM glutamine was included to substantially increase the total tissue free glutamine concentration since studies have shown the importance of this amino acid as a preferred fuel for enterocytes (Souba et al. 1985, Windmueller 1982), and in stimulating intestinal growth and protein synthesis under a variety of patho-physiological conditions (O'Dwyer et al. 1989, Stein et al. 1994). Luminal delivery of the 30 mM amino acid mixture or 30 mM glutamine (Adegoke et al. 2003) significantly decreased mRNA levels of components of the ubiquitin-proteasome system. Results from this study indicate that luminal amino acids may have a direct effect on regulating small intestinal protein degradation, since amino acid perfusion had no apparent systemic effect on accumulation of free amino acids and did not increase hormone levels. These luminal perfusions significantly increased the concentration of most amino acids in tissue compared to phosphate buffered saline (PBS) perfusion. It was noted that no one single amino acid seemed to be responsible for suppressive affect of amino acid mixture on mRNA levels for components of the ubiquitin-proteasome system. Also it was noted that as the concentration of total mucosal amino acids increased, the mRNA levels for components of the ubiquitin-proteasome system decreased.

The effect of intravenous delivery of amino acid mixtures on small intestinal protein degradation has indirectly been studied by Adegoke et al. (2003). In this study, the amino acid mixture was given as a bolus equal to about 1/3 of the daily protein requirement for pigs. This approach substantially increased plasma amino acid concentrations to a level seen after a meal. Intravenous delivery of the amino acid mixture to over-night fasted piglets, compared to saline significantly decreased mRNA levels for components of the ubiquitin-proteasome system.

It was shown that when amino acids were given both luminaly and intravenously and the highest mucosal level of amino acids was achieved, the effect of amino acids on reducing mRNA levels for ubiquitin-proteasome system was greater than when they were given either luminaly or intravenously (Adegoke et al. 2003). The in situ system used by Adegoke et al. (2003) implies a direct effect of amino acids on mRNA levels and that amino acids directly reduce mucosal protein degradation. In conclusion, we need to have a method to measure the direct effect of amino acids on the rate of small intestinal protein degradation.

1.5.2 Glucose

Glucose is the main product formed in hydrolysis of most carbohydrates after digestion. It is the main monosaccharide in the bloodstream and an important source of energy for cells. Glucose is an important fuel for the small intestine in fed state (McBurney 1994). Despite the potential importance of glucose as an energy source for the small intestine, to my knowledge, the specific effect of glucose on small intestinal mass has not been studied. I am not aware of any study on the effect of glucose on small intestinal protein mass.

1.5.2.1 Glucose and small intestinal protein synthesis

The effect of luminal and/or intravenous glucose perfusion on small intestinal protein synthesis has been studied. Luminal glucose has been shown to increase the rate of jejunal protein synthesis in the rat. Weber and colleagues (1989) demonstrated that, in fed rats, luminal perfusion of a 10-cm jejunal segment of rats for 1.75 h with 56 mM glucose increased the rate of jejunal mucosal protein synthesis. In this study, the size of the perfused segment relative to the total absorptive capacity of the small intestine was large. As a result this would likely have raised plasma glucose and insulin levels and would have had a

systemic effect. Therefore it is difficult to distinguish between the direct effect of glucose and the systemic effect of hormones released in response to glucose. However, using an in situ method where the systemic affect was avoided, luminal delivery of 50 mM glucose did not affect small intestinal protein synthesis (Adegoke et al. 1999b). In this study, because of the small size of the perfused segments relative to the total absorptive capacity of the small intestine, systemic levels of perfused nutrients and plasma levels of hormone were not changed. However, intravenous delivery of glucose to overnight fasted piglets, increased mucosal protein synthesis rate by 16% (Adegoke et al. 2003). The failure of luminal but not intravenous glucose to affect protein synthesis, suggests that glucose might indirectly increase small intestinal protein synthesis, since plasma glucose and insulin levels were increased (Adegoke et al. 2003).

1.5.2.2 Glucose and small intestinal protein degradation

The effect of glucose on small intestinal protein degradation has only been indirectly studied. Adegoke and colleagues (2003) measured the mRNA levels of components of ubiquitin-proteasome and calcium-activated proteolytic systems to indirectly assess the effect of glucose on small intestinal protein degradation in piglets. Results from this study showed that intravenous glucose delivery had no effect on the mRNA levels of components of these proteolytic systems. This suggests that glucose may not influence mucosal protein degradation. It must be noted that mRNA levels of proteolytic systems do not measure proteolysis. The need for developing a method to directly measure the effect of glucose on small intestinal protein degradation is necessary.

Luminal co-perfusion of glucose plus amino acids abolished the suppressive affect of amino acids on the mRNA levels of components for ubiquitin-proteasome system. Addition

of 50 mM glucose to 30 mM amino acids significantly lowered the total mucosal free amino acid concentration in the corresponding intestinal segments compared to segments perfused with the amino acid mixture alone (Adegoke et al. 2003). Again, this suggests that when the intracellular amino acid concentration is higher, the mRNA level for components of the proteolytic system is lower.

1.5.3 Short-chain fatty acids (SCFA)

Short-chain fatty acids (acetate, propionate, and butyrate) are produced in the cecum and colon of non-ruminant animals and humans by fermentation of unabsorbed carbohydrates and dietary fiber. Short-chain fatty acids are metabolized to CO_2 and other metabolites by the colonic epithelial cells. Butyrate is known to influence the proliferation and differentiation of cells such as colonocytes. It is an important fuel for colonic epithelial cells. It has been shown that butyrate is metabolized more readily to CO_2 than other potential substrates such as acetate, propionate, glucose, glutamine, long-chain fatty acids, and ketone bodies (Fitch and Fleming 1999). Despite the fact that SCFA are important as an energy source and for cell growth, little is known about how and to what extent they affect small intestinal protein mass and turnover. Inclusion of SCFA to TPN feeding to rats increased ileal RNA and DNA content compared to a TPN solution lacking SCFA (Tappenden et al. 1997). Short-chain fatty acids suppressed the rate of protein synthesis in isolated rat colonocytes (Marsman and McBurney 1996). However, an iso-osmotic solution of NaCl decreased the rate of protein synthesis by the same amount, suggesting that the effect of SCFA was non-specific. Luminal delivery of 50 mM SCFA had no effect on mucosal protein synthesis in overnight fasted piglets compared to an iso-osmotic saline solution (Adegoke 1999b). Stein and colleagues (1994) showed that addition of butyrate to TPN solution

stimulated protein synthesis in the jejunum from rats receiving standard TPN solution for 5 days. Also, butyrate had a rapid effect on intestinal adaptation and growth in TPN fed piglets after jejunoileal resection (Bartholome et al. 2004). Although, butyrate has a positive effect in TPN as an energy source, its effect on intestinal protein synthesis is not clear. It is worthwhile to investigate the effect of SCFA and butyrate in particular on the rate of small intestinal protein degradation to obtain the whole picture of their effect on small intestinal protein metabolism. The effect of SCFA on small intestinal protein degradation has not been studied yet.

1.5.4 Ketone bodies

β-hydroxybutyrate and acetoacetate are known as ketone bodies. They are made in the liver from partial oxidation of fatty acids. They can be used by most aerobic tissues as an energy source including the small intestine (McBurney 1994). Ketone bodies are an excellent energy substrate for the small intestine because they are easily transported into the peripheral tissue. They act as major energy substrates when carbohydrate supply is limited. For example, in prolonged starvation, ketone bodies become major energy substrates (Katayama et al. 1994). In the post-absorptive state, acetoacetate and β-hydroxybutyrate provide 47% of energy to the small intestine (Windmueller and Spaeth 1978). Their effect on the small intestinal protein synthesis and mass has been studied. Luminal delivery of 20 mM β-hydroxybutyrate had no effect on mucosal protein synthesis in overnight fasted piglets (Adegoke et al. 1999b). Addition of β-hydroxybutyrate to total parenteral nutrition (TPN) increases small intestinal mass and inhibits its atrophy (Kripke et al. 1988). Therefore, it might influence small intestinal growth. The effect of ketone bodies on small intestinal protein degradation is unknown. In skeletal muscles, the direct effect of ketone bodies on the

rate of protein degradation has been studied in a variety of *in vitro* systems, using tyrosine as an indicator of protein degradation. It was shown that 4 and 6 mM β-hydroxybutyrate significantly decreased intracellular tyrosine concentration in isolated quarter diaphragms from 48 h fasted rats (Palaiologos and Felig 1976). Also Tischler and colleagues (1982) provided evidence that addition of a mixture of 1 mM acetoacetate, 4 mM β-hydroxybutyrate, and 5 mM glucose to incubation media decreased the rate of protein degradation in 48 h fasted rat diaphragms. In 24 h fasted chicks, 4 mM acetoacetate or β-hydroxybutyrate significantly decreased the rate of muscle protein degradation in extensor digitorum muscles (Wu and Thompson 1990). These studies provide evidence that ketone bodies are effective in reducing the rate of skeletal muscle protein degradation. The above findings are supported by an *in vivo* experiment that showed that in stressed human subjects, ketone bodies significantly reduced net body protein loss by decreasing urinary nitrogen and 3-methylhistidine excretion (Pawan and Semple 1983).

In summary, to understand how nutrients and nutritional status affect small intestinal protein mass, the focus of studies so far, has been on investigating the effect of nutrition or nutrients on the rate of small intestinal protein synthesis. The results of these studies have not clearly explained the extent and direction of this effect. The change in small intestinal protein synthesis (either slight increase or no change) was not totally clear to explain the decrease in small intestinal mass. It seems more likely that a process other than protein synthesis might be involved to account for the loss of small intestinal mass. On the other hand, studies on the effect of nutrients or nutrition on the rate of small intestinal protein degradation are very limited due to lack of a satisfactory direct method to measure the rate of small intestinal protein degradation. Developing a method to directly measure the rate of small intestinal

protein degradation is necessary and very important. This will give the opportunity to look at the direct effect of nutrients or nutritional status on small intestinal protein degradation.

1.6 HYPOTHESES

The hypotheses of my thesis are that:

1. the rate of protein degradation in isolated small intestinal enterocytes from rats can be determined by measuring tyrosine release;
2. amino acids, butyrate, and β-hydroxybutyrate decrease the rate of protein degradation in isolated small intestinal enterocytes from rats; and
3. glucose or mixture of a mix of amino acids plus glucose will not affect the rate of protein degradation in isolated small intestinal enterocytes from rats.

1.7 OBJECTIVES

The objectives of my thesis are:

1. to establish a method to measure the rate of protein degradation in isolated small intestinal enterocytes from rats by determining viability and linear tyrosine release; and
2. to measure the effect of specific nutrients on the rate of protein degradation in isolated small intestinal enterocytes from rats.

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CHAPTER TWO: effect of energy substrates on protein degradation in isolated small intestinal enterocytes from rats

2.1 INTRODUCTION

Specific nutrients affect small intestinal protein mass and metabolism. Studies suggest that energy yielding nutrients such as β-hydroxybutyrate, glucose, short-chain fatty acids, and glutamine may influence small intestinal protein growth (Kripke et al. 1988, Baracos et al. 2000). Inclusion of energy nutrients to total parenteral nutrition (TPN) increases small intestinal mass and inhibits its atrophy (Kripke et al. 1988, Burke et al. 1989, Koruda et al. 1990). Ketone bodies (β-hydroxybutyrate and acetoacetate) are an excellent source of energy. They act as an important energy fuel during prolonged starvation or when carbohydrate supply is limited (Katayama et al. 1994). Glucose is an important energy substrate for the small intestine in the fed state (McBurney 1994). Butyrate is the preferred oxidative fuel for colonocytes. In colonic epithelial cells, it is utilized to a greater extent than other short-chain fatty acids such as acetate and propionate (Roediger 1982, Fleming et al. 1991). Inclusion of SCFA to TPN enhances functional recovery from intestinal resection in rats (Tappenden et al. 1997). Glutamine is the major fuel for enterocytes (Windmueller and Spaeth 1978, Windmueller and Spaeth 1980, Windmueller 1982, Souba et al. 1985) and promotes growth, metabolism, structure, and function of the intestinal mucosa (Windmueller 1982). The small intestine has a vital role in maintaining the nutritional and health status of

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the entire animal. In animal models, the protein mass of this organ is easily affected by diet and stressors and is rapidly lost during catabolic conditions such as starvation, cancer cachexia, infection, and the use of TPN (Baracos et al. 2000). For instance, TPN resulted in decreased digestive capacity (Burrin et al. 1994, Bertolo et al. 1999), weakened intestinal barrier to bacterial translocation (Li et al. 1994), and decreased mucosal protein synthesis (Dudley et al. 1999). Therefore, loss of small intestinal mass not only impairs small intestinal function, but also affects the health and normal function of the entire animal.

A clear understanding of the processes that regulate small intestinal mass is needed when investigating the effect of energy nutrients on protein mass. Protein mass of the small intestine is affected by two independent mechanisms; protein synthesis and degradation. Most studies have focused on the effect of energy nutrients on small intestinal protein synthesis. Results of these studies have shown that nutrients positively affect small intestinal mass but have not given a clear explanation to the extent and direction of the effect of nutrients on small intestinal protein synthesis (Baracos et al. 2000). β-hydroxybutyrate had no direct effect on small intestinal protein synthesis (Adegoke et al. 1999). Butyrate did not affect protein synthesis in isolated rat colonocytes (Marsman and McBurney 1996), but when added to standard TPN solution, it increased mucosal protein synthesis (Stein et al. 1994). Luminal delivery of glucose to piglets did not affect small intestinal protein synthesis (Adegoke et al. 1999).

Protein degradation is a catabolic process that likely affects small intestinal mass. Evidence suggests a regulatory role for protein degradation in small intestine (Samuels et al. 1996, Burrin et al. 1991, Bouteloup-Demange et al. 1998, Halseth et al. 1997). These studies have suggested that protein degradation might account for the loss of small intestinal mass

during a variety of catabolic conditions such as starvation, TPN, and exercise. Studies on the direct effect of energy nutrients on the rate of small intestinal protein degradation are very limited due to the lack of a method. Furthermore, the direct effect of energy yielding substrates on small intestinal protein degradation has not yet been reported. Therefore, the focus of this study was to establish and test a method to directly estimate protein degradation in isolated enterocytes from rat small intestine, and to test the effect of energy yielding nutrients (β-hydroxybutyrate, glucose, butyrate, and glutamine) on protein degradation. Also, since the addition of nutrients increased the osmolarity of incubation media, another objective of this study was to test the effect of high osmotic pressure on protein degradation in isolated enterocytes.

2.2 MATERIALS AND METHODS

2.2.1 Chemicals

All chemicals used were from Sigma-Aldrich (St. Louis MO. USA) unless otherwise stated.

2.2.2 Animals

Male Sprague-Dawley rats (150-200 g) were obtained from the University of British Columbia Animal Care Centre (Vancouver, B. C. Canada). For the initial validation experiments, rats were obtained on the day of the experiment. For the experiments to determine the effects of nutrients on protein degradation, rats were obtained 2 to 3 days in advance to allow them to adjust to their new environment before they were used. These rats were housed in group polycarbonate cages. Rats were maintained at room temperature (22°C-24°C) on a 12 h light dark cycle (lights on at 0800 h). Rats were given free access to water and food (standard rodent chow, UBC Animal Care Centre). Rats were provided with nesting material and a nesting box. All animal studies were carried out in accordance with the National Research Council Guide for the care and use of laboratory animals (see appendix one).

2.2.3 Preparation of enterocytes

Enterocytes were prepared from rat small intestine based on the methods used by Watford and colleagues (1979) and Wu and colleagues (1994). Briefly, rats were killed using Halothane. The entire small intestine was removed and rinsed two times with (37°C) oxygenated (95% O_2-5% CO_2) luminal perfusate solution. Luminal perfusate solution contained Ca^{2+} free Krebs-Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 mM KCl,

1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$, pH 7.4), 20 mM HEPES (pH 7.4), 7 mM EDTA (pH 7.4), 5 mM glucose, and 0.67 mM glutamine. Glutamine and glucose are important energy sources in the fed and immediate post-absorptive state. Enterocytes in vivo never face an environment with zero amounts of glutamine and glucose. I chose to include a basal amount of glutamine and glucose in the media to supply enterocytes with a basal source of energy. The small intestine was then rinsed once with (37°C) oxygenated luminal perfusate filling solution containing 25% bovine serum albumin (BSA)(Sigma A-7030) and 1 mM DL-dithiothreitol (DTT). The small intestine was ligated about 10 cm from the pyloric sphincter. The next 50 to 60 cm of the small intestine was filled with 37°C oxygenated luminal perfusate filling solution until it was slightly distended and then was ligated. This intestinal segment was placed in 37°C oxygenated luminal perfusate solution. The intestine was incubated at 37°C in shaking water bath (70 oscillations/min) for 20 min. At the end of incubation, the intestine was removed and gently patted with fingertips for 2 min to help cells to come off. The lumen was drained into a 50 ml polypropylene tube. From this and subsequent points no glassware was used to prevent clumping and all the plastic-ware was siliconized.

The cells were centrifuged (Sorvall RC 5B Plus) at 1500 rpm for 3 min at room temperature, washed with about 20 ml of 37°C oxygenated cell wash solution containing KHB buffer, 2.5 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, and 0.67 mM glutamine but no EDTA, and then gently resuspended three to four times. Enterocytes were centrifuged, resuspended, and washed three times in all. The supernatant was discarded after each wash. After the third wash, cells were resuspended in a volume of cell wash solution so that there were about 10^7 cells/ml. One ml of resuspended cells was used for incubation experiments.

At least three 1 ml samples of resuspended cells were taken for determination of protein content and stored at -24°C until assayed.

2.2.4 Incubation of enterocytes

Incubations were performed at 37°C in 15 ml polypropylene tubes in a shaking water bath (70 oscillations/min). One ml of prepared enterocytes was added to each tube containing 1 ml of 37°C oxygenated incubation media at time 0 min. These tubes were individually and continuously gassed (95% O_2-5% CO_2) while shaking. The final basal incubation media was cell wash solution plus 2% BSA and 0.5 mM cycloheximide. This was considered the control group. Cycloheximide was added to prevent reincorporation of tyrosine into protein (Fulks et al. 1975). Cells were incubated up to 45 min.

2.2.5 Tyrosine release and rate of protein degradation

Tyrosine release was used as an indicator of protein degradation, since tyrosine is not metabolized nor degraded in the small intestine (Lin and Knox 1958, Gillam et al. 1974, Rodwell 1988, Fitzpatrick 2003). To determine the amount of tyrosine in incubated enterocytes, 0.5 ml ice-cold 25% TCA was added to each incubation tube to stop all metabolic activity and tyrosine release from protein. These tubes were stored at -24°C until assayed. To determine the amount of tyrosine released from enterocytes during the actual incubation, the amount of tyrosine in the time 0 min sample was subtracted from the amount of tyrosine in incubation tubes after the specified incubation times. The rate of protein degradation was calculated by dividing the amount of tyrosine released by the period of incubation and by the protein content. The results are expressed as nmol tyrosine/30 min/mg protein.

2.2.6 Experiment one: Establishment of a method to measure protein degradation in isolated small intestinal enterocytes from rats.

To determine if cells were viable, viability was measured using trypan blue exclusion. Viability of isolated enterocytes was measured in cells from 10 rats after 0, 15, 30, and 45 min of incubation. Tyrosine release was measured in cells from 12 rats at times 0, 10, 20, 30, and 40 min in the initial validation experiment to ensure the linearity of released tyrosine during the period of incubation. Linearity of tyrosine release indicates constant protein degradation over the course of the experiment.

The addition of nutrients will increase the osmolarity of the incubation medium. In hepatocytes, changes in osmotic pressure and consequently cellular hydration states, influence protein degradation (Häussinger 1996). Since the effect of osmolarity on protein degradation in the small intestine is not known, the effect of high osmolarity was tested to determine if it affected protein degradation in isolated enterocytes. Protein degradation was measured when cells were exposed to control (360 mosm/L) or high osmotic pressure (460 mosm/L). Addition of nutrients increased the osmolarity of the incubation media up to 410 mosm/L. In light of this, I decided to test the effect of high osmolarity (460 mosm/L) on protein degradation in isolated enterocytes because small intestinal luminal osmolarity fluctuates about 100 mosm/L (Ferraris et al. 1990). High osmotic pressure was induced by increasing the amount of NaCl in the incubation medium.

2.2.7 Experiment two: The effect of β-hydroxybutyrate, glucose, butyrate, and glutamine on protein degradation in isolated small intestinal enterocytes from rats

Tyrosine release was measured after 30 min incubation using eight rats per treatment. The effect of 20 mM β-hydroxybutyrate, 50 mM glucose, 4.7 mM butyrate, and 30 mM

glutamine was tested in triplicate within any individual rat. The chosen amount of β-hydroxybutyrate is within the physiological range that is found in plasma during ketoacidosis in diabetes and had no effect on the small intestinal mucosal protein synthesis in piglets (Adegoke et al. 1999). Fifty mM glucose represents the concentration found in digesta after a meal (Ferraris et al. 1990). Butyrate is the preferred oxidative fuel for colonocytes and is utilized to a greater extent than other SCFA; acetate and propionate (Roediger 1982, Fleming et al. 1991). Also, this amount of butyrate in a mix of SCFA did not affect the rate of protein synthesis in piglets (Adegoke et al. 1999). Luminal delivery of 30 mM glutamine to piglets significantly decreased mRNA levels of components for the ubiquitin-proteasome system (Adegoke et al. 2003). The result of each treatment within a rat was averaged. Viability was determined at the beginning and after 30 min incubation time. Three rats were used per day.

2.2.8 Quantification of tyrosine and protein

The amount of tyrosine was determined using a fluorometric method (Waalkes and Udenfriend 1957). Fluorescence was determined using a Shimadzu fluorimeter (Shimadzu Spectrofluorophotometer RF-540. Shimadzu Corporation. Kyoto, Japan). Protein content of the enterocytes samples was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich). Samples were thawed and dissolved by adding 1 ml of 1 M NaOH to each tube at 37°C. A triplicate sample of 20 µl of each dissolved protein sample was used to determine the amount of protein in each tube using a spectrophotometer (Shimadzu UV-160 Spectrophotometer).

2.2.9 Statistical Analyses

The effect of nutrients and osmolarity was analyzed by ANOVA using Randomized Complete Block Design. In this design, rats were the individual blocks. Therefore, effects of

treatments were tested within each individual animal. Differences between means of each treatment and the control group were tested using Dunnett's test. The linearity of tyrosine release was tested using linear regression analysis. Data are means ± SEM. Probability values less than 0.05 were defined as being statistically significant.

2.3 RESULTS

2.3.1 Experiment one: Establishment of a method to measure protein degradation in isolated small intestinal enterocytes from rats

The average viability was 88.8% at 0 min (Figure 2.1). Viability was high at the beginning of the experiment and remained high up to 30 min. At 30 min of incubation the average viability was 85.8% (range 81-94%). At 45 min, viability noticeably decreased. In the treatment experiments, average viability was about 87% at 30 min (range 85-97%), (data not shown). Viability was never lower than 81% at 30 min of incubation in any of the experiments. Tyrosine release was linear over the period of incubation (R^2= 0.995) ($p<0.05$) (Figure 2.2). From the viability and tyrosine release data, we concluded that it was appropriate to measure protein degradation in subsequent experiments at 30 min. The average rate of protein degradation in isolated enterocytes in the initial validation and the nutrients experiments was 3.86 nmol tyrosine/30 min/mg protein. High osmotic pressure did not affect the rate of protein degradation in isolated enterocytes from rats compared with control ($p<0.05$) (Figure 2.3).

2.3.2 Experiment 2: The effect of β-hydroxybutyrate, glucose, butyrate, and glutamine on protein degradation in isolated small intestinal enterocytes from rats

β-hydroxybutyrate significantly decreased (-16%; $p<0.05$) protein degradation in isolated enterocytes compared with control (Figure 2.4). Glucose and glutamine did not affect protein degradation in isolated enterocytes, whereas butyrate slightly increased it compared to control (+5%; $p<0.05$).

2.4 DISCUSSION

Protein degradation is a catabolic process that may make an important contribution to the regulation of small intestinal protein mass and metabolism. There is limited knowledge and understanding about the possible regulatory effect of protein degradation on small intestinal protein mass. This is largely due to a lack of a suitable method to directly estimate protein degradation in the small intestine. I successfully adapted and established a method to directly measure protein degradation in isolated enterocytes. This was achieved by a high viability and linear tyrosine release. Viability of isolated enterocytes in my experiments remained high during incubation time and is comparable to the literature values (Higashiguchi et al. 1993). Tyrosine release was linear during the incubation time, indicating a constant rate of protein degradation. Tyrosine release has been widely used to estimate protein degradation in incubated skeletal muscles (Attaix et al. 1994, Sugden and Fuller 1991), but in small intestine to my knowledge, this study is the first to directly measure protein degradation in isolated enterocytes. Protein degradation in isolated enterocytes was 3.86 nmol tyrosine/mg protein/30 min, which is at least 300% higher than the rate of protein degradation in skeletal muscle from rats (Temparis et al. 1994). This high rate of protein degradation in the small intestine, gives more direct and stronger evidence that protein degradation may significantly contribute to and play an important role in regulating small intestinal protein mass.

I did not observe any effect of osmolarity on protein degradation in isolated enterocytes. This is a significant finding because if an effect is seen with the addition of nutrients that in parallel increases the osmolarity of the basal incubation media, this could be attributed to the nutrient itself not to the increased osmolarity. Cellular hydration states play

an important role in cellular metabolic function to such an extent that it is believed that hormones and nutrients exert their effects on metabolism partially by modifying cell volume. In liver, this role has been extensively studied showing that cell swelling inhibits protein degradation, whereas cell shrinkage stimulates protein degradation (Häussinger et al. 1990, Häussinger et al. 1991). My study suggests that unlike liver, small intestine's protein degradation is not influenced by changes in osmotic pressure. No other study had looked at the effect of osmotic pressure on protein degradation in small intestine.

Using our model system, I measured the effect of energy yielding substrates on protein degradation in isolated enterocytes from rat small intestine. This is the first study to look at the direct effect of β-hydroxybutyrate, glucose, butyrate, and glutamine on protein degradation in isolated enterocytes. β-hydroxybutyrate significantly decreased protein degradation in isolated enterocytes. There is no other study on the effect of ketone bodies on small intestinal protein degradation with which to compare my results. However, using tyrosine as an indicator of protein degradation, the direct effect of ketone bodies on the rate of protein degradation in skeletal muscles has been studied in a variety of *in vitro* systems. It was shown that 4 and 6 mM β-hydroxybutyrate significantly decreased intracellular tyrosine concentration in isolated quarter diaphragms from 48 h fasted rats (Palaiologos and Felig 1976). Also my finding is in agreement with the study of Tischler and colleagues (1982) who provided evidence that addition of a mixture of 1 mM acetoacetate, 4 mM β-hydroxybutyrate, and 5 mM glucose to incubation media decreased the rate of protein degradation in 48 h fasted rat diaphragms. In 24 h fasted chicks, 4 mM acetoacetate or β-hydroxybutyrate significantly decreased the rate of muscle protein degradation in extensor digitorum muscles (Wu and Thompson 1990). These studies provide evidence that ketone

bodies are effective in reducing the rate of skeletal muscle protein degradation. The above findings are supported by an *in vivo* experiment that showed that in stressed human subjects, ketone bodies significantly reduced net body protein loss by decreasing urinary nitrogen and 3-methylhistidine excretion (Pawan and Semple 1983). In conditions such as TPN or when food intake is decreased, ketone bodies may have a potential role in modulating the small intestinal protein mass, a role that has been shown in skeletal muscle. My data suggest that β-hydroxybutyrate may reduce loss of protein mass in the small intestine by decreasing protein degradation, since it had no stimulatory effect on the rate of small intestinal protein synthesis (Adegoke et al. 1999). This effect might give ketone bodies a potential significant use in TPN, in which the large loss of protein mass in stressed subjects could be limited by decreasing small intestinal protein degradation.

Glucose did not affect protein degradation in isolated enterocytes. Glucose is an important fuel for the small intestine in the fed state (McBurney 1994). Despite the potential importance of glucose as an energy source for the small intestine, to my knowledge, the specific effect of glucose on small intestinal protein degradation has not been studied yet. However, the indirect effect of glucose on protein degradation was tested and showed that a large bolus of intravenous glucose had no effect on the mucosal mRNA levels for proteolytic systems (Adegoke et al. 2003). The lack of effect of glucose on protein degradation in isolated enterocytes in my experiments, and on the mucosal mRNA levels in the study of Adegoke and colleagues (2003), suggests that glucose may not influence small intestinal protein degradation.

Butyrate unexpectedly increased protein degradation in isolated enterocytes. I chose to test the effect of butyrate on protein degradation since it did not influence protein

synthesis in colonocytes or in the small intestine. Marsman and McBurney (1996) showed that 5 mM butyrate in a 15 mM mix of SCFA did not affect the rate of protein synthesis in isolated rat colonocytes. Also, 4.7 mM butyrate in a 50 mM mixture of SCFA had no effect on the mucosal protein synthesis in piglets (Adegoke et al. 1999). The effect of butyrate seen in my experiment was small and may not be biologically relevant. My finding is surprising given the clear and rapid effect of butyrate on intestinal adaptation and growth in TPN fed piglets after jejunoileal resection (Bartholome et al. 2004). However, the difference could be attributed to the difference in species, health status of the animal, and the route of delivery employed in this study and in that of mine. It may also be that butyrate affects intestinal growth by a mechanism other than by protein degradation. Also, another explanation could be that butyrate may have provided a little extra energy to stimulate the activity of existing and highly energy dependent proteolytic systems in the small intestine.

I did not observe any effect of glutamine on protein degradation in our experiment. The effect of glutamine on small intestinal protein degradation had not directly been studied previously. However, luminal delivery of 30 mM glutamine to piglets, significantly decreased mRNA levels of components of the ubiquitin-proteasome system (Adegoke et al. 2003). Interestingly, enteral glutamine decreased mRNA levels of ubiquitin proteasome systems, but did not alter mRNA levels for cathepsin D or m-calpain (Coëffier et al. 2003). However, it should be noted that measuring mRNA levels of proteolytic systems is an indirect method that may suggest a decrease in protein degradation; whereas, my approach is a direct measurement of protein degradation.

In summary, I achieved high viability and linear tyrosine release in my experiments. This indicates a successful establishment of a method that can be used to estimate protein

degradation in isolated enterocytes from the small intestine in rats. One strength of our system is that in any individual animal, the effect of different factors such as hormones or nutrients could be tested at the same time. Nevertheless, it must be remembered that my experiments were done in an *in vitro* system, which is an artificial environment and in a short period of time, since cells remained viable for only 30 min. Also, this method only addresses protein degradation in the epithelium and not other layers of the small intestine. My findings suggest that butyrate, glucose, and glutamine might not directly contribute to the maintenance of gut protein mass via decreasing protein degradation. If these nutrients do affect protein degradation, my data suggest that they would have to act indirectly. Among energy yielding substrates, the large and significant effect of β-hydroxybutyrate indicates a potential positive role for ketone bodies to limit atrophy and loss of small intestinal protein mass by decreasing protein degradation. This gives ketone bodies a possible significant use in catabolic conditions such as TPN or in conditions where food intake is compromised. In such conditions, loss of small intestinal protein mass is substantial and life threatening. Further studies are needed to investigate the effect of ketone bodies on protein degradation under different catabolic conditions.

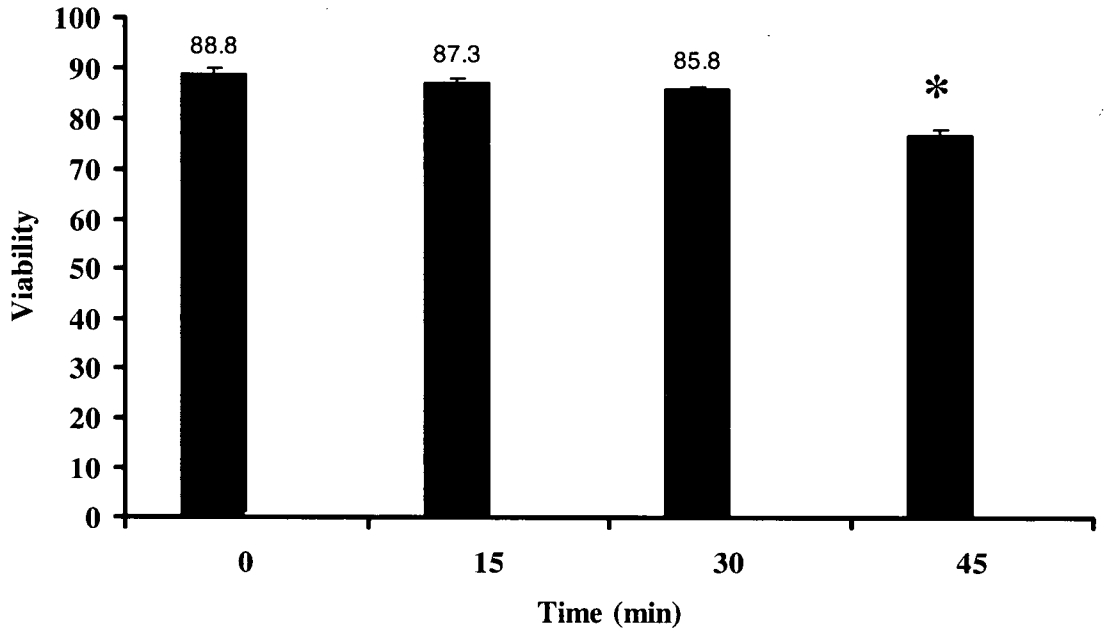

Figure 2.1: Viability of isolated small intestinal enterocytes from rats over time.

Enterocytes were isolated from rat small intestine. Viability of cells from 12 rats was measured using trypan blue exclusion by adding 0.5 ml of incubated cells to viability tubes containing 1.3 ml saline and 0.3 ml trypan blue solution. * significantly different from other times ($p<0.05$).

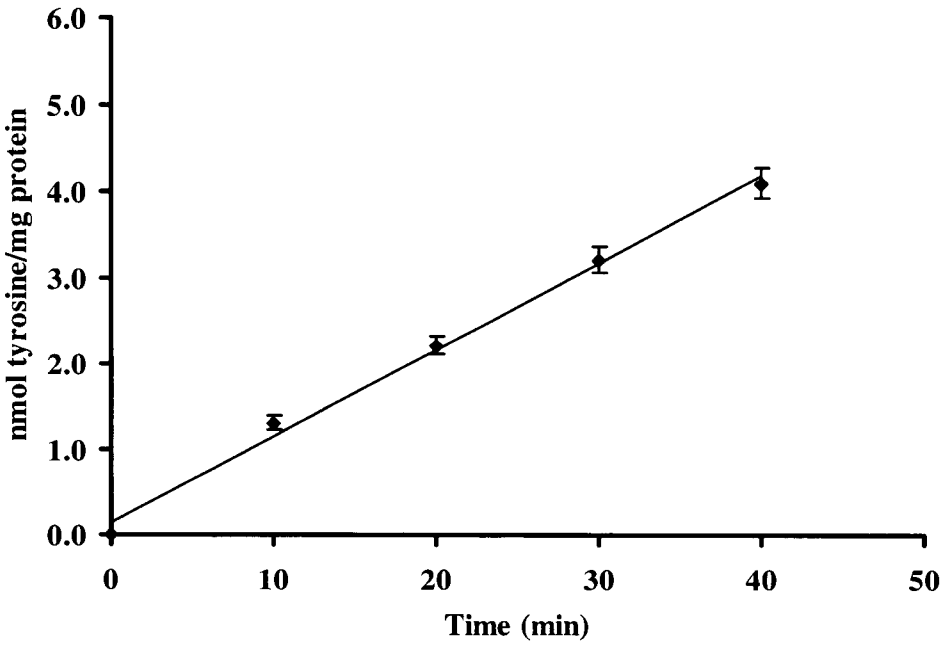

Figure 2.2: Tyrosine release in isolated small intestinal enterocytes from rats over time.

Tyrosine release was measured in cells from 12 rats at times 0, 10, 20, 30, and 40 min in the presence of 0.5 mM cycloheximide and it was linear over the period of incubation. Cycloheximide was added to prevent re-incorporation of tyrosine into protein. One ml of cells supernatant was used to determine the amount of tyrosine release using a fluorometric method. Fluorescence was determined using Shimadzu fluorimeter. Tyrosine release is expressed as nmol tyrosine/mg protein.

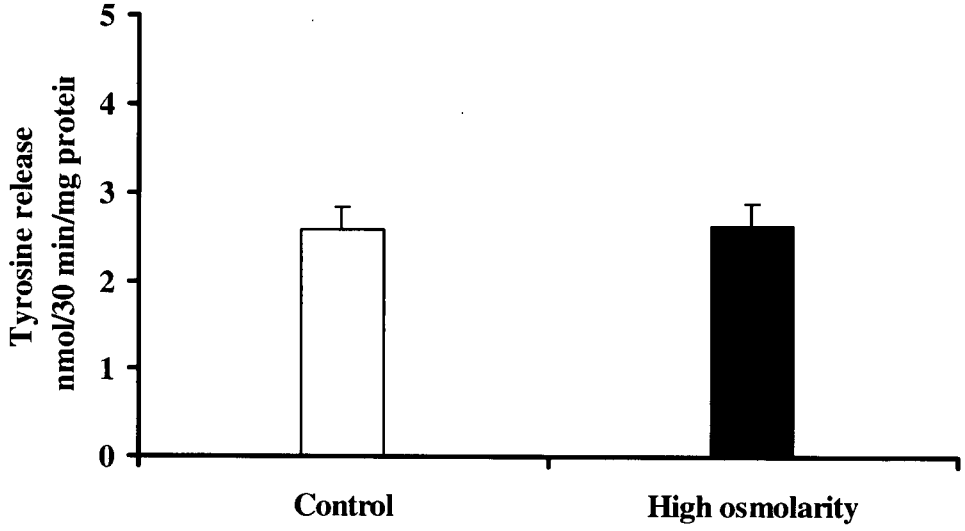

Figure 2.3: Effect of osmolarity on protein degradation in isolated small intestinal enterocytes from rats. Isolated enterocytes were incubated in the presence of different osmotic pressures: control (360 mosml/l), and high osmolarity (460 mosmol/l) and 0.5 mM cycloheximide. Cycloheximide was added to prevent re-incorporation of tyrosine into protein. Tyrosine release was used as an indicator of protein degradation. Values are nmol tyrosine release/30 min/mg protein.

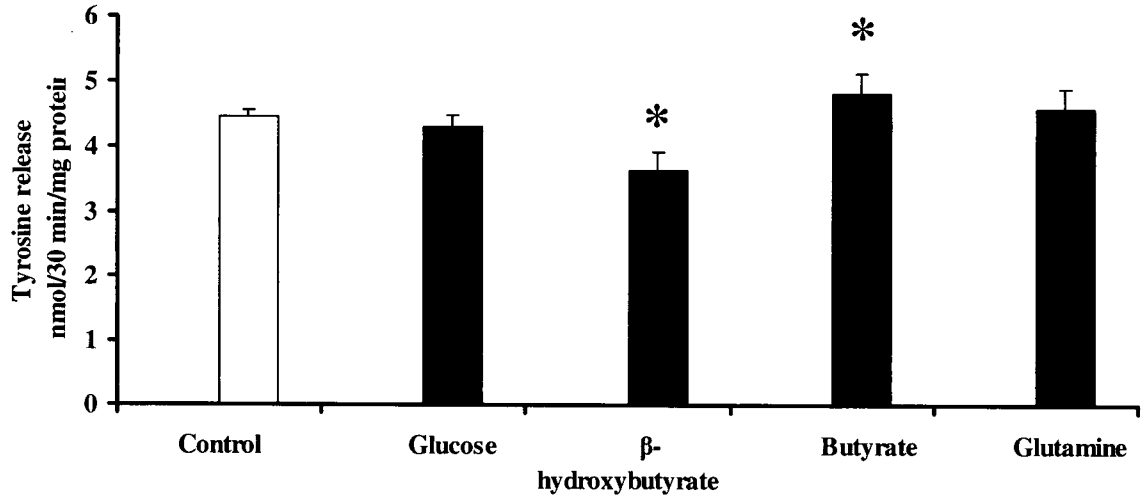

Figure 2.4: Effect of energy substrates on protein degradation in isolated small intestinal enterocytes from rats. Isolated enterocytes were incubated in the presence of 50 mM glucose, 20 mM β-hydroxy-butyrate, 4.7 mM butyrate, and 30 mM glutamine and 0.5 mM cycloheximide. Cycloheximide was added to prevent re-incorporation of tyrosine into protein. Tyrosine release was used as an indicator of protein degradation. Values are nmol tyrosine release/30 min/mg protein. * significantly different from control ($p<0.05$). Eight rats were used to test the effect of energy substrates.

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CHAPTER THREE: Effect of amino acids on protein degradation in isolated small intestinal enterocytes from rats

3.1 INTRODUCTION

Amino acids are the main metabolic fuels for the small intestine and are considered essential nutrients in its metabolism (Stoll et al. 1998, Wu 1998). Glutamine is the major fuel for enterocytes (Windmueller and Spaeth 1978 and 1980, Windmueller 1982, Souba et al. 1985). The small intestine is the main organ for glutamine utilization in postabsorptive rats (Windmueller and Spaeth 1980) and pigs (Rerat et al. 1992). Glutamine promotes growth, metabolism, structure, and function of the intestinal mucosa (Windmueller 1982). Glutamate is produced from glutamine by glutaminase (Newsholme et al. 1994). In healthy animals almost all dietary glutamate is absorbed and catabolized by the enterocytes (Wu 1998). Glutamate might be a preferable fuel for enterocytes and increased mucosal protein synthesis in burned rats, when added to enteral nutrition (Hasebe et al. 1999) but has no effect on small intestinal growth in healthy piglets (Burrin et al. 1991). Studies have shown that a mixture of amino acids or individual amino acids such as glutamine or glutamate may influence small intestinal protein growth (Higashiguchi et al. 1993, Adegoke et al. 1999b, Hasebe et al. 1999, Marchini et al. 1999, Baracos et al. 2000, Adegoke et al. 2003).

The small intestine is an important organ in the body. It plays an important role in maintaining the nutritional and health status of the entire animal. Its protein mass is sensitive to diet and stressors and is rapidly lost during catabolic conditions such as starvation, protein deficiency, cancer cachexia, infection, and during the use of TPN (Baracos et al. 2000). Starvation greatly decreases the protein mass of the small intestine (Emery et al. 1986,

Samuels et al. 1996). Protein deficiency causes an overall reduction in small intestinal mass in pigs (Wykes et al. 1996). Cancer cachexia has been shown to reduce protein mass of the small intestine in mice (Samuels et al. 2000). Infection is associated with a decrease in small intestinal protein mass (Samuels and Baracos 1995). In animals, TPN resulted in decreased digestive capacity (Burrin et al. 1994, Bertolo et al. 1999), weakened intestinal barrier to bacterial translocation (Li et al. 1994), and decreased mucosal protein synthesis (Dudley et al. 1998). TPN compared to enteral feeding, leads to a substantial loss of small intestinal mass in rats (O'Dwyer et al. 1989). Therefore, loss of small intestinal mass not only impairs small intestinal function, but also affects the health status and normal function of the entire animal.

A clear understanding of the processes that regulate small intestinal mass is needed when investigating the effect of nutrients on its protein mass. Protein mass of the small intestine is affected by two independent mechanisms; protein synthesis and degradation. Most studies have focused on the effect of nutrients on small intestinal protein synthesis. Protein degradation is a catabolic process that likely affects small intestinal mass. Evidence suggests a regulatory role for protein degradation in the small intestine (Burrin et al. 1991, Samuels et al. 1996, Halseth et al. 1997, Bouteloup-Demange et al. 1998). These studies have suggested that protein degradation might account for the loss of small intestinal mass during a variety of catabolic conditions such as starvation, TPN, and exercise. I have recently developed a method to directly measure protein degradation in isolated small intestinal enterocytes from rats. I showed that isolated enterocytes remain highly viable up to 30 min and that tyrosine release was linear during this time, indicating constant protein degradation in enterocytes. My data also suggested that β-hydroxybutyrate but not other energy substrates decreased protein degradation (Saberi et al. 2006). This finding might be

significant in catabolic conditions where loss of protein mass is substantial. The direct effect of amino acids on small intestinal protein degradation has not yet been reported. However, using their in-situ method, Adegoke and colleagues (2003) showed that luminal or intravenous delivery of a mixture of amino acids or luminal delivery of glutamine significantly decreased mRNA levels for the components of the ubiquitin-proteasome system in piglets. Interestingly, when glucose was added to the mix of amino acids, the suppressive effect of amino acids was abolished. Ammonium chloride at a concentration of 1 mmol/L had no effect on small intestinal protein synthesis in piglets (Adegoke et al. 1999b). To my knowledge there is no published data on the direct effect of amino acids on small intestinal protein degradation. This is largely due to the lack of a proper method to measure protein degradation in the small intestine. I used our newly developed method to test the effect of a mixture of amino acids, glutamine, glutamate, combination of mixture of amino acids plus glucose, and ammonium on protein degradation in isolated small intestinal enterocytes from rats.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All chemicals used were from Sigma-Aldrich (St. Louis MO. USA) unless otherwise stated.

3.2.2 Animals

Male Sprague-Dawley rats (150-200 g) were obtained from the University of British Columbia Animal Care Centre (Vancouver, B. C. Canada). For the initial validation experiments, rats were obtained on the day of the experiment. For the experiments to determine the effects of nutrients on protein degradation, rats were obtained 2 to 3 days in advance to allow them to adjust to their new environment before they were used. These rats were housed in group polycarbonate cages. Rats were maintained at room temperature (22°C-24°C) on a 12 h light dark cycle (lights on at 0800 h). Rats were given free access to water and food (standard rodent chow, UBC Animal Care Centre). Rats were provided with nesting material and a nesting box. All animal studies were carried out in accordance with the National Research Council Guide for the care and use of laboratory animals.

3.2.3 Preparation of enterocytes

Enterocytes were prepared from the rat small intestine based on the methods used by Watford and colleagues (1979) and Wu and colleagues (1994). Briefly, rats were killed using Halothane. The entire small intestine was removed and rinsed two times with (37°C) oxygenated (95% O_2-5% CO_2) luminal perfusate solution. Luminal perfusate solution contained Ca^{2+} free Krebs-Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$, pH 7.4), 20 mM HEPES (pH 7.4),

7 mM EDTA (pH 7.4), 5 mM glucose, and 0.67 mM glutamine. Glutamine and glucose are important energy sources in the fed and immediate post-absorptive state. Enterocytes in vivo never face an environment with zero amounts of glutamine and glucose. I chose to include a basal amount of glutamine and glucose in the media to supply enterocytes with a basal source of energy. The small intestine was then rinsed once with (37°C) oxygenated luminal perfusate filling solution containing luminal perfusate solution plus 25% bovine serum albumin (BSA)(Sigma A-7030) and 1 mM DL-dithiothreitol (DTT). The small intestine was ligated about 10 cm from the pyloric sphincter. The next 50 to 60 cm of the small intestine was filled with 37°C oxygenated luminal perfusate filling solution until it was slightly distended and then was ligated. This intestinal segment was placed in 37°C oxygenated luminal perfusate solution. The intestine was incubated at 37°C in a shaking water bath (70 oscillation/min) for 20 min. At the end of incubation, the intestine was removed and gently patted with fingertips for 2 min to help cells to come off. The lumen was drained into a 50 ml polypropylene tube. From this and subsequent points no glassware was used to prevent clumping and all the plastic-ware was siliconized.

The cells were centrifuged (Sorvall RC 5B Plus) at 1500 rpm for 3 min at room temperature, washed with about 20 ml of 37°C oxygenated cell wash solution containing KHB buffer, 2.5 mM $CaCl_2$, 20 mM HEPES, 5 mM glucose, and 0.67 mM glutamine but no EDTA, and then gently resuspended three to four times. Enterocytes were centrifuged, resuspended, and washed three times in all. The supernatant was discarded after each wash. After the third wash, cells were resuspended in a volume of cell wash solution so that there were about 10^7 cells/ml. One ml of resuspended cells was used for incubation experiments.

At least three 1 ml samples of resuspended cells were taken for determination of protein content and stored at -24°C until assayed.

3.2.4 Incubation of enterocytes

Incubations were performed at 37°C in 15 ml polypropylene tubes in a shaking water bath (70 oscillations/min). One ml of prepared enterocytes was added to each tube containing 1 ml of 37°C oxygenated incubation media at time 0 min. These tubes were individually and continuously gassed (95% O_2-5% CO_2) while shaking. The final basal incubation media was cell wash solution plus 2% BSA and 0.5 mM cycloheximide. This was considered the control group. Cycloheximide was added to prevent reincorporation of tyrosine into protein (Fulks et al. 1975). Cells were incubated up to 45 min.

3.2.5 Tyrosine release and rate of protein degradation

Tyrosine release was used as an indicator of protein degradation, since tyrosine is not metabolized nor degraded in the small intestine (Lin and Knox 1958, Gillam et al. 1974, Rodwell 1988, Fitzpatrick 2003). To determine the amount of tyrosine in incubated enterocytes, 0.5 ml ice-cold 25% TCA was added to each incubation tube to stop all metabolic activity and tyrosine release from protein. These tubes were stored at -24°C until assayed. To determine the amount of tyrosine released from enterocytes during the actual incubation, the amount of tyrosine in the time 0 min sample was subtracted from the amount of tyrosine in incubation tubes after the specified incubation times. The rate of protein degradation was calculated by dividing the amount of tyrosine released by the length of the incubation period and by the protein content. The results are expressed as nmol tyrosine/30 min/mg protein.

3.2.6 The effect of amino acids, ammonium, and mixture of amino acids plus glucose on protein degradation in isolated small intestinal enterocytes from rats

Tyrosine release was measured after 30 min incubation using at least eight rats per treatment. The effect of 30 mM mixture of amino acids, 30 mM glutamine, 30 mM glutamate, 1% ammonium, and a combination of 30 mM mixture of amino acids plus 50 mM glucose was tested in triplicate within any individual rat. The concentration and the composition of the 30 mM mixture of amino acids reflects what is found in digesta after a meal (Adibi and Mercer 1973) and significantly decreased mRNA levels of components for ubiquitin-proteasome system in the small intestine in piglets (Adegoke et al. 2003). Thirty mM glutamine significantly decreased the mRNA levels of the proteolytic pathway in overnight fasted pigs (Adegoke et al. 2003). Considering the fact that glutamate has similar characteristics to glutamine for enterocytes, this high concentration of glutamate was tested to investigate its possible effect on small intestinal protein degradation. We chose to test the possibility that the potential effect of amino acids or glutamine on protein degradation might be mediated by ammonium as it is largely produced in the metabolism of amino acids and specifically in the conversion of glutamine to glutamate. Also, this concentration of ammonium chloride had no effect on protein synthesis in overnight fasted piglets (Adegoke et al. 1999b). Perfusion of small intestinal segments with a combination of a mixture of amino acids plus glucose significantly reduced intracellular levels of most amino acids compared to perfusate containing amino acids alone. In this system, short and isolated intestinal segments comprised only less than 4% of total absorptive surface area of the small intestine of pigs (Adegoke et al. 2003). This luminal co-perfusion of amino acids and glucose abolished the suppressive affect of amino acids on the mRNA levels for components of the

ubiquitin-proteasome system (Adegoke et al. 2003). The result of each treatment within a rat was averaged. Viability was determined at the beginning and after 30 min incubation time. Three rats were used per day.

3.2.7 Quantification of tyrosine and protein

The amount of tyrosine was determined using a fluorometric method (Waalkes et al. 1957). Fluorescence was determined using a Shimadzu fluorimeter (Shimadzu Spectrofluorophotometer RF-540. Shimadzu Corporation. Kyoto, Japan). Protein content of the enterocyte samples was determined using a bicinchoninic acid protein assay kit. Samples were thawed and dissolved by adding 1 ml of 1 M NaOH to each tube at 37°C. A triplicate sample of 20 µl of each dissolved protein sample was used to determine the amount of protein in each tube using a spectrophotometer (Shimadzu UV-160 Spectrophotometer).

3.2.8 Statistical Analyses

The effect of nutrients and osmolarity was analyzed by ANOVA using Randomized Complete Block Design. In this design, rats were the individual blocks. Therefore, effects of treatments were tested within each individual animal. Differences between means of treatments and the control were tested using *Dunnett's test. Student's t test* was used to compare the effect of a mixture of amino acids to the combination of a mixture of amino acids plus glucose. Data are means ± SEM. Probability values less than 0.05 were defined as being statistically significant.

3.3 RESULTS

3.3.1 Effect of amino acids, ammonium, and a mixture of amino acids plus glucose on protein degradation in isolated small intestinal enterocytes from rats

Glutamate and a mixture of amino acids increased protein degradation in isolated enterocytes compared to control (+10%; $p<0.05$) (Figure 3.1). The combination of a mixture of amino acids plus glucose decreased protein degradation compared to the mixture of amino acids only (-13%; $p<0.05$) (Figure 3.2). Glutamine and ammonium had no effect on protein degradation in isolated small intestinal enterocytes from rats (Figure 3.1).

3.4 DISCUSSION

Protein degradation is a catabolic process that may make an important contribution to the regulation of small intestinal protein mass and metabolism. There is limited knowledge and understanding about the possible regulatory effect of protein degradation on small intestinal protein mass. Also, to my knowledge there are no published data on the direct effect of nutrients on the rate of small intestinal protein degradation. Using our model system, I measured the effect of amino acids on protein degradation in isolated enterocytes from the rat small intestine. This is the first study to look at the direct effect of glutamate, glutamine, a mixture of amino acids, and a combination of a mixture of amino acids plus glucose, and ammonium on protein degradation in isolated enterocytes.

Glutamate increased protein degradation in isolated enterocytes. Enterally delivered glutamate may enhance protein synthesis in burned rats (Hasebe et al. 1999), but in healthy piglets, it does not affect small intestinal growth and development (Burrin et al. 1991). However, in fed piglets, enteral glutamate has been shown to be the preferred source for mucosal glutathione synthesis. Glutathione plays an important role in maintaining the defense of the mucosa against toxic damage (Aw et al. 1992, Aw 1994). This suggests that glutamate might affect protein turnover by controlling the production of glutathione. Glutamate can also act as an energy source, as Hasebe and colleges (1999) showed that enteral glutamate might be the preferred fuel for enterocytes in burned rats. One explanation for the effect of glutamate in our study is that it may have acted as an energy source for the existing and energy dependent ubiquitin-proteasome system in the small intestine. We previously showed that energy substrates such as butyrate might act as a source of energy for

the ubiquitin-proteasome system in the small intestine and increase protein degradation in isolated small intestinal enterocyte from rats (Saberi et al. 2006).

I did not observe any effect of glutamine on protein degradation in my experiment. It had been previously shown that luminal delivery of 30 mM glutamine to piglets, significantly decreased mRNA levels of components for the ubiquitin-proteasome system (Adegoke et al. 2003). Interestingly, enteral glutamine decreased mRNA levels of only ubiquitin proteasome systems, but did not alter mRNA levels for cathepsin D or m-calpain (Coëffier et al. 2003). Glutamine has been shown to promote growth, metabolism, structure, and function of the intestinal mucosa (Windmueller 1982). In rats recovering from starvation, the addition of glutamine to TPN increased small intestinal mass (Inoue et al. 1993). Also in healthy rats, addition of glutamine to TPN solution, increased jejunal mucosal weight, DNA content, and villus height (O'Dwyer et al. 1989). Intravenous delivery of glutamine to patients fed standard TPN solution, prevented intestinal permeability and improved mucosal structure (Van Der Hulst et al. 1993). However, intravenous glutamine failed to influence small intestinal mass in healthy animals. Addition of glutamine to TPN solution had no affect on small intestinal mass in healthy young piglets after 7 days exclusive TPN feeding (Burrin et al. 1991b). It might be speculated that glutamine affects small intestinal mass and metabolism in sick or stressed animals. My animals were healthy and not stressed. Another explanation is that nutrients might indirectly affect small intestinal metabolism. My approach measures the direct effect of nutrients.

The mixture of amino acids increased protein degradation in isolated enterocytes. The only study that had indirectly looked at the effect of amino acids on protein degradation has shown a substantial decrease in the mRNA levels of components of the ubiquitin-proteasome

system with luminal or intravenous delivery of a mixture of amino acids (Adegoke et al. 2003). However, it should be noted that measuring mRNA levels of proteolytic systems is an indirect method that may suggest a decrease in protein degradation; whereas my approach is a direct measurement of protein degradation. Amino acids are the main metabolic fuels for the small intestine and are considered essential nutrients in its metabolism (Stoll et al. 1998, Wu 1998). Another explanation for the effect of amino acids seen in my experiment could be that the mixture of amino acids acted as energy source for the existing and highly energy dependent ubiquitin-proteasome systems in the small intestine.

The effect of addition of glucose to the mixture of amino acids seen in my experiment is consistent with that of Adegoke and colleagues (2003) who showed that glucose abolished the effect of amino acids. The direction of this effect is different but the net effect is that addition of glucose to the mix of amino acids abolishes their effect. When glucose was added to a mix of amino acids, it abolished the effect of amino acids on reducing the mRNA levels of components of ubiquitin-proteasome systems. In my study, glucose abolished the effect of amino acid on increasing the protein degradation. My data may suggest that glucose may have exerted its effect by decreasing the concentration of amino acids. However, amino acid concentrations were not measured in the present study.

In summary, I used our newly developed method to test the effect of amino acids on protein degradation in isolated enterocytes from the small intestine in rats. One strength of my system is that in any individual animal, the effect of different amino acids or amino acid mixtures could be tested at the same time. Nevertheless, it must be remembered that my experiments were done in an *in vitro* system, which is an artificial environment and in a short period of time, since cells remained viable for only 30 min. Also, this method only addresses

protein degradation in the epithelium and not other layers of small intestine. My data suggest that amino acids might contribute to small intestinal protein metabolism by their role as energy substrates. The effect of glutamine requires further investigation particularly in subjects under stress conditions and in sick animals.

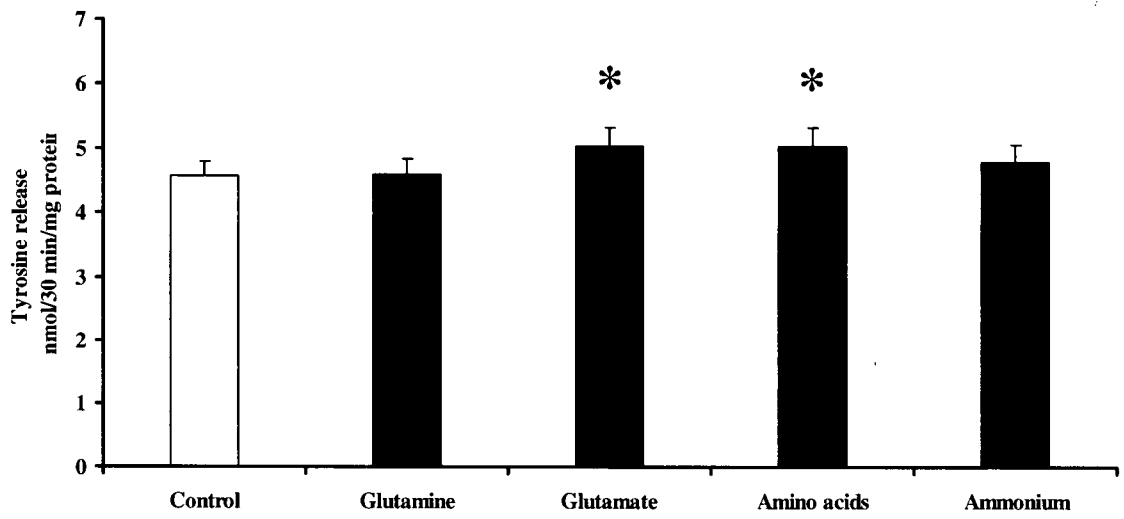

Figure 3.1: Effect of amino acids and ammonium on protein degradation in isolated small intestinal enterocytes from rats. Isolated enterocytes were incubated in the presence of a 30 mM mixture of amino acids, 30 mM glutamine, 30 mM glutamate or 1% ammonium and 0.5 mM cycloheximide. Cycloheximide was added to prevent re-incorporation of tyrosine into protein. Tyrosine release was used as an indicator of protein degradation. Values are nmol tyrosine release/30 min/mg protein. * significantly different from control ($p<0.05$). Eight rats were used to test the effect of each treatment.

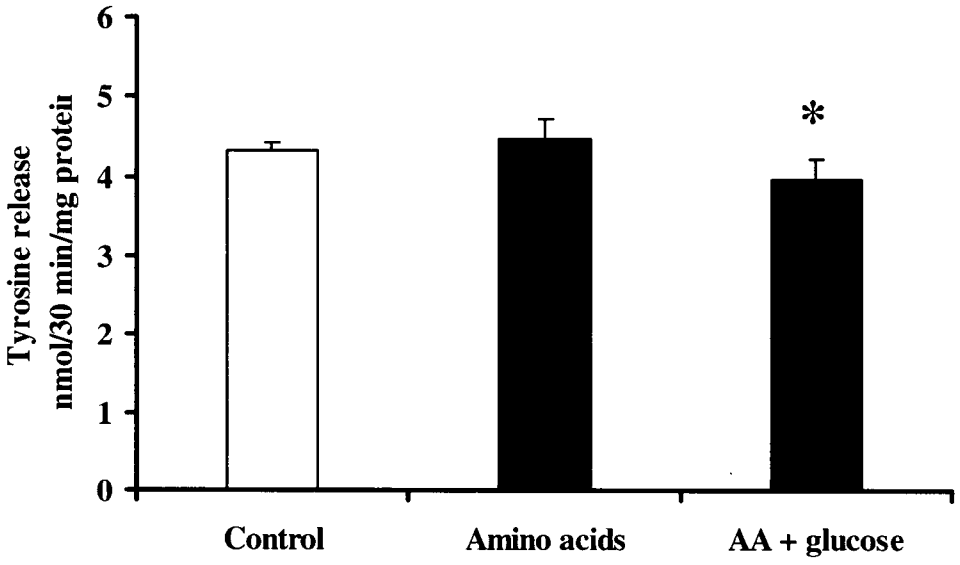

Figure 3.2: Effect of amino acids or amino acids plus glucose on protein degradation in isolated small intestinal enterocytes from rats. Isolated enterocytes were incubated in the presence of a 30 mM mixture of amino acids or a combination of a 30 mM mixture of amino acids plus 50 mM glucose and 0.5 mM cycloheximide. Cycloheximide was added to prevent re-incorporation of tyrosine into protein. Tyrosine release was used as an indicator of protein degradation. Values are nmol tyrosine release/30 min/mg protein. * significantly different from amino acids. Eight rats were used to test the effect of a mixture of amino acids and a combination of a mixture of amino acids plus glucose.

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CHAPTER FOUR: General discussion and conclusions

4.1 SMALL INTESTINE

The small intestine is an important organ in the body. It plays a vital role in maintaining the health status of the entire animal (McBurney 1994). The protein mass of this vital organ is easily affected by diet and stressors. Loss of small intestinal mass will not only lead to change and loss of small intestinal function, but also affect the health and well being of the entire animal. To understand changes in the small intestinal protein mass and factors that affect it, studies have focused on the effect of nutrients or nutritional status on the rate of small intestinal protein synthesis (Samuels et al. 1996, Burrin et al. 2000, Vann et al. 2000). In these studies changes in the rate of small intestinal protein synthesis did not completely explain the changes in the small intestinal mass. This suggests that there may be another process, other than protein synthesis, involved to account for the loss of small intestinal mass.

4.2 PROTEIN DEGRADATION

Protein degradation is a catabolic process that may make an important contribution to the regulation of small intestinal protein mass and metabolism. There is limited knowledge and understanding about the possible regulatory effect of protein degradation on small intestinal protein mass. This is largely due to a lack of a suitable method to directly estimate protein degradation in the small intestine. Measuring protein degradation in the small intestine is difficult and complicated. Small intestinal protein turnover is dependent not only on the rate of protein synthesis and protein degradation, but also upon other factors such as cell sloughing and exporting synthesized proteins. In the small intestine, cells are continuously sloughing off into the lumen (McBurney 1994), significantly reducing the

protein content of the small intestine. In addition, large proportions of the synthesized protein in the small intestine are secreted lumenally or systemically and therefore reduce the protein content of the small intestine (Reeds et al. 1993). These factors limit the choices in measuring small intestinal protein degradation. In the small intestine, protein degradation cannot be simply measured by the difference between the growth rate and the rate of protein synthesis, as indirect in vivo measurements. This method assumes that the difference between the growth rate and the rate of protein synthesis is the rate of protein degradation. This assumption is not true in the small intestine, as small intestinal protein content is reduced by factors other than protein degradation. Protein degradation can be directly estimated in vitro by measuring the amount of released amino acid such as tyrosine (Sugden and Fuller 1991). However, to my knowledge, there are no published data on measuring the rate of protein degradation in the intestine using in vitro techniques. To understand changes in the small intestinal mass, it is necessary to determine protein degradation itself using an appropriate method. Developing a method to directly measure small intestinal protein degradation is necessary and important. This will give the opportunity to look at the direct effect of nutrients or nutritional status on small intestinal protein degradation.

4.3 METHODOLOGY

A method was developed to directly measure protein degradation in isolated enterocytes. High viability and linear tyrosine release in my experiments indicate a successful establishment of the method. Viability remained high during incubation time and is comparable to the literature values (Higashiguchi et al. 1993). Tyrosine release was linear during the incubation time, indicating a constant rate of protein degradation. Tyrosine release has been widely used to estimate protein degradation in incubated skeletal muscles (Sugden

and Fuller 1991, Attaix et al. 1994), but in the small intestine to my knowledge, my studies are the first to directly measure protein degradation in isolated small intestinal enterocytes from rats. Protein degradation in isolated enterocytes was 3.86 nmol tyrosine/mg protein/30 min, which is at least 300% higher than the rate of protein degradation in rats' skeletal muscle (Temparis et al. 1994). This high rate of protein degradation in the small intestine, gives more direct and stronger evidence of the importance of the small intestine and the fact that protein degradation may significantly contribute to and play an important role in regulating small intestinal protein mass.

4.4 OSMOLARITY

Osmolarity had no effect on protein degradation in isolated enterocytes. This is a significant finding because if an effect is seen with the addition of nutrients that in parallel increases the osmolarity of the basal incubation media, this could be attributed to the nutrient itself not to the increased osmolarity. Cellular hydration states play an important role in cellular metabolic function to such an extent that it is believed that hormones and nutrients exert their effects on metabolism partially by modifying cell volume. Although, studies in the liver have shown that cell swelling inhibits protein degradation, whereas cell shrinkage stimulates it (Häussinger et al. 1990, Häussinger et al. 1991), my study suggests that unlike the liver, the small intestine's protein degradation is not influenced by changes in osmolarity. No other study had looked at the effect of osmotic pressure on protein degradation in the small intestine.

4.5 NUTRIENTS

This is the first study to look at the direct effect of energy substrates and amino acids on protein degradation in isolated small intestinal enterocytes from rats. β-hydroxybutyrate significantly decreased protein degradation in isolated enterocytes. There is no other study on the effect of ketone bodies on small intestinal protein degradation with which to compare my results. However, using tyrosine as an indicator of protein degradation, Palaiologos and Felig (1976) showed that 4 and 6 mM β-hydroxybutyrate significantly decreased intracellular tyrosine concentration in isolated quarter diaphragms from rats. Also, Tischler and colleagues (1982) showed that addition of a mixture of 1 mM acetoacetate, 4 mM β-hydroxybutyrate, and 5 mM glucose to incubation media decreased the rate of protein degradation in 48 h fasted rat diaphragms. In fasted chicks, 4 mM acetoacetate or β-hydroxybutyrate significantly decreased the rate of muscle protein degradation in extensor digitorum muscles (Wu and Thompson 1990). These studies provide evidence that ketone bodies are effective in reducing the rate of skeletal muscle protein degradation. The above findings are supported by an *in vivo* experiment that showed that in stressed human subjects, ketone bodies significantly reduced net body protein loss by decreasing urinary nitrogen and 3-methylhistidine excretion (Pawan and Semple 1983). In conditions such as TPN or when food intake is decreased, ketone bodies may have a potential role in modulating the small intestinal protein mass, a role that has been shown in skeletal muscle. My findings suggest that β-hydroxybutyrate may reduce loss of protein mass in the small intestine by decreasing the rate of protein degradation. This effect might give ketone bodies a potential significant use in catabolic conditions such as cancer cachexia, TPN, or when food intake is

compromised. In these conditions, the large loss of protein mass in stressed subjects could be limited by decreasing small intestinal protein degradation.

Glucose had no effect on protein degradation. Despite the potential importance of glucose as an energy source for the small intestine in the fed state, to my knowledge, its direct effect on small intestinal protein degradation has not been studied yet. However, Adegoke and colleagues (2003) tested the indirect effect of glucose on protein degradation and showed that a large bolus of intravenous glucose had no effect on the mucosal mRNA levels for proteolytic systems. The lack of effect of glucose on protein degradation in isolated enterocytes seen in my experiments, and on the mucosal mRNA levels in the study of Adegoke and colleagues (2003), suggests that glucose may not influence small intestinal protein degradation.

Butyrate unexpectedly increased protein degradation in isolated enterocytes. I chose to test the effect of butyrate on protein degradation since it did not influence protein synthesis in colonocytes or in the small intestine (Marsman and McBurney 1996, Adegoke et al. 1999). The effect of butyrate seen in my experiment was small and may not be biologically relevant. It may be that butyrate affects intestinal growth by a mechanism other than by protein degradation. Also, another explanation could be that butyrate may have provided a little extra energy to stimulate the activity of existing and highly energy dependent proteolytic systems in small intestine.

Glutamine did not affect protein degradation in isolated small intestinal enterocytes. The effect of glutamine on small intestinal protein degradation had not directly been studied yet. Studies on the indirect effect of glutamine on small intestinal protein degradation have given different results. Luminal delivery of glutamine to piglets significantly decreased

mRNA levels of components for the ubiquitin-proteasome system (Adegoke et al. 2003). Interestingly, enteral glutamine decreased mRNA levels of ubiquitin proteasome systems, but did not alter mRNA levels for cathepsin D or m-calpain (Coëffier et al. 2003). It should be noted that measuring mRNA levels of proteolytic systems is an indirect method that may suggest a decrease in protein degradation; whereas, my approach is a direct measurement of protein degradation. Among energy yielding substrates, the large and significant effect of β-hydroxybutyrate indicates a potential positive role for ketone bodies to limit atrophy and loss of small intestinal protein mass by decreasing protein degradation. This gives ketone bodies a possible significant use in catabolic conditions where food intake is compromised. In such conditions, loss of small intestinal protein mass is substantial and life threatening. Further animal studies are needed to investigate the effect of ketone bodies on protein degradation under different catabolic conditions.

Glutamate increased protein degradation in isolated enterocytes. Enterally delivered glutamate may enhance protein synthesis in burned rats (Hasebe et al. 1999), but in healthy piglets, it does not affect small intestinal growth and development (Burrin et al. 1991). In fed piglets, enteral glutamate has been shown to be the preferred source for mucosal glutathione synthesis. Glutathione plays an important role in maintaining the defense of mucosa against toxic damage (Aw et al. 1992, Aw 1994). This suggests that glutamate might affect protein turnover by controlling the production of glutathione. Glutamate can also act as an energy source, as it was shown by Hasebe and colleges (1999) that enteral glutamate might be the preferred fuel for enterocytes in burned rats. One explanation for the effect of glutamate in my study is that it may have acted as an energy source for the existing and highly energy dependent ubiquitin-proteasome system in the small intestine. I previously showed that

energy substrates such as butyrate may act as a source of energy for ubiquitin-proteasome system in small intestine and increase protein degradation in isolated small intestinal enterocyte from rats (Saberi et al. 2006).

I did not observe any effect of glutamine on protein degradation in my experiment. Luminal delivery of 30 mM glutamine to piglets significantly decreased mRNA levels of components for the ubiquitin-proteasome system (Adegoke et al. 2003). Interestingly, enteral glutamine decreased mRNA levels of ubiquitin proteasome systems, but did not alter mRNA levels for cathepsin D or m-calpain (Coëffier et al. 2003). Glutamine has been shown to promote growth, metabolism, structure, and function of the intestinal mucosa (Windmueller 1982). Addition of glutamine to TPN solution given to rats recovering from starvation, increased small intestinal mass (Inoue et al. 1993). Also in healthy rats, addition of glutamine to TPN solution, increased jejunal mucosal weight, DNA content, and villus height (O'Dwyer et al. 1989). Intravenous delivery of glutamine to patients fed standard TPN solution prevented intestinal permeability and improved mucosal structure (Van Der Hulst et al. 1993). However, intravenous glutamine failed to influence small intestinal mass in healthy animals. Addition of glutamine to TPN solution had no affect on small intestinal mass in healthy young piglets after 7 days exclusive TPN feeding (Burrin et al. 1991b). It might be speculated that glutamine affects small intestinal mass and metabolism in sick or stressed animals. My animals were healthy and not stressed. Another explanation is that nutrients might indirectly affect small intestinal metabolism. My approach measures the direct effect of nutrients. Further studies needed to investigate the effect of glutamine on small intestinal protein degradation.

The mixture of amino acids increased protein degradation in isolated enterocytes. The only study that had indirectly looked at the effect of amino acids on protein degradation in the small intestine has shown a substantial decrease in the mRNA levels of components of the ubiquitin-proteasome system with luminal or intravenous delivery of a mixture of amino acids (Adegoke et al. 2003). However, it should be noted that measuring mRNA levels of proteolytic systems is an indirect method that may suggest a decrease in protein degradation; whereas, my approach is a direct measurement of protein degradation. Amino acids are the main metabolic fuels for the small intestine and are considered essential nutrients in its metabolism (Stoll et al. 1998, Wu 1998). One explanation for the effect of amino acids seen in my experiment could be that mixture of amino acids acted as an energy source for the existing and energy dependent ubiquitin-proteasome systems in the small intestine.

The effect of addition of glucose to a mixture of amino acids seen in my experiment is consistent with that of Adegoke and colleges (2003) in that glucose abolished the effect of amino acids. The direction of this effect is different but the net effect is that addition of glucose to the mix of amino acids abolishes their effect. When glucose was added to a mix of amino acids, it abolished the effect of amino acids on reducing the mRNA levels of components of ubiquitin-proteasome systems. In my study, glucose abolished the effect of amino acid on increasing the protein degradation. My data may suggest that glucose might exert its effect by decreasing the concentration of amino acids.

4.6 SUMMARY AND FUTURE DIRECTIONS

In summary, I achieved high viability and linear tyrosine release in my experiments. This indicates a successful establishment of a method that can be used to estimate protein degradation in isolated enterocytes from small intestine in rats. My findings suggest that

butyrate, glucose, and glutamine might not directly contribute to the maintenance of gut protein mass via decreasing protein degradation. If these nutrients do affect protein degradation, my data suggest that they would have to act indirectly. The mixture of amino acids or glutamate may have acted as an energy source for the highly energy dependent enterocyte. This suggests that amino acids might contribute to small intestinal protein metabolism by their role as energy substrate. The addition of glucose to the mixture of amino acids may have decreased the concentration of amino acids and therefore, abolished their effect. The large effect of β-hydroxybutyrate and the mixture of amino acids plus glucose suggest a potential positive role for ketone bodies and combination of amino acids plus glucose to limit the loss of small intestinal protein mass by decreasing protein degradation. These finding are significant, particularly in catabolic conditions such as starvation, protein deficiency, and TPN, where the loss of small intestinal protein mass is substantial. The effect of glutamine requires further investigation particularly under stress conditions and in sick animals.

I used our newly developed method to test the effect of nutrients on protein degradation in isolated small intestinal enterocytes from rats. One strength of our system is that in any individual animal, the effect of different factors such as hormones or nutrients could be tested at the same time. Nevertheless, it must be remembered that my experiments were done in an *in vitro* system, which is an artificial environment and in a short period of time, since cells remained viable for only 30 min. Also, this method only addresses protein degradation in the epithelium and not other layers of small intestine. Another limitation is that I only looked at protein degradation and not both processes; protein degradation and

protein synthesis in isolated small intestinal enterocytes. Further studies needed to test the effect of different nutrients or hormones in a variety of conditions.

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APPENDIX ONE: Animal Care Certificate