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# RUMINANT NUTRITION SYMPOSIUM: Molecular adaptation of ruminal epithelia to highly fermentable diets<sup>1</sup>

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**ABSTRACT:** Feeding highly fermentable diets to ruminants is one strategy to increase energy intake. The increase in short-chain fatty acid (SCFA) production and reduced ruminal pH associated with highly fermentable diets imposes a challenge to the metabolism and the regulation of intracellular pH homeostasis of ruminal epithelia. The ruminal epithelia respond to these challenges in a coordinated manner. Whereas the enlargement of absorptive surface area is well documented, emerging evidence at the mRNA and transporter and enzyme activity levels indicate that changes in epithelial cell function may be the initial response. It is not surprising that gene expression analysis has identified pathways involved in fatty acid metabolism, ion transport, and intracellular homeostasis to be the pathways dominantly affected during adaptation and after adaptation to a highly fermentable diet. These findings are important because the intraepithelial metabolism of SCFA, particularly butyrate, helps to maintain the concentration gradient between the cytosol and lumen,

thereby facilitating absorption. Butyrate metabolism also controls the intracellular availability of butyrate, which is widely regarded as a signaling molecule. Current data indicate that for butyrate metabolism, 3-hydroxy-3-methylglutaryl-CoA synthase and acetyl-CoA acetyltransferase are potential regulatory points with transient up- and downregulation during diet adaptation. In addition to nutrient transport and utilization, genes involved in the maintenance of cellular tight junction integrity and induction of inflammation have been identified as differentially expressed genes during adaptation to highly fermentable diets. This may have important implications on ruminal epithelial barrier function and the inflammatory response often associated with subacute ruminal acidosis. The objective of this review is to summarize ruminal epithelial adaptation to highly fermentable diets focusing on the changes at the enzyme and transporter activity levels, as well as the underlying molecular changes at the mRNA and protein expression levels.

**Key words:** adaptation, gene expression, rumen, ruminal acidosis, ruminal epithelia

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## INTRODUCTION

Feedlot cattle often gain BW in excess of 1.5 kg/d (May et al., 2010; Schoonmaker et al., 2010) and may achieve rates of BW gain over 2 kg/d during the finishing period (Wierenga et al., 2010). Likewise, the level of productivity for lactating Holstein cows is great, with daily milk yield regularly exceeding 40 kg/d even into

mid-lactation (Penner et al., 2009d). To achieve these levels of productivity, highly fermentable diets are required to promote short-chain fatty acid (SCFA) production within the rumen, thereby increasing the total ME supply. Gradual transition from diets with low to moderate fermentability to those with a high fermentability is required to minimize the risk for digestive disorders, such as ruminal acidosis (Bevans et al., 2005; Penner et al., 2007; Steele et al., 2009a), and associated disorders, such as liver abscesses (Nagaraja et al., 2005) and laminitis (Nocek, 1997). Because ruminants derive a significant proportion of their ME supply via absorption of SCFA across the ruminal epithelia, it is important to understand mechanisms regulating changes in epithelial function during dietary adaptation.

The complete sequencing of the bovine genome has provided a new opportunity to investigate regulatory mechanisms involved in ruminal epithelial function and

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adaptation at the molecular level. A recent review has described the molecular basis for digestive processes across the gastrointestinal tract in ruminants (Connor et al., 2010). The intent of the present review was to summarize the current understanding of ruminal epithelial adaptation to highly fermentable diets at the transport and enzyme activity levels and to relate those findings to more recent research examining epithelial adaptation at the molecular level.

### CHANGES IN THE RUMINAL ENVIRONMENT IMPOSED BY HIGHLY FERMENTABLE DIETS

Strategies to allow for adaptation to highly fermentable diets differ in dairy and beef production schemes; however, the end results are similar: increased SCFA production (Sutton et al., 2003) and concentration (Penner et al., 2009c), decreased ruminal pH (Penner et al., 2007, 2009c), and greater toxin concentration in ruminal fluid (Gozho et al., 2006; Plaizier et al., 2008). In dairy settings, it is recommended to feed a diet with low to moderate fermentability during the far-off dry period (i.e., 60 to 28 d prepartum) and to increase diet fermentability during the close-up period (i.e., for the last 28 d prepartum; NRC, 2001). After parturition, diet fermentability is further increased to meet the energy demands arising from the onset of lactation. In addition to providing a highly fermentable diet, increases in DMI also occur during the first 28 d of lactation (Penner et al., 2007; Penner and Oba, 2009). This increase in DMI and, thus, an increase in the intake of fermentable OM constitutes an equally important trigger and stimulates the dietary adaptation process. In contrast, beef cattle transitioning to increased energy intake are exposed to a series of diets with incremental increases in the proportion of concentrate. These sequential increases are designed to minimize the negative impact of increasing diet fermentability on ruminal fermentation (Owens et al., 1998; Brown et al., 2006). Total DMI often does not differ during diet adaptation in beef cattle although concentrate intake increases (Bevans et al., 2005). However, the high fermentability of the diets and the associated shifts in ruminal pH may cause substantial variation in daily DMI (Bevans et al., 2005; Brown et al., 2006).

A study evaluating ruminal fermentation in Holstein heifers during early lactation reported increases in the total SCFA concentration from nearly 108 mmol/L for the 5 d preceding parturition to more than 125 mmol/L within 58 d postpartum (Penner et al., 2007). Accompanying the increase in SCFA concentration was a quadratic decrease in the molar proportion of acetate, a quadratic increase in the molar proportion of propionate with no change in the molar proportion of butyrate. These changes in fermentation products corresponded to an increased severity of ruminal acidosis that has been commonly observed during early lactation (Gröhn and Bruss, 1990; Fairfield et al., 2007; Penner

et al., 2007). Similarly, transitioning beef cattle to a high concentrate finishing diet with abrupt increases in the proportion of dietary concentrate has been reported to increase SCFA concentration and reduce ruminal pH (Bevans et al., 2005). For example, G. B. Penner, J. R. Aschenbach, and M. Oba (University of Alberta, Edmonton, Alberta, Canada; unpublished data) rapidly transitioned beef heifers to a 92% concentrate diet over a period of 17 d. In that study, another cohort of heifers was maintained on the control diet (i.e., 100% hay). Heifers exposed to the rapid diet transition protocol had greater concentrations of total SCFA with a decreased molar proportion of acetate and greater proportions of propionate and butyrate. Although SCFA concentration does not serve as a measure for SCFA production, increased SCFA concentration does pose a challenge to ruminal epithelia and stimulates an adaptive response.

In addition to the nutritionally desired increase in SCFA production, ruminal lipopolysaccharide (LPS) concentration increases with increasing proportions of dietary concentrate (Gozho et al., 2006; Zebeli and Ametaj, 2009). More specifically, as the proportion of barley grain increased in 15% increments from 0 to 45% (on a DM basis), the concentration of LPS in ruminal increased quadratically from 781 to 8,890 ng/mL (Zebeli and Ametaj, 2009). Although previous studies focused on the increase in total LPS concentration, it should be acknowledged that other toxins, such as biogenic amines, also become more abundant in ruminal digesta (Irwin et al., 1979a,b; Underwood, 1992; Plaizier et al., 2008). The increase in ruminal toxin concentration may be a particular problem because the permeability of ruminal epithelia increases with decreasing pH (Aschenbach and Gäbel, 2000; Penner et al., 2010). It is clear that highly fermentable diets provide a greater availability of SCFA, but the negative effects of low pH and increased toxin concentration also need to be evaluated.

### ADAPTIVE RESPONSES OF THE RUMINAL EPITHELIUM

#### *Epithelial Proliferation*

Epithelial adaptation encompasses morphological adaptations associated with tissue proliferation and changes in the function of individual cells (Gäbel and Aschenbach, 2007). Previous studies have largely focused on the proliferative response as part of ruminal development in calves during the transition from preruminants to functional ruminants (Tamate et al., 1962), and in dairy cattle during the periparturient period (Dirksen et al., 1985; Penner et al., 2006; Bannink et al., 2008). It should be acknowledged that dietary transition from backgrounding diets to finishing diets for feedlot cattle should also elicit such a response. Despite this, there is a paucity of data regarding ruminal epithelial proliferation in feedlot cattle.

The duration of time required for maximal increases for ruminal papillae surface area was previously reported to require 6 to 8 wk after an increase in the dietary energy density (Dirksen et al., 1985). More recently, Bannink et al. (2008) reported that cows fed rapid increases in dietary concentrate (i.e., greater fermentability) after parturition had a greater rate of epithelial proliferation, resulting in maximal surface area approximately 3 to 4 wk postpartum compared with the 7 to 8 wk required for cows receiving more gradual increases in the dietary concentrate. Thus, differences in the timeline required for epithelial proliferation likely depends on the energy density of the prepartum diet and the rate of increase in diet fermentability in the subsequent diets. Supporting this notion, a study conducted using primiparous Holstein cows fed pre- and postpartum diets in alignment or exceeding the recommendations of the NRC (2001) found that maximal papillae surface area was achieved prepartum and was not affected by the forage-to-concentrate ratio of the prepartum diets (Penner et al., 2006). Likewise, Reynolds et al. (2004) reported no differences in papillae surface area between the pre- and postpartum phases when collected from Holstein cows fed diets based on NRC (1989) recommendations. Ruminal epithelial proliferation is presumably a response that maximizes the absorptive surface area for nutrient absorption (Dirksen et al., 1985; Baldwin, 1999; Mentschel et al., 2001) but the current data indicate that the importance of epithelial proliferation may have been overemphasized for periparturient dairy cattle, at least when fed diets based on NRC (1989, 2001) recommendations. To date, there has been little advancement in describing the molecular basis of epithelial proliferation.

Increasing the diet fermentability changes the rate of production of specific SCFA, namely acetate, propionate, and butyrate (Sutton et al., 2003; Plaizier et al., 2008). Short-chain fatty acids are commonly regarded as luminal growth factors and increasing their production alters digestive tract proliferation in ruminant and nonruminant models (Sakata and Yajima, 1984). Of the SCFA, butyrate has been reported to be the most potent stimulator of epithelial proliferation in colonic epithelial cells (Blottière et al., 2003) and intraruminal infusion of butyrate has long been known to induce ruminal epithelial proliferation in vivo (Sakata and Tamate, 1978; Sakata et al., 1980; Nozière et al., 2000). In accordance with these results, young calves fed a milk replacer fortified with butyrate exhibited increased ruminal papillae length, width, and surface area (Gorka et al., 2009). An inhibitory effect of butyrate on ruminal epithelial apoptosis has been demonstrated in vitro (Neogrády et al., 1989a,b; Mentschel et al., 2001), thereby identifying a new putative mechanism for the role of butyrate in promoting epithelial growth.

The direct effect of butyrate on intestinal epithelial gene expression is well documented, and candidate molecular mechanisms are now being proposed for its action. Considerable evidence has accumulated describing

the inhibitory effect of butyrate on histone deacetylation and the hyperexpression of genes regulating the cell cycle, such as cyclin D3 (Blottière et al., 2003). To date, only one known study (Wang and Jiang, 2010) has investigated the role of cyclins D1, D2, D3, E1, and E2 and cyclin-dependent kinase inhibitors 1A, 1B, 2A, and 2B with respect to ruminal epithelial proliferation. In contrast to previous studies, Wang and Jiang (2010) reported that in isolation, acetate, propionate, and butyrate did not affect epithelial proliferation based on the number of viable cells after 24, 48, or 72 h in culture, or apoptosis based on DNA fragmentation. Their study highlighted that incubation of epithelial cells in ruminal fluid, but not SCFA, had a strong inhibitory effect on proliferation but not apoptosis, which was regulated by increased expression of bovine cyclin-dependant kinase inhibitor 1A and 2B. Future studies are needed to determine the molecular basis for epithelial proliferation during diet adaptation. A directed focus on known pathways characterized in the intestinal epithelium is the ideal starting point for future studies.

In contrast to in vivo studies, butyrate repeatedly has been shown to elicit an inhibitory effect on ruminal epithelial proliferation in vitro (Gálfi et al., 1981, 1993; Neogrády et al., 1989a,b; Baldwin, 1999). These results support the concept that butyrate does not promote epithelial proliferation directly, but rather acts through the release of hormones and growth factors. Principle candidates include IGF-1, GH, insulin, and glucagon because their plasma concentrations increase in a dose-dependent manner in response to ruminal infusions of mixed SCFA containing 10% butyrate (Zhao and Sun, 2010). Of these hormones, insulin (Sakata et al., 1980; Gálfi et al., 1993), glucagon (Gálfi et al., 1993), and IGF-1 (Baldwin, 1999) have indeed induced proliferation in vitro. A growth-promoting effect in vitro has also been shown for epidermal growth factor (EGF; Baldwin, 1999), which is supplied to the rumen primarily from the luminal-side via saliva (Onaga et al., 2006). Epidermal growth factor would thus couple epithelial proliferation to the intake of physically effective fiber (peNDF) rather than the rates of butyrate production because salivary secretion increases with increasing intake of peNDF (Yang and Beauchemin, 2006; Beauchemin et al., 2008), whereas butyrate fermentation decreases with increasing peNDF (Zebeli et al., 2008).

Among the hormones and growth factors promoting epithelial growth, special interest has been directed toward the role of IGF-1 and EGF (Baldwin, 1999). These growth factors induce their cellular response by binding to respective cellular membrane receptors. In Caco-2 and DLD-1 cell lines, this binding activates EGF-receptor and IGF-1-receptor-tyrosine kinases, thereby, initiating a signal transduction cascade that ultimately enhances the transcription of extracellular related protein kinases that are involved in cellular proliferation, for example, the serine/threonine protein kinase, ATK (Kaulfuss et al., 2009). In calves orally supplemented

with butyrate, plasma IGF-1 concentrations have been positively correlated with papillae growth (Zitnan et al., 2005). Moreover, ruminal papillae proliferation increased when a highly fermentable diet was fed to goats and was associated with more IGF-1 receptors in ruminal epithelial cells and increased plasma IGF-1 concentrations (Shen et al., 2004). Because IGFBP modulate IGF-stimulated cellular events (Firth and Baxter, 2002), they may play a role in the regulation of ruminal epithelial proliferation. Results from Steele et al. (2009c) support this notion and showed that transcription of mRNA for IGFBP-5 is upregulated in ruminal papillae when cattle were transitioned from a 0 to 65% grain diet, but the expression of IGFBP-3 and IGFBP-6 in ruminal papillae were downregulated during the grain challenge. On the contrary, in another study (Zhao and Sun, 2010), the protein expression of IGF-1 and IGFBP-3 were increased in the dorsal and ventral ruminal sacs when increasing amounts of SCFA were infused into the rumen of young sheep over 8 to 12 d. Further investigations on how EGF, IGF, as well as their receptors and binding proteins, interact to adapt ruminal epithelial growth to the physical structure and energy density of the diet have the potential to greatly improve our understanding of absorptive area plasticity under changing dietary conditions.

### *Changes in Cellular Function*

As noted previously, drastic changes in the ruminal environment occur during dietary adaptation to highly fermentable diets. Among these changes, the increased supply of SCFA, reduction in ruminal pH, and increased toxin concentrations pose significant challenges to ruminal epithelia. The importance of SCFA absorption for supplying ME in ruminants is well established (Bergman, 1990), especially with the increase SCFA production associated with greater diet fermentability (Sutton et al., 2003). Although a previous review (Gálfi et al., 1991) concluded that epithelial adaptation was largely a result of increased absorptive surface area via increases in cell number (i.e., hyperplastic growth), emerging results point to the notion that the adaptation of individual cells is the initial response to changes in diet fermentability. Specifically, Sehested et al. (2000) reported that cows fed diets resulting in short-term increases in ruminal SCFA concentrations, achieved by feeding additional concentrate once per day, increased the rate of butyrate transport measured in Ussing chambers. Moreover, this increase in transport occurred without corresponding increases in the absorptive surface area of the ruminal papillae (Andersen et al., 1999). Other studies have also shown drastic changes in transporter activity without corresponding changes in absorptive surface area. For example, Gäbel et al. (1993) and Gäbel and Aschenbach (2002) demonstrated that the fractional absorption rates of SCFA and minerals in vivo, as well as the net absorption of the glucose derivative (i.e., 3-*o*-methylglucose) in vitro,

were reduced by more than 40% after 48 h of complete feed withdrawal. The changes observed for nutrient and ion transport in those studies could not be accounted for by equivalent changes in epithelial surface area.

It is well established that the rate of SCFA (i.e., acetate, propionate, and butyrate) absorption across the ruminal wall is greater for sheep fed a high-concentrate (i.e., 81% concentrate) diet compared with sheep fed 100% hay (Gäbel et al., 1991). It is also known that the apical uptake of SCFA is mediated by passive diffusion and facilitated transport mechanisms, as reviewed by Aschenbach et al. (2011). The marked increase in SCFA absorption for concentrate-fed animals indicates that the capability of the ruminal epithelia to absorb SCFA increases in response to increasing diet fermentability. Whereas many of the transporters involved in SCFA absorption have yet to be characterized, potential candidate transporters have been identified (Bilk et al., 2005). These candidate transporters include downregulated-in-adenoma (**DRA**) and the putative anion transporter 1 on the apical membrane, and anion exchanger 2 on the basolateral membrane. Few studies have evaluated the relative expression of these transporters during dietary adaptation, but based on quantitative real-time PCR, Connor et al. (2010) reported that calves fed hay or grain for 14 d after receiving milk replacer for 42 d had a 142-fold increase in the relative expression of DRA compared with calves fed milk replacer only. Thus, it is possible that DRA, a dominantly expressed SCFA<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger at the mRNA level, may have a prominent role in SCFA absorption. The potential increase in bicarbonate-dependent transport mediated via DRA for ruminants fed highly fermentable diets would also help to regulate ruminal pH (Gäbel et al., 2002; Penner et al., 2009a; Aschenbach et al., 2011).

Low ruminal pH enhances the passive diffusion of undissociated SCFA (**HSCFA**; Dijkstra et al., 1993) as well as SCFA<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange (Aschenbach et al., 2009). Intracellular dissociation of HSCFA, as well as HCO<sub>3</sub><sup>-</sup> export from cells in exchange for SCFA<sup>-</sup> will decrease intracellular pH (Müller et al., 2000; Aschenbach et al., 2011). Thus, to regulate intracellular pH, upregulation of the Na<sup>+</sup>/H<sup>+</sup> exchangers (**NHE**) occurs. The identified isoforms of ruminal NHE include NHE1, NHE2, and NHE3 (Graham et al., 2007), which secrete H<sup>+</sup> in apical or basolateral directions. Etschmann et al. (2009) examined Na<sup>+</sup> transport across the isolated ruminal epithelium in Ussing chambers when collected from sheep fed supplemental concentrate for 0, 1, 2, 4, 6, or 12 wk. As could be expected, supplemental concentrate increased the net absorption of Na<sup>+</sup> in Ussing chambers; however, more interestingly, they found that 73% of the increase in Na<sup>+</sup> transport occurred during the first week of concentrate feeding. These results further confirm that functional adaptations of the ruminal epithelia occur much more rapidly than the morphological adaptation. These results are in alignment with the results of Yang et al. (2009), where increased fermentable energy intake resulted in greater mRNA abundance

for NHE1 and NHE3 in ruminal tissue collected from goats. Furthermore, this study also indicated that the timing of sampling is crucial when investigating the ruminal epithelial adaptation at the transcriptional level because goats fed diets with high or low fermentability, but fasted for 16 h before slaughter, exhibited no differences in mRNA abundance for NHE1 and NHE3, whereas differences were found for goats not subjected to fasting. Future studies should examine the role of fermentation products for the regulation of transporter expression and function and the potential variation in response to feeding management.

One additional method to increase dietary fermentability is to alter the type of nonstructural carbohydrate present in the diet rather than altering the concentration of nonstructural carbohydrate. Numerous studies have been conducted to evaluate the impact of including sugar as a partial replacement for corn starch with most studies reporting that sugar inclusion did not decrease ruminal pH *in vitro* (Vallimont et al., 2004) or *in vivo* (Broderick et al., 2008; Penner et al., 2009b; Penner and Oba, 2009) despite the theoretical increase in the rate of fermentation with sugar inclusion (Weisbjerg et al., 1998). The reason behind the lack of response in ruminal pH for diets containing a greater proportion of sugar is still not fully understood but may be related to glucose transport across the ruminal epithelia. Although still a controversial topic, numerous studies have consistently demonstrated the presence of glucose transporters in ruminal tissue at the mRNA and protein expression levels (Zhao et al., 1998; Aschenbach et al., 2000a,b; 2005), as well as at the transporter activity level (Aschenbach et al., 2000a,b, 2002, 2005). Immunohistochemical studies localized the sodium-dependent glucose-linked transporter-1 and facilitated glucose transporter (GLUT) 2 proteins to the apical membrane of ruminal epithelial cells [I. Frydryck (University of Leipzig, Leipzig, Germany), G. Gäbel (University of Leipzig, Leipzig, Germany), and J. R. Aschenbach; unpublished results]. Based on apical uptake measurements using Ussing chambers and based on experiments using the washed reticulo-rumen technique it is clear that luminal glucose can be absorbed very efficiently from the ruminal content of sheep and dairy cattle (Aschenbach et al., 2000a,b, 2002, 2005, 2006).

Although it is not very likely that ruminal glucose absorption contributes substantially toward meeting the energy requirements of ruminants under most dietary settings, glucose absorption may serve as a mechanism to regulate fermentation and, thus, intraruminal pH. Supporting this theory, Aschenbach et al. (2005) found increasing activity for GLUT transport proteins after inclusion of molasses into diets fed to sheep. Moreover, this group also demonstrated short-term regulation of glucose absorption by epinephrine and glucagon-37 (Aschenbach et al., 2002; Frydryck et al., 2004), which demonstrates another mode of transport modulation beyond the adaptation at the mRNA and protein ex-

pression levels. Future work should be conducted to determine the localization of GLUT isoforms other than GLUT-2 in ruminal tissue and further elucidate the physiological relevance of glucose absorption in ruminants, particularly during the adaptation to highly fermentable diets.

### *Metabolic Adaptation of Ruminal Epithelia*

The ruminal epithelium is metabolically active and is the greatest consumer of energy of the total viscera (Huntington, 1990; Britton and Krehbiel, 1993). Using immunocytochemistry, Graham and Simmons (2005) determined that the localization of Na<sup>+</sup>/K<sup>+</sup>-ATPase was in the stratum basale and stratum spinosum. In whole ruminal papillae, Na<sup>+</sup>/K<sup>+</sup>-ATPase has been estimated to account for one-fifth of the total O<sub>2</sub> consumption (Kelly et al., 1993). Unlike the small intestine, which relies on glucose, glutamine, and ketone bodies for energy, the ruminal epithelium captures the majority of its energy from the oxidation of fermentation end products, namely SCFA. Bergman (1990) approximated that 30% of acetate, 50% of propionate, and 90% of butyrate is metabolized within the ruminal epithelium. It is, however, likely that these are overestimates because Kristensen et al. (2000a,b) reported negligible metabolism of acetate and propionate across the portal-drained viscera. Nevertheless, the literature consistently reports that butyrate is the preferred substrate (Britton and Krehbiel, 1993; Kristensen and Harmon, 2004) and its metabolism will be the focus of this section.

After intraepithelial SCFA uptake, metabolism of SCFA is initiated by the addition of a CoA ester by a family of acyl-CoA synthetases (Ash and Baird, 1973). Several acyl-CoA synthetases exist in animal tissues, each with its own substrate specificities and kinetic characteristics, and their properties may be key regulatory factors in SCFA epithelial metabolism (Ash and Baird, 1973). Penner et al. (2009c) evaluated the relative expression of 3 acyl-CoA synthetase isoforms in ruminal tissue collected from cows fed a 64%-concentrate diet compared with cows fed an 8% concentrate diet. No changes were observed in the relative expression for any of the acyl-CoA synthetase isoforms. Thus far, only a long-chain family member, 1 acyl-CoA synthetase, has been shown to have its mRNA transcription down-regulated after 3 wk of feeding a high-grain diet (Steele et al., 2009b).

Although SCFA can be catabolized within the epithelium through oxidative pathways, the dominant pathway of butyrate metabolism in the ruminal epithelium is ketogenesis. It is important to note that in the fed state, the ruminal epithelium synthesizes more ketone bodies than the liver, thereby providing a quantitatively significant amount of energy substrates for peripheral tissues (Pennington, 1952). The enzymes of ketogenesis have not been localized within the ruminal epithelium using immunohistochemistry; however, the primary presence of mitochondria in the stratum basale

and, to a lesser extent, the stratum spinosum (Graham and Simmons, 2005) strongly indicates that ketogenesis is restricted to the most basolateral epithelial strata.

Acetyl-CoA acetyl transferase (**ACAT**) and 3-hydroxy, 3-methylglutaryl CoA synthase (**HMGCS**) convert acetyl-CoA to 3-hydroxy, 3-methylglutaryl CoA (**HMG-CoA**), a central metabolite of the ruminal epithelium (Baldwin, 1998). Both ACAT and HMGCS have been considered to be rate-limiting enzymes for ruminal epithelial ketogenesis (Lane et al., 2002). Correspondingly, G. B. Penner, J. R. Aschenbach, and M. Oba (University of Alberta, Edmonton, Alberta, Canada; unpublished data) found that heifers exposed to a rapid diet transition from an all-hay diet to a high-concentrate diet (i.e., 92% concentrate) over a period of 17 d had greater ( $P = 0.018$ ) expression of ACAT than heifers fed all-hay over the whole experimental period (relative expression of  $1.62 \pm 0.19$  vs.  $0.82 \pm 0.26$ , for high-concentrate and hay-fed heifers, respectively). Although it could not be confirmed from the present data, it is speculated that upregulation of ACAT may be an indicator for increased metabolism of even-chain SCFA (Lane et al., 2002). However, the relationship between mRNA abundance and activity of ACAT and HMGCS, as well as their relationships to ketogenesis and SCFA absorption, have not been investigated in detail.

It is known that HMG-CoA is present in both mitochondrial and cytoplasmic fractions (Leighton et al., 1983). Exclusively in the mitochondria, HMG-CoA can be converted by a HMG-CoA lyase to synthesize the ketone bodies acetoacetate and  $\beta$ -hydroxybutyrate (Rémond et al., 1995; Baldwin, 1998). Based upon studies using hepatocytes, we can extrapolate that 2 isoforms of HMGCS exist: HMGCS1, which is located within the cytoplasm, and HMGCS2, which is exclusively found in the mitochondria (Hegardt, 1999). The expression of HMGCS2 is regulated at the transcriptional level (Lane et al., 2002); its promoter region contains a peroxisome proliferator response element that is under the transcriptional regulation of the PPAR- $\alpha$  (Meertens et al., 1998). It is well established that SCFA can influence the expression of genes under the control of PPAR in intestinal tissue (Kinoshita et al., 2002); therefore, one may hypothesize that a shift in ruminal SCFA during a grain challenge would influence the expression of ketogenic genes. The long-known increase in ketogenesis when glucose replaces butyrate as an energy source (Giesecke et al., 1979) also may be promoted by this mechanism if one assumed that preferential glucose metabolism in ruminal epithelial cells would increase intracellular butyrate availability as a PPAR- $\alpha$  ligand. However, the possible relevance of this mechanism is yet to be shown because the immediate ketogenic effect of glucose is currently well explained by the release of reducing equivalents from glucose that need to be disposed into  $\beta$ -hydroxybutyrate (Giesecke et al., 1985).

Although increasing the amount of rapidly fermentable carbohydrates is associated with increased concentrations of blood  $\beta$ -hydroxybutyrate, presumably from

increased ruminal epithelial ketogenesis or possibly increased glucose availability on the luminal side, no changes were found by Penner et al. (2009c) in ketogenic gene expression after at least 28 d of diet adaptation. Acetyl-CoA acetyl transferase and HMGCS were downregulated between the initial (i.e., wk 1) and adapted phases (i.e., wk 3) of a grain challenge (Steele et al., 2009b), which corresponded with a reduction in plasma  $\beta$ -hydroxybutyrate, thereby supporting the hypothesis that the flux of this pathway may be controlled by enzyme mRNA transcription or altered substrate availability during the initial phases of dietary adaptation. These results also support the previous discussion that adaptations at the cellular level are the initial and rapidly occurring changes in ruminal epithelia with enhancement of the absorptive surface area accounting for long-term adaptation (Etschmann et al., 2009).

In addition to being a substrate for ketogenesis, HMG-CoA may also proceed into the cholesterol biosynthesis pathway in the cytoplasm (Dempsey, 1974), which has been almost ignored as a relevant metabolic pathway in the ruminal epithelium. Although cholesterol is an essential component of mammalian cell membranes, hypercholesterolemia is associated with inflammation, and increased cellular proliferation and migration (Liao and Laufs, 2005). The flux of metabolites through this pathway is under the control of the HMGCS1 isoform, which is an enzymatic control point of the cholesterol and isoprenoid biosynthetic pathways. Steele et al. (2009b) reported a slight increase in the abundance of HMGCS1 mRNA in ruminal papillae during the first week of the grain challenge before a downregulation from baseline to wk 3 of the high-grain diet in mature dairy cows. The extent of the downregulation was such that the baseline expression was 2-fold greater than during wk 3. In the same study, the mRNA transcription of cholesterolgenic genes, such as ACAT2, HMG-CoA reductase, farnesyl-diphosphate farnesyltransferase 1, farnesyl diphosphate synthase, and lanosterol synthase were unchanged or slightly elevated (i.e., ACAT2 and farnesyl diphosphate synthase) during the first week of the grain challenge and downregulated from the first to third week of the high-grain diet such that the expression during the first week was 1.5- to 2.2-fold greater than during wk 3 [M. A. Steele, G. Vandervoort (University of Guelph, Ontario, Canada), O. AlZahal (University of Guelph, Ontario, Canada), S. E. Hook (University of Guelph, Ontario, Canada), J. C. Matthews (University of Kentucky, Lexington), and B. W. McBride; unpublished results]. The change in the expression over time in the grain challenge and downregulation of this pathway between the baseline and third week of the grain challenge may be involved in the suppression of the inflammatory response. The hypothesis regarding suppression of the immune system from wk 1 to 3 is supported by ruminal papillae lesion scores and tissue damage, which were elevated during the first week of the grain challenge but decreased from the first to third week [M. A. Steele, G. Vandervoort

(University of Guelph, Ontario, Canada), O. AlZahal (University of Guelph, Ontario, Canada), S. E. Hook (University of Guelph, Ontario, Canada), J. C. Matthews (University of Kentucky, Lexington), and B. W. McBride; unpublished results]. Because the amount of substrate (SCFA) available for epithelial metabolism is increased during a grain challenge, it can be hypothesized that long-term ruminal epithelial adaptation to highly fermentable diets involves the downregulation of this pathway presumably from an accumulation of intracellular sterols.

The sterol regulatory element binding protein (**SREBP**) family of transcriptional factors controls cholesterolgenic and lipogenic gene expression in the bovine liver and mammary glands (Harvatine and Bauman, 2006; Viturro et al., 2009). The bovine genome encodes 3 SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2. Of these isoforms, SREBP-2 preferentially activates cholesterol biosynthesis (Dempsey, 1974) and its activation requires the cleavage of the inactive precursor bound to the endoplasmic reticulum to allow transmigration to the nucleus, thereby influencing gene expression (Horton, 2002; Horton et al., 2002). An accumulation of sterols can inhibit cleavage and transmigration and can be assumed to be a potential mechanism for shifting cytoplasmic metabolism of SCFA in the ruminal epithelium.

In summary, we hypothesize that increases in the production of SCFA, especially butyric acid, in the rumen during a grain challenge are the primary trigger for alterations in the expression of genes involved in the metabolism of SCFA. It is likely that the regulation of these events is coordinated through transcription factors, such as PPAR- $\alpha$  and SREBP. Studying these transcription factors in combination with the genes they influence is the next logical step in molecular-based ruminal epithelial metabolic research.

### *Tissue Permeability and Inflammation*

Epithelial barrier function is a critical component of the immune system in ruminants. The ruminal barrier is responsible for maintaining concentration gradients required for ion absorption, preventing the translocation of LPS and other toxins, and preventing translocation of bacteria into the portal circulation. Ruminal acidosis is the major insult leading to barrier failure in the rumen. Impairment of barrier function is mainly linked to low pH (Gaebel et al., 1987; Aschenbach and Gäbel, 2000; Penner et al., 2010; Aschenbach et al., 2011) and, temporarily, to hyperosmolarity during episodes of rapid fermentation (Schweigel et al., 2005; Lodemann and Martens, 2006; Penner et al., 2010). Additionally, barrier function may be compromised after an acidotic insult by toxins, such as histamine (Aschenbach et al., 1998), or inflammatory responses (Bruewer et al., 2003).

Proteins involved in epithelial barrier function have been characterized in ruminal epithelia (Steven and

Marshall, 1969; Graham and Simmons, 2005) and other epithelia (Stevenson and Keon, 1998; D'Atri and Citi, 2002). For ruminal epithelia, the stratum granulosum and corneum play essential roles as the dominant barriers to the translocation of compounds across the ruminal epithelium. The stratum corneum is the outermost layer of the epithelium and is composed of dead keratinocytes that act as a physical barrier shielding the lower strata (Graham and Simmons, 2005). Beneath the stratum corneum is the stratum granulosum, which is composed of granular cells with tight junctions (Steven and Marshall, 1969; Graham and Simmons, 2005). Graham and Simmons (2005) demonstrated the localization of the tight-cell junction protein, claudin-1, and the junction-associated protein, zona occludin-1, with dominant expression in the stratum granulosum. The continuous array of tight junctions in the upper stratum granulosum divides the multilayered epithelium into apical and basolateral compartments, making the stratum granulosum the dominant stratum for barrier function. In contrast to simple epithelia, the basolateral compartment of the ruminal epithelium comprises not only the basolateral pole of upper stratum granulosum cells but also the several cell layers beneath the tight junction that are linked to the stratum granulosum by gap junctions to form a functional syncytium (Steven and Marshall, 1969; Graham and Simmons, 2005).

Information on the regulation for tight cell junctions in ruminal epithelia is extremely limited. Based on functional studies using in vivo (i.e., temporarily isolated and washed reticulo-rumen) or in vitro approaches (i.e., Ussing chambers), the relationship between dietary fermentability and epithelial permeability has been elucidated. Early work by Gaebel et al. (1987) showed that rapidly reducing buffer solution pH from 6.8 to 4.8 reduced the net absorption of Na<sup>+</sup>, Cl<sup>-</sup>, and Mg<sup>2+</sup>. However, the reduction in the net absorption of the measured ions was less severe when animals had been prefed a diet high in concentrates. Likewise, Lodemann and Martens (2006) reported that the short-term barrier-disrupting effect of hyperosmolarity was not as severe for sheep prefed greater proportions of dietary concentrate.

The molecular regulation for epithelial barrier function is not well understood in ruminal tissue, and to date, no studies known to the authors have investigated the expression of genes involved in barrier function during adaptation to highly fermentable diets. In intestinal tissue, the role of butyrate in promoting barrier function has been examined (Bordin et al., 2004; Hamer et al., 2008; Peng et al., 2009; Lewis et al., 2010). If results from intestinal tissue can be applied to ruminal tissue, it appears that butyrate modulates epithelial barrier function by promoting tight-cell junction protein expression (Bordin et al., 2004) and by enhancing the assembly of tight cell junctions (Peng et al., 2009). This is likely regulated via AMP-activated protein kinase. Supporting this hypothesis, using a 24K bovine genome-specific microarray, Taniguchi et al. (2010) found

that of the 5,200 differentially expressed genes, 96 were multi-functional genes. Gene ontology analysis elucidated that mitogen-activated protein kinase signaling, regulation of the actin cytoskeleton, and focal adhesion were dominantly affected pathways. These pathways are all involved in epithelial barrier function (<http://www.genome.jp/kegg/pathway.html>) and, as such, future studies should be conducted to elucidate the regulation of tight-cell junctions and, as a whole, barrier function in ruminal tissue during dietary adaptation.

The rate of epithelial differentiation is altered during shifts in rapidly fermentable carbohydrates. When extremely large amounts of easily fermentable carbohydrates are included in the diet, the ruminal epithelium can become parakeratotic (Tamate and Kikuchi, 1978; Steele et al., 2009a). Parakeratosis and hyperkeratosis are commonly associated with high-grain feeding or rapid increases in the proportion of dietary concentrate and are characterized by acute alterations in tissue ultrastructure and integrity, and severely compromised SCFA absorption (Bull et al., 1965; Hinders and Owen, 1965; Dirksen et al., 1985, 1997). In addition to highly fermentable diets, intraruminal infusion of specific SCFA can also cause erosion of the tight junctions between cells and the formation of lesions (Costa et al., 2008a,b; Steele et al., 2009a). The local effects on ruminal epithelial tissue damage become systemic when compounds or organisms, such as endotoxins or microbes, translocate into the portal blood stream, thus causing a systemic immune response (Nagaraja and Titgemeyer, 2007).

The principle mechanisms responsible for triggering an immune response during dietary adaptation are unknown. The influence of intraluminal SCFA on inflammation is well documented in the colon and is likely the mechanism that triggers the inflammatory response in the ruminal epithelium (Mortensen and Clausen, 1996). Moreover, it has been shown that increasing dietary fermentability by increasing the proportion of concentrate in the diet increases the concentration of ruminal LPS that are shed from the cell walls of gram-negative bacteria (Andersen et al., 1994; Gozho et al., 2006). The endotoxic effects of LPS on the ruminal epithelium may be responsible for the initial local inflammatory reaction, leading to an immune-mediated cascade of events to repair the ruminal epithelium (Thibault et al., 2010). As well, LPS can translocate through the ruminal epithelium into blood (Emmanuel et al., 2007), causing concentrations of proinflammatory cytokines and acute phase proteins to be increased (Gozho et al., 2006; Khafipour et al., 2009). It should be acknowledged that other toxins, such as biological amines, are present in ruminal fluid (Nagaraja et al., 2005; Plaizier et al., 2008). These compounds may also contribute to initiation of the immune response and disruption of barrier function.

An ideal starting point for the study of ruminal epithelial inflammation at the molecular level should be focused upon ubiquitous transcription factors that play

a key role in the immune response. To this end, nuclear factor  $\kappa$ B (NF- $\kappa$ B) and PPAR- $\gamma$ , 2 candidate transcriptional factors that are crucial in intestinal inflammation (Hoffmann and Baltimore, 2006; Calder, 2008), should receive more attention in ruminal epithelial research. Nuclear factor  $\kappa$ B is present in all animal cell types and its stimulation by antigens, cytokines, and reactive oxygen species causes the phosphorylation of the inhibitory subunit of the NF- $\kappa$ B complex (Hoffmann and Baltimore, 2006). This process enables NF- $\kappa$ B to transverse the nuclear envelope and bind the promoter sequence of genes encoding for pro-inflammatory cytokines and inflammatory enzymes, thus initiating the immune response. On the other hand, PPAR- $\gamma$  is an important transcription factor mitigating gastrointestinal inflammation and deserves more attention as a potential mediator of the ruminal epithelial immune response (Guri et al., 2010). Most evidence for the anti-inflammatory effect of PPAR- $\gamma$  comes from studies where subjects with advanced inflammatory bowel disease generally show decreased PPAR- $\gamma$  mRNA transcription (Desreumaux et al., 1999) and treatment with a PPAR- $\gamma$  agonist reduced symptoms of colitis (Desreumaux et al., 2001). In conclusion, focused efforts upon the ubiquitous transcriptional machinery orchestrating the inflammatory response is essential for further understanding ruminal epithelial adaptation to high-grain diets.

## SUMMARY AND CONCLUSIONS

In response to highly fermentable diets and the resulting decrease in ruminal pH, increase in SCFA concentrations, and increase in toxin concentration within ruminal contents, the ruminal epithelium adapts with coordinated changes in epithelial proliferation, cellular function, and tissue permeability. The molecular regulation of ruminal epithelial proliferation seems to be mediated through growth factors such as EGF and IGF-1. Future studies should evaluate the linkage between these growth factors and their receptors to elucidate the mechanisms involved in epithelial proliferation. The increased ruminal ketogenic activity for cows fed highly fermentable diets corresponds to increased mRNA abundance for ACAT and HMGCS, both of which are thought to be rate-limiting enzymes for ketogenesis, and decreased expression of genes involved in cholesterol synthesis. Until recently, the cholesterol synthesis pathway has been overlooked in ruminal epithelia, and thus, future studies should evaluate the relationship between cholesterol synthesis, dietary adaptation, and inflammation. Moreover, the regulatory events controlling these changes have not been elucidated in ruminal tissue. In addition to changes in proliferation and cellular activity, there is evidence that epithelial barrier function increases with increasing diet fermentability. Past studies have revealed the localization of claudin-1 and zona occludin-1 in the stratum granulosum, but the role of these tight junction proteins in the adaptive

response remains to be elucidated. Further information regarding formation of tight junctions could be used to develop strategies to minimize the potential for the translocation of bacteria and toxins across the ruminal epithelia.

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