Short communication: Glucagon-like peptide-2 and coccidiosis alter tight junction gene expression in the gastrointestinal tract of dairy calves¹

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ABSTRACT

Tight junction (TJ) proteins are integral factors involved in gut barrier function, and therapy with glucagon-like peptide-2 (GLP-2) enhances gut integrity. Our aim was to assess effects of GLP-2 treatment on mRNA expression of 8 TJ complex proteins in the intestine of dairy calves not infected or infected with *Eimeria bovis* at 11 ± 3 d of age. Mucosal epithelium from jejunum, ileum, and cecum was collected at slaughter from Holstein bull calves assigned to 4 groups: noninfected, buffer-treated (n = 5); noninfected, GLP-2 treated (n = 4); *E. bovis*-infected, buffer-treated (n = 5); and *E. bovis*-infected, GLP-2-treated (n = 4). Infected calves were orally dosed with 100,000 to 200,000 sporulated *E. bovis* oocysts on d 0; GLP-2-treated calves received 50 µg of GLP-2/kg of body weight subcutaneously twice daily for 10 d beginning on d 18; and buffer-treated calves received an equal injection volume of 0.01 M Na bicarbonate buffer. All calves were killed on d 28. The mRNA expression of coxsackie and adenovirus receptor (*CXADR*), claudins 1, 2, and 4 (*CLDN1*, *CLDN2*, and *CLDN4*), F11 receptor (*F11R*), junction adhesion molecule 2 (*JAM2*), occludin (*OCN*), and tight junction protein ZO-1 (*TJP1*) was determined by real-time quantitative PCR. In jejunum and ileum, an interaction of *E. bovis* infection and GLP-2 treatment on gene expression was noted. In jejunum of noninfected calves, GLP-2 increased *CXADR*, *CLDN2*, *OCN*, and *TJP1* mRNA expression but had no effect on mRNA expression in infected calves. Treatment with GLP-2 also increased tight junction protein ZO-1 protein expression in jejunum of noninfected calves as determined by immunohistochemistry. In ileum, *E. bovis* decreased expression of *JAM2*, *OCN*, and *TJP1* in buffer-treated calves, and GLP-2 increased *TJP1* expression in infected calves. In cecum, *E. bovis* infection reduced

expression of *CXADR*, *CLDN4*, *F11R*, and *OCN*, and GLP-2 therapy increased expression of *CLDN4*, *F11R*, *OCN*, and *TJP1*. Results are consistent with studies in nonruminants showing decreased expression of TJ complex proteins in the intestinal tract during pathogen-induced diarrhea and increased TJ protein expression in intestinal tissues in response to GLP-2 treatment. In conclusion, *E. bovis* reduces gene expression of TJ proteins primarily in cecum of calves 28 d postinfection, and GLP-2 increases expression of selected TJ genes in intestinal tissues. Use of GLP-2 to improve gut barrier function in ruminants during pathogen-induced diarrhea warrants additional study.

Key words: coccidiosis, dairy calf, gene expression, tight junction protein

Short Communication

Acute diarrhea due to intestinal pathogens is a common cause of calf mortality (Uetake, 2013). Furthermore, diarrhea in early life of dairy heifers reduces milk production in the first lactation (Heinrichs and Heinrichs, 2011). Hence, benefits to calf and milk production may be achieved by enhancing intestinal health and stabilizing gut integrity and barrier function of young dairy calves. The intestinotrophic hormone, glucagon-like peptide 2 (GLP-2), is reported to increase tight junction (TJ) protein expression and enhance intestinal barrier function in cultured human intestinal cells (Moran et al., 2012) and to increase mRNA and protein expression of TJ proteins in the jejunum of pigs (Yu et al., 2014). Recent studies suggest the potential for GLP-2 therapies to benefit gut development and health in cattle (Connor et al., 2010, 2013; Taylor-Edwards et al., 2010, 2011). However, the effects of GLP-2 on intestinal epithelial barrier function of ruminants have not been investigated.

The protozoan *Eimeria bovis* is a common pathogen of young calves and causes severe diarrhea and tissue damage within the distal ileum, cecum, and colon during its life cycle within its host (Friend and Stockdale, 1980). *Eimeria bovis* infection impairs intestinal integrity (Friend and Stockdale, 1980) and, thus, could serve as an experimental model to examine the capac-

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ity for GLP-2 treatment to restore barrier function in vivo. Intestinal barrier function is regulated primarily through the action of the TJ complex, which includes transmembrane proteins and TJ-associated cytoplasmic members that control selective flux of large macromolecules, small molecules, and ions (Liang and Weber, 2014). Claudin proteins are responsible for flux of small molecules and ions through pores in the TJ (Van Itallie et al., 2008), whereas the leak of macromolecules is attributed to tight junction protein ZO-1, occludin members, and other associated proteins (Van Itallie et al., 2009). Thus, our aim was to assess the effects of *E. bovis* infection and GLP-2 therapy on gene expression of select proteins of the TJ complex in the small intestine and cecum of calves. Our hypotheses were that expression of TJ complex proteins, particularly in ileocecum, is reduced by *E. bovis* infection, and that GLP-2 treatment increases TJ gene expression and ameliorates reductions in expression of TJ complex proteins of *E. bovis*-infected calves.

To test our hypotheses, mRNA and protein expression of selected TJ complex proteins was examined in intestinal tissues from a previous study by Connor et al. (2013). Briefly, calves were assigned to 4 groups including, noninfected calves injected (s.c.) twice daily for 10 d with sodium bicarbonate buffer (n = 5), or GLP-2 (50 µg/kg of BW; n = 4), and *E. bovis*-infected calves injected (s.c.) twice daily for 10 d with sodium bicarbonate buffer (n = 5) or GLP-2 (50 µg/kg of BW; n = 4). Calves in the infected groups were orally infected with 100,000 to 200,000 sporulated *E. bovis* oocysts on study d 0, GLP-2 or buffer injections began on study d 18, and calves were killed on d 28 for tissue collection. Mean (±SD) calf age was 11 (±3) d on study d 0.

Mucosa from jejunum, ileum, and cecum was collected and stabilized in RNAlater (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Samples were stored at -80°C until RNA extraction and evaluation of mRNA expression for coxsackie adenovirus receptor (*CXADR*), claudins 1, 2, and 4 (*CLDN1*, *CLDN2*, and *CLDN4*), F11 receptor (*F11R*; also known as *JAMA*), junctional adhesion molecule

2 (*JAM2*), occludin (*OCN*), and TJ protein ZO-1 (*TJP1*) by real-time quantitative PCR. Whole tissue from jejunum of noninfected calves also was formalin fixed and stored in 70% ethanol at 4°C until sectioned for immunohistochemical analysis of TJ protein ZO-1 and occludin.

Total RNA was extracted and its integrity and quality were assessed as previously described (Connor et al., 2010). All samples had an RNA integrity number of 7.0 or greater. Reverse transcription was performed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) using 1.0 µg of total RNA, according to the manufacturer's instructions. Real-time quantitative PCR was performed in triplicate 20-µL reactions using the iCycler iQ, SsoAdvanced Universal SYBR Green Super Mix (Bio-Rad Laboratories), and a concentration of 400 nM for each primer. Cycling conditions were 95°C for 3 min, followed by 45 cycles of 63°C for 30 s, 70°C for 30 s, and 95°C for 30 s. Primer sequences (Table 1) were designed using primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) except those for *OCN*, which were based on Malmuthuge et al. (2013). Primer specificity was validated by gel purification (Qiagen, Valencia, CA) and DNA sequencing (CEQ 8000 GeXP genetic analysis system, Beckman Coulter Inc., Brea, CA) of amplicons from a standard end-point PCR reaction. Quantification of gene copy numbers in samples was based on interpolation from a standard curve analyzed simultaneously with samples as described by Connor et al. (2005). Assay amplification efficiencies ranged from 87 to 115%, and linearity (r^2) of standard curves ranged from 0.995 to 1.000. Comparisons of least squares means of gene expression level were made using the GLM procedure of SAS (SAS Institute Inc., Cary, NC). The model included the fixed effects of treatment (buffer vs. GLP-2), infection status (noninfected vs. infected with *E. bovis*), and the interaction of treatment and infection status. Further pairwise comparisons of least squares means within treatment and infection were made using the PDIF option of SAS.

Antibodies used for immunohistochemical analysis of jejunal sections were rabbit anti-occludin (cat. no. 71–

Table 1. Primer sequences and annealing temperatures used for real-time quantitative PCR

Gene symbol	Gene name	Sense primer (5' → 3')	Antisense primer (5' → 3')
<i>CXADR</i>	Coxsackie and adenovirus receptor	TCCGACTCACAGAACTGCC	CCGTACAGGTGTATGTCCCG
<i>CLDN1</i>	Claudin 1	AAGACGACGAGGCACAGAAG	CAGCCCAGCCAATGAAGAGA
<i>CLDN2</i>	Claudin 2	CCAGGCCATGATGGTGACAT	GAAGAAGACTCCGCCACAA
<i>CLDN4</i>	Claudin 4	GTGCCCTCATCGTCATCTGT	TTGTTAGCCGTCCAGGACAC
<i>F11R</i>	F11 receptor	TCAGAGAGAGATGGCTCCCC	TCCAGTATCAGAGGCCGACA
<i>JAM2</i>	Junction adhesion molecule 2	CCCCATCGGAACAAGGTCAA	GACATCGCAGCTCTACCACA
<i>OCN</i>	Occludin	ACGCAGGAAGTGCCTTTGGTAGC	GCAGCCATGGCCAGCAGGAA
<i>TJP1</i>	Tight junction protein ZO-1	AATGCATCCTGACCACCAGG	GATGGTGCCGGGTTTGTTC

500, Life Technologies; 2.5 µg/mL), and rabbit anti-TJ protein ZO-1 (cat. no. 61-7300, Life Technologies; 5.0 µg/mL). Antibody signal was detected using the SuperPicture Polymer Detection (DAB, broad spectrum) Kit (Life Technologies) following the manufacturer's instructions. Sections of jejunum were photographed using an Olympus IX81 microscope (Olympus America Inc., Center Valley, PA) equipped with an Olympus DP-71 camera. Captured images of 7 to 10 nonoverlapping fields containing intact villi for each animal were manually evaluated for positive labeling of epithelial cells as previously described by Connor et al. (2008) for quantification of proliferating mammary endothelial cells. A minimum of 2,500 epithelial cells was evaluated per calf for each target protein ($4,268 \pm 189$ cells; mean $\pm$ SE). Figure 1 shows a representative image of TJ protein ZO-1 and occludin staining in calf jejunum. The mean percentages of labeled cells for occludin and TJ protein ZO-1 were compared between buffer- and GLP-2-treated groups using the TTEST procedure of SAS. A P -value ≤ 0.05 was considered significant and $0.05 < P \leq 0.10$ was considered a tendency for all statistical inferences.

Table 2 summarizes the effects of *E. bovis* infection, GLP-2 treatment, and the interaction of infection and GLP-2 on gene transcript abundance of TJ complex proteins in each intestinal tissue. In jejunum, no main effects of *E. bovis* or GLP-2 on mRNA expression of *CLDN1*, *CLDN4*, *F11R*, or *JAM2* were observed, but an interaction of GLP-2 and infection status on mRNA expression of *CXADR*, *CLDN2*, *OCN* ($P \leq 0.05$), and *TJP1* ($P \leq 0.06$) was noted. Among noninfected calves, GLP-2 increased ($P < 0.05$) expression of these 4 genes

by 1.8- to 5.1-fold relative to buffer treatment. Our results were similar to those of Yu et al. (2014), who observed increased *TJP1*, *CLDN1*, and *OCN* mRNA, as well as protein expressions, with GLP-2 treatment in porcine jejunal explants.

Although *E. bovis* typically causes the greatest damage to the distal small intestine and colon (Friend and Stockdale, 1980), expression of *CLDN2*, *TJP1*, and *OCN* mRNA was decreased in jejunum of GLP-2-treated calves by *E. bovis* infection. This was not entirely unexpected, as multiple pathogenic organisms target and disrupt TJ complex proteins in the gut, causing diarrhea (reviewed by Guttman and Finlay, 2009). For example, in jejunum of newborn pigs, rotavirus reduces barrier function and alters occludin localization (Jacobi et al., 2013), and enterotoxigenic *Escherichia coli* reduces *TJP1* and *OCN* mRNA and protein expression (Yang et al., 2014). It is not clear, however, why the effect of *E. bovis* on expression of these transcripts was more pronounced in GLP-2-treated calves.

To determine whether increases in mRNA expression of *OCN* and *TJP1* mRNA in jejunum in response to GLP-2 reflected similar increases at the protein level, TJ protein ZO-1 and occludin were measured in noninfected calves treated with GLP-2 or buffer by immunohistochemistry. Tissue from only noninfected animals was used to remove any confounding effects of *E. bovis* infection on tissue integrity, which can make immunohistochemical analysis difficult. Greater ($P < 0.002$) TJ protein ZO-1 abundance was detected in GLP-2-treated calves versus buffer-treated calves, but abundance of occludin was only numerically increased ($P < 0.15$) by GLP-2 treatment (Figure 2). In a hu-

Figure 1. Representative immunohistochemical detection of tight junction protein ZO-1 and occludin in jejunum of noninfected calves treated with 0.01 M sodium carbonate/bicarbonate buffer. (A) Villus containing tight junction protein-ZO-1-positive cells. (B) Villus containing occludin-positive cells. Examples of positive staining are indicated by arrows. (C) Negative control for unspecific secondary antibody staining. Negative control slides were incubated without primary antibody and showed no staining. Magnification bar = 50 microns.

Table 2. The effects of *Eimeria bovis* infection (Eim) and glucagon-like peptide 2 (GLP-2) treatment on LSM ($\pm$ SEM) for tight junction gene transcript abundance in jejunum, ileum, and cecum of Holstein bull calves

Gene ¹	Treatment ²				P-value ³				
	Con-Buffer (n = 5)	Con-GLP2 (n = 4)	Eim-Buffer (n = 5)	Eim-GLP2 (n = 4)	SEM ⁴	SEM ⁵	Eim	GLP-2	Eim $\times$ GLP-2
Jejunum									
<i>CXADR</i>	216,380 ^a	574,927 ^{b,A}	291,200 ^{ab,B}	223,791 ^a	89,050	102,916	0.18	0.16	0.05
<i>CLDN1</i>	11,987	8,351	14,204	18,077	5,668	6,550	0.36	0.98	0.56
<i>CLDN2</i>	6,276 ^a	23,759 ^{b,A}	17,752 ^{ab}	7,770 ^{ab,B}	4,614	5,333	0.66	0.47	0.02
<i>CLDN4</i>	207,840	340,452	310,300	461,784	94,754	109,509	0.30	0.19	0.93
<i>F11R</i>	119,730	222,162	194,580	5,598	77,492	89,558	0.42	0.61	0.11
<i>JAM2</i>	23,680	21,126	14,416	18,354	7,394	8,545	0.47	0.93	0.69
<i>OCN</i>	9,030 ^a	46,369 ^b	10,453 ^a	6,124 ^a	8,371	9,674	0.06	0.10	0.05
<i>TJP1</i>	465,700 ^a	827,046 ^b	471,200 ^a	498,046 ^a	72,467	83,751	0.07	0.03	0.06
Ileum									
<i>CXADR</i>	45,410	42,637	35,060	46,837	5,607	6,480	0.62	0.47	0.26
<i>CLDN1</i>	6,422	7,347	7,568	9,170	2,080	2,404	0.53	0.58	0.88
<i>CLDN2</i>	380,180	328,001	157,770	213,387	94,841	109,609	0.13	0.99	0.61
<i>CLDN4</i>	175,670	178,883	110,080	118,501	37,169	42,957	0.15	0.89	0.94
<i>F11R</i>	283,600	257,895	177,400	240,759	59,688	68,982	0.36	0.78	0.51
<i>JAM2</i>	63,500 ^a	48,413 ^{ab}	43,000 ^b	55,091 ^{ab}	6,459	7,465	0.35	0.83	0.08
<i>OCN</i>	394,800 ^a	307,174 ^{ab}	219,300 ^b	337,810 ^{ab}	51,500	59,519	0.22	0.79	0.10
<i>TJP1</i>	40,500 ^A	28,414 ^{AB}	23,800 ^B	41,732 ^A	5,941	6,867	0.80	0.66	0.04
Cecum									
<i>CXADR</i>	775,500	967,975	502,100	649,702	103,726	119,877	0.03	0.16	0.85
<i>CLDN1</i>	3,088	3,383	4,097	6,347	1,285	1,485	0.19	0.38	0.50
<i>CLDN2</i>	442,600	756,725	137,200	447,852	212,125	245,155	0.21	0.20	0.99
<i>CLDN4</i>	138,060	206,711	72,940	122,666	28,536	32,980	0.04	0.08	0.77
<i>F11R</i>	20,090	35,899	10,530	19,498	4,125	4,767	0.02	0.02	0.46
<i>JAM2</i>	115,520	137,239	136,260	115,253	10,316	11,922	0.96	0.98	0.09
<i>OCN</i>	115,960	195,195	67,260	101,840	19,380	22,398	0.01	0.02	0.31
<i>TJP1</i>	3,766	7,320	3,225	6,329	1,169	1,351	0.56	0.02	0.86

^{a,b}Within a row, means without a common superscript differ ($P \leq 0.05$).^{A,B}Within a row, means without a common superscript differ ($P \leq 0.10$).¹*CXADR* = coxsackie and adenovirus receptor; *CLDN1* = claudin 1; *CLDN2* = claudin 2; *CLDN4* = claudin 4; *F11R* = F11 receptor; *JAM2* = junction adhesion molecule 2; *OCN* = occludin; and *TJP1* = tight junction protein 1.²Con-Buffer = noninfected calves injected subcutaneously with 0.01 M sodium carbonate/bicarbonate buffer for 10 d; Con-GLP2 = noninfected calves injected subcutaneously every 12 h with 50 μ g of GLP-2/kg of BW for 10 d; Eim-Buffer = *E. bovis*-infected calves injected with 0.01 M sodium carbonate/bicarbonate buffer; Eim-GLP2 = *E. bovis*-infected calves injected every 12 h with 50 μ g of GLP-2/kg of BW for 10 d.³P-values ≤ 0.10 are indicated in bold font.⁴SEM for Con-Buffer and Eim-Buffer groups where n = 5.⁵SEM for Con-GLP2 and Eim-GLP2 groups where n = 4.

man colorectal cell line, Moran et al. (2012) observed increased protein expression of TJ protein ZO-1 and occludin, and a corresponding reduction in a measure of TJ permeability, in response to GLP-2 treatment. In mice, treatment with GLP-2 reduced permeability of the jejunum, which was associated with increased protein expression of claudins 3 and 7, but not claudin 15 or TJ protein ZO-1 (Dong et al., 2014). Thus, variation exists across species and experimental conditions regarding which proteins of the TJ respond to GLP-2 treatment. Increased expression of *CXADR*, *CLDN2*, *OCN*, and *TJP1* genes in calf jejunum in response to GLP-2 treatment may ultimately serve to enhance barrier function in this tissue, but additional study is needed in cattle to determine whether increases in transcript levels of specific members of the TJ complex

translate into increases in their protein levels and reduction in tissue permeability.

In the ileum, an interaction of *E. bovis* infection by GLP-2 treatment was noted for expression of *JAM2*, *OCN* ($P \leq 0.10$), and *TJP1* ($P \leq 0.04$) mRNA, but there was no main effect of infection status or GLP-2 treatment on mRNA expression among the other TJ complex genes evaluated (Table 2). Among buffer-treated calves, *E. bovis* infection decreased expression of *JAM2*, *OCN* ($P < 0.05$), and *TJP1* ($P < 0.10$) mRNA in ileum. These findings are consistent with studies showing reduced expression of TJ complex proteins, such as occludin, in ileum of animals with pathogen-induced diarrhea (Zhang et al., 2012; Xu et al., 2014). Of interest, in *E. bovis*-infected calves, GLP-2 treatment increased ($P < 0.10$) *TJP1* mRNA expression in ileum

Figure 2. Effect of glucagon-like peptide 2 (GLP-2) treatment on mean ($\pm$ SE) percentage of cells positively labeled for tight junction protein ZO-1 (TJP ZO-1) and occludin protein expression in the jejunum of noninfected Holstein bull calves as determined by immunohistochemistry. Con-buffer = noninfected, injected (s.c.) twice daily with 0.01 M sodium carbonate/bicarbonate buffer ($n = 5$); Con-GLP2 = noninfected, injected (s.c.) twice daily with 50 μ g of GLP-2/kg of BW ($n = 4$). ** $P < 0.002$.

to levels similar to those of noninfected calves receiving buffer treatment, suggesting a potential therapeutic use of GLP-2 during intestinal infections.

Of the 3 intestinal tissues sampled, most treatment effects were observed in the cecum where mRNA expression of *CXADR*, *CLDN4*, *F11R*, and *OCN* was decreased ($P < 0.05$) by *E. bovis* infection by 1.5- to 1.9-fold, and treatment with GLP-2 increased expression of *CLDN4* ($P \leq 0.08$), *F11R*, *OCN*, and *TJP1* mRNA ($P \leq 0.02$) by 1.6- to 2.0-fold (Table 2). The effects of *E. bovis* on TJ gene expression are consistent with the known damage that occurs in this tissue during *E. bovis* replication in the gut (Friend and Stockdale, 1980). Similarly, altered mRNA expression of TJ complex proteins *OCN*, *CLDN2*, and *CLDN3* was reported in the colon of mice infected with *Escherichia coli* O157:H7, which adhered primarily in the cecum and colon and significantly impaired colonic barrier function (Roxas et al., 2010).

In conclusion, subcutaneous GLP-2 administration increases gene expression in various members of the TJ complex in distal small intestine and cecum of calves. Infection with *E. bovis* downregulates mRNA expression of several genes associated with the TJ complex, particularly in cecum. Glucagon-like peptide-2 therapy may help to restore transcription of these genes to basal levels and ultimately reduce permeability of intestinal tissues. These findings lay the groundwork for functional studies involving manipulations of TJ complex

members that are regulated by GLP-2, and provide further evidence for the potential use of GLP-2 therapies to improve intestinal health of dairy cattle and other ruminants.

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