Characterization of glucagon-like peptide 2 pathway member expression in bovine gastrointestinal tract

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ABSTRACT

Glucagon-like peptide 2 (GLP-2), secreted by enteroendocrine cells, has several physiological effects on the intestine of monogastric species, including promotion of growth of intestinal epithelium, reduction of epithelial cell apoptosis, and enhancement of intestinal blood flow, nutrient absorption, and epithelial barrier function. The regulatory functions of GLP-2 in the ruminant gastrointestinal tract (GIT) have not been well studied. The objectives of this investigation were to characterize the mRNA expression of 4 members of the GLP-2 pathway throughout the bovine GIT, including (1) proglucagon (*GCG*), the parent peptide from which GLP-2 is derived through cleavage by prohormone convertase; (2) prohormone convertase (*PCSK1*); (3) GLP-2 receptor (*GLP2R*); and (4) dipeptidyl peptidase IV (*DPP4*), the enzyme that inactivates GLP-2. Gene expression was evaluated in rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, and rectum collected at slaughter from prepubertal heifers, mature cows in early, mid, and late lactation, and non-lactating cows ($n = 3$ per stage) by a gene expression profiling assay. In addition, mRNA expression of 14 genes involved in nutrient transport, enzyme activity, blood flow, apoptosis, and proliferation were evaluated in the 9 GIT tissues for their association with *GCG* and *GLP2R* mRNA expression. Immunohistochemistry was used to localize GLP2R protein in tissues of the lower GIT. Results indicated that mRNA expression of *GCG*, *PCSK1*, *GLP2R*, and *DPP4* varies across the 9 GIT tissues, with greatest expression in small and large intestines, and generally nondetectable levels in forestomachs. Expression of *DPP4* and *GLP2R* mRNA varied by developmental stage or lactational state in intestinal tissues. Expression of *GCG* or *GLP2R* mRNA was correlated with molecular markers of proliferation, apoptosis, blood flow, enzyme activity, and urea transport, depending on the tissue examined, which suggests

a potential for involvement of GLP-2 in these physiological processes in the ruminant GIT. The GLP2R protein was expressed in intestinal crypts of the bovine GIT, which is consistent with the distribution in monogastric species. Our findings support a functional role of the GLP-2 pathway in bovine GIT and the potential for use of GLP-2 as a therapy to improve intestinal function and nutrient absorption in ruminants.

Key words: cattle, gastrointestinal tract, gene expression, glucagon-like peptide 2

INTRODUCTION

Glucagon-like peptide 2 (GLP-2) is a hormone secreted by intestinal L cells, which has been shown in monogastric species to increase cell proliferation in the intestinal crypts, reduce apoptosis of intestinal mucosa, suppress inflammation, and enhance nutrient absorption in the gut (Lovshin and Drucker, 2000; Burrin et al., 2003; Drucker, 2005). The peptide has been used to improve intestinal growth and integrity after injury or after regression resulting from parenteral nutrition in pigs, and may be a viable treatment to improve intestinal function in premature infants (Burrin et al., 2007), as well as adults with short-bowel syndrome (Jeppesen, 2003).

The biological actions of GLP-2 are determined by the liberation of the peptide from its parent protein proglucagon (*GCG*) via the enzyme prohormone convertase (*PCSK1*), its degradation by dipeptidyl peptidase IV (*DPP4*), and interaction with its G protein-coupled receptor, GLP-2 receptor (*GLP2R*). To date, functional studies of the GLP-2 pathway in livestock have been limited to swine, although preliminary reports indicate that *GCG* and *GLP2R* mRNA are expressed in bovine gastrointestinal tract (GIT; Taylor-Edwards et al., 2008), and that GLP-2 treatment enhances splanchnic blood flow and mass of the small intestine in calves (Taylor-Edwards et al., 2009a,b). The complete functions and therapeutic potential of GLP-2 in ruminants are unknown, but its use as a treatment for intestinal diseases such as calf scours has been suggested (Burrin et al., 2003).

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The purpose of this study was to evaluate mRNA expression of *GCG*, *PCSK1*, *DPP4*, and *GLP2R*, as well as mRNA for nutrient transporters, markers of apoptosis, proliferation, growth, enzyme activity, and blood flow in the bovine GIT. Gene expression was evaluated during 5 stages of development or lactation, including prepubertal heifers, lactating cows during early, mid, and late lactation, and nonlactating cows. A 22-gene multiplex expression assay was used to test the hypothesis that changes in lactational stage are associated with changes in expression of GLP-2 pathway members. In addition, we tested the hypothesis that expression of *GCG* or *GLP2R* mRNA is correlated to changes in expression of mRNA for markers of apoptosis, proliferation, growth, enzyme activity, and blood flow, because GLP-2 is known to affect these processes in other species. Last, cellular expression of GLP2R protein was characterized in the lower bovine gut by immunohistochemistry.

MATERIALS AND METHODS

Bovine Tissues

Intestinal tissues including rumen, reticulum, omasum, abomasum, duodenum, jejunum, ileum, cecum, and rectum were collected at slaughter from culled adult Holstein cows from the Beltsville Agricultural Research Center dairy herd (Beltsville, MD) at the institutional abattoir, and from prepubertal Holstein heifers (100 to 130 d of age) from a study conducted at Clemson University (Clemson, SC). All animal procedures associated with the heifer samples were approved by the Clemson University Animal Care and Use Committee. For each developmental stage examined, gene expression was based on 3 animals. Among the lactating Holsteins, cows were categorized as early lactation [19 to 92 DIM; 3.7 ± 1.4 yr of age (mean $\pm$ SD); lactation number of 2.3 ± 1.5 (mean $\pm$ SD)], mid lactation (138 to 201 DIM; 4.9 ± 4.1 yr of age; 3.0 ± 3.5 lactations), or late lactation (250 to 414 DIM; 4.9 ± 2.1 yr of age; 2.7 ± 2.1 lactations). Nonlactating cows were 5.3 ± 2.1 yr of age, nonpregnant, and killed either 59 or 77 d after cessation of milking.

At slaughter, each tissue was dissected and rinsed in tap water (rumen, reticulum, omasum, and abomasum) or physiological saline (duodenum, jejunum, ileum, cecum, and rectum) to remove residual feed and GIT contents. In an attempt to maintain consistency for tissue sampling, rumen was sampled from the anterior portion beneath the reticulum and below the rumen fluid layer; abomasum was sampled from the lower, ventral surface of the posterior compartment; duodenum was sampled posterior to the bile ducts; ileum was sampled anterior to the ileo-cecal junction; cecum was sampled from the

most distal region of the enclosed sack; and rectum was sampled anterior to the anus.

For RNA analysis, sampling was limited to the epithelial layers. For the small and large intestinal segments, a glass slide was used to scrape the epithelial mucosal layer for sample collection. Samples were then immediately immersed in RNeasy RNA stabilization solution (Ambion, Austin, TX) and stored at 4°C overnight. Excess RNeasy was then removed and preserved tissues were stored at -80°C until RNA extraction. Whole tissues were also sampled for sectioning and subsequent immunohistochemical analysis. Tissue pieces were placed in glass scintillation vials with 15 mL of 10% neutral buffered formalin (pH 6.8 to 7.2; Richard-Allan Scientific, Kalamazoo, MI) and stored at 4°C overnight. Excess neutral buffered formalin was removed and tissue pieces were rinsed once with 70% ethanol, and then stored at 4°C in fresh 70% ethanol until paraffin embedding and tissue sectioning.

RNA Extraction

Frozen tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted using the RNeasy Midi Kit (Qiagen, Valencia, CA) with on-column DNase digestion. Quality of RNA was assessed using a 2100 Bioanalyzer and RNA 6000 Nano LabChip kits (Agilent Technologies, Palo Alto, CA), and RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE).

22-Gene Multiplex Expression Assay

Expression of 22 genes was quantified simultaneously in each RNA sample using a custom multiplex gene expression assay, based on traditional reverse transcription-PCR, developed for the Genome Lab GeXP Genetic Analysis System and GeXP Start Kit (Beckman Coulter, Inc., Brea, CA). Genes of interest are summarized in Table 1, including 4 potential reference genes for normalization: ATP synthase (*ATP5B*), hypoxanthine guanine phosphoribosyl transferase 1 (*HPRT1*), *GAPDH*, and β -2 microglobulin (*B2M*). Primer selection was conducted using eXpress Designer software (Beckman Coulter, Inc.) and GenBank accession numbers (<http://www.ncbi.nlm.nih.gov/>) of bovine transcripts of interest. In each case, we confirmed that the primers used for each gene produced a single product of the expected size as measured by capillary electrophoresis. Primers were evaluated in both singlet and multiplex reactions to verify that the same amplicons appeared (at the same sizes) in the electropherogram in the multiplex reaction as they appeared in the singlet

reactions, and that no extraneous bands appeared due to unexpected interaction of the pooled, multiplexed primers.

Procedures for assay optimization, generation of standard curves, reverse transcription, PCR, and capillary electrophoresis on the GeXP Genetic Analysis System were performed as recommended by the manufacturer (GenomeLab GeXP Protocol A29143 AB; December 2007). Additional details on the methodology can be found in Rai et al. (2009). Briefly, 100 ng of total RNA was used in each 20- μ L reverse transcription reaction containing 20 U of reverse transcriptase (provided), 1,000 pg of KanR RNA (internal control provided with the GeXP start kit), 1 $\times$ reverse transcription buffer, 2 μ L of pooled gene-specific reverse primers (concentrations optimized according to manufacturer's guidelines), and reaction conditions consisting of 1 min at 48°C, 60 min at 42°C, and 5 min at 95°C. A no-template (minus RNA) control reaction was performed with each assay to ensure no contamination of reagents, and a negative control in the absence of reverse transcriptase was performed for each RNA sample to ensure no genomic DNA contamination. Amplification by PCR was performed in duplicate using 9.3 μ L of cDNA, 1 $\times$ PCR buffer, pooled forward primers (at 20 nM each), and 3.5 U of Thermo-Start DNA polymerase (ABgene, Surrey, UK) under the following conditions: 10 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 68°C. Resulting PCR products were diluted in sample loading solution and size standard provided in the GeXP kit and separated by capillary electrophoresis in the GeXP Genetic Analysis System using the Frag-3 protocol. Standard curves were generated for each gene in the 22-gene multiplex using a 10-point standard curve ranging from 1 to 500 ng of template RNA.

Results including the peak height and area under the curve for each fragment in the electropherograms were exported to the eXpress Profiler program (Beckman Coulter, Inc.) for analysis and normalization to the internal control (KanR). Stability of 4 potential genes for normalization was assessed using the GeNorm program (<http://medgen.ugent.be/~jvdesomp/genorm/>) and the most stably expressed genes were used for each comparison of interest (Table 2). Interpolation from the standard curves and final normalization to reference genes were performed using the GeneQuant Tool (Beckman Coulter, Inc.). Thus, normalized expression of each gene transcript was reported as the quantity relative to the geometric mean expression of the selected reference genes.

Immunohistochemistry

Formalin-fixed bovine GIT tissues were dehydrated in ethanol and embedded in paraffin for subsequent

sectioning and staining. Tissues were serially sectioned and mounted onto Superfrost glass slides by Histoserv Inc. (Gaithersburg, MD). The slides were processed for GLP2R protein detection by use of a rabbit polyclonal antibody (MBL Intl., Woburn, MA) raised against human and porcine amino acid sequences. A section of porcine duodenum collected from a 1-yr-old, first-parity gilt at 110 d of gestation at slaughter was also used as a positive and negative control for the staining procedure. Briefly, slides were deparaffinized and hydrated through sequential alcohol washes. After quenching in 3% hydrogen peroxide, antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH 6.0) for 2 $\times$ 5-min pulses with a 5-min rest period between each pulse period, and a final 30-min cooling period. Nonspecific binding was blocked by incubating in CAS block (Invitrogen) for 10 min at room temperature. The GLP2R antibody was applied at a 1:500 dilution in CAS block and incubated at room temperature for 2 h. A negative control was also performed on tissue from the rectum of 1 animal in the absence of GLP2R antibody. The presence of GLP2R was visualized with a 4-min incubation with diaminobenzidine (DAB) using the Super Picture HRP Polymer Conjugate Broad Spectrum staining kit according to the manufacturer's instructions (Invitrogen). Finally, slides were counterstained with Carrazi's hematoxylin for 1 min, dehydrated, and mounted with Permaslip (Alban Scientific Inc., St. Louis, MO).

Statistical Analyses

For nondetectable gene expression values, a random number between 0 and the lowest detectable value measured among the samples for that particular transcript was assigned to obtain a mean and variance for mean comparisons. Mean comparisons of gene expression across tissues or developmental stages were made by ANOVA using the GLM procedure in SAS (SAS Institute Inc., Cary, NC). For analysis of overall mRNA expression differences across tissues for *GCG*, *PCSK1*, *GLP2R*, and *DPP4*, tissue was the CLASS variable and the MODEL statement included each gene as a dependent variable and tissue as the independent effect. To evaluate differential mRNA expression across developmental and lactational stages, tissue and stage were the CLASS variables and the MODEL statement included each gene as a dependent variable and tissue, developmental stage, and their interaction as the independent effects. Further pairwise comparisons of least squares means across tissues or developmental stages within each gene were performed with SAS using the PDIF option in the LSMEANS statement for each CLASS variable. To determine if expression of

Table 1. Genes included in the 22-gene multiplex used to assess gene expression, their primer sequences (forward, F, and reverse, R), GenBank accession numbers used in primer design, and standard curve fit (r^2)

Gene symbol	Primer	Primer sequence (5' → 3') including 5' <u>adapter sequence</u>	Gene name	GenBank accession no.	Linearity of standard curve
Glucagon-like peptide 2 (GLP2) activity					
<i>GCG</i>	F	<u>AGGTGACACTATAGAATAGCCTTTACTTTGTGGCTGGA</u>	Pre-pro glucagon	NM_173916	0.9977
	R	<u>GTACGACTCACTATAGGGAATTCATCAACCACTGCACGA</u>			
<i>GLP2R</i>	F	<u>AGGTGACACTATAGAATACTCTCCATCGTCTCGCTCTT</u>	GLP2 receptor	XM_589370	0.9995
	R	<u>GTACGACTCACTATAGGGATGAGCAGCCACGAGTAATTG</u>			
<i>PCSK1</i>	F	<u>AGGTGACACTATAGAATATGGAAGCAAATCCCAATCTC</u>	Prohormone convertase 1	NM_174412	0.9995
	R	<u>GTACGACTCACTATAGGGATCGACTGTTCAACCATCAAGC</u>			
<i>DPP4</i>	F	<u>AGGTGACACTATAGAATACAGGCCCTGTAGTCAAAAA</u>	Dipeptidyl peptidase IV	NM_174039	0.9998
	R	<u>GTACGACTCACTATAGGGAGGTAACCACTTCCTCTGCCA</u>			
Normalization					
<i>ATP5B</i>	F	<u>AGGTGACACTATAGAATACCCCTCAAGGAGACCATCAAA</u>	ATP synthase	NM_175796	0.9992
	R	<u>GTACGACTCACTATAGGGAGGACACCATGGAGGATGAGT</u>			
<i>HPRT1</i>	F	<u>AGGTGACACTATAGAATAGCCGACCTGTTGGATTACAT</u>	Hypoxanthine guanine phosphoribosyl transferase 1	NM_001034035	0.9985
	R	<u>GTACGACTCACTATAGGGAGCATTGTCTTCCCAGTGTCA</u>			
<i>GAPDH</i>	F	<u>AGGTGACACTATAGAATGATTGTCTCAGCAATGCCTCCT</u>	Glyceraldehyde-3-phosphate dehydrogenase	NM_001034034	0.9994
	R	<u>GTACGACTCACTATAGGGACCATCCACAGTCTTCTGGGT</u>			
<i>B2M</i>	F	<u>AGGTGACACTATAGAATAAGCGTCCTCCAAAGATTCAA</u>	β-2 Microglobulin	NM_173893	0.9995
	R	<u>GTACGACTCACTATAGGGAACAGGTCTGACTGCTCCGAT</u>			
Transporter					
<i>SGLT1</i>	F	<u>AGGTGACACTATAGAATAACACAGACACCTTGCAGACG</u>	Sodium-glucose cotransporter	NM_174606	0.9984
	R	<u>GTACGACTCACTATAGGGAAGATGATGGTCAGCCCAAAG</u>			
<i>GLUT2</i>	F	<u>AGGTGACACTATAGAATATTGGTGTGATCAATGCACCT</u>	Facilitated glucose transporter	NM_001103222	0.9989
	R	<u>GTACGACTCACTATAGGGAAGACAGGACAGAACATGG</u>			
<i>SLC14A1</i>	F	<u>AGGTGACACTATAGAATACTTGAACCTCCGTGTTGAGCA</u>	Urea transporter	NM_001008666	0.9998
	R	<u>GTACGACTCACTATAGGGAGATCAAGGTGCTTGGGAAAA</u>			
<i>PEPT1</i>	F	<u>AGGTGACACTATAGAATAAGCAAAGCCTTTCGAAGACA</u>	Oligopeptide transporter	NM_001099378	0.9988
	R	<u>GTACGACTCACTATAGGGAACAGGCATCCTGCTTGAAGT</u>			
Apoptosis					
<i>CASP3</i>	F	<u>AGGTGACACTATAGAATAAATGCAACTCTTCCACCAGC</u>	Caspase 3	XM_001256573	0.9990
	R	<u>GTACGACTCACTATAGGGACTGTGAAGAATCTAGCCCGC</u>			
<i>CASP6</i>	F	<u>AGGTGACACTATAGAATATGCTAGCCACGTAGATGCAG</u>	Caspase 6	NM_001035419	0.9994
	R	<u>GTACGACTCACTATAGGGACGACACGCCTGAATGATAAA</u>			
Proliferation					
<i>SOX9</i>	F	<u>AGGTGACACTATAGAATAAATCTCCTGGACCCCTTCAT</u>	Transcription factor SOX9	AF278703	0.9938
	R	<u>GTACGACTCACTATAGGGATCGCTCTCCTTCTTCAGGTC</u>			
<i>PCNA</i>	F	<u>AGGTGACACTATAGAATAAGGACAGTGCCTTCATTTGG</u>	Proliferating cell nuclear antigen	NM_001034494	0.9995
	R	<u>GTACGACTCACTATAGGGAAAACACTGCCCTACAACGCT</u>			
<i>CCND1</i>	F	<u>AGGTGACACTATAGAATACATGAACCTACCTGGACCGCT</u>	Cyclin D1	NM_001046273	0.9987
	R	<u>GTACGACTCACTATAGGGAGCATCTTGGAGAGGAAGTGC</u>			
<i>IGF1</i>	F	<u>AGGTGACACTATAGAATAGTTGGTGGATGCTCTCCAGT</u>	Insulin-like growth factor 1	NM_001077828	0.9996
	R	<u>GTACGACTCACTATAGGGACCTCCTCAGATCACAGCTCC</u>			
Enzyme activity					
<i>SI</i>	F	<u>AGGTGACACTATAGAATACGAAGAACGTGGCTGCTTAT</u>	Sucrase-isomaltase	NM_001114189	0.9994
	R	<u>GTACGACTCACTATAGGGAACCTCCACACGAAGAGACGA</u>			
<i>MGAM</i>	F	<u>AGGTGACACTATAGAATACAGTACCGCCACAACATGAC</u>	Maltase-glucoamylase	XM_594452	0.9991
	R	<u>GTACGACTCACTATAGGGAATTTCCAGGAACACATAGAAGTC</u>			
<i>ANPEP</i>	F	<u>AGGTGACACTATAGAATAGCCCATGTTCACTCCACTCT</u>	Aminopeptidase	NM_001075144	0.9996
	R	<u>GTACGACTCACTATAGGGACCTGAGATCAGACCCAGAGC</u>			

(Continued)

Table 1 (Continued). Genes included in the 22-gene multiplex used to assess gene expression, their primer sequences (forward, F, and reverse, R), GenBank accession numbers used in primer design, and standard curve fit (r^2)

Gene symbol	Primer	Primer sequence (5' → 3') including 5' adapter sequence	Gene name	GenBank accession no.	Linearity of standard curve
Blood flow <i>NOS3</i>	F	AGGTGACACTATAGAAATGATCAGCAACGCTATCACGA	Nitric oxide synthase 3	NM_181037	0.9989
	R	GTACGACTCACTATAGGGACCATAGTGGATGACAGCCC			

GCG or *GLP2R* might influence apoptosis, proliferation, growth, enzyme activity, and blood flow in GIT, Pearson correlation coefficients were determined for *GCG* and *GLP2R* mRNA versus mRNA expression of molecular markers for the processes of interest, using normalized expression values obtained across all developmental stages in each GIT tissue. A *P*-value of 0.05 was considered significant for all statistical inferences excluding correlation analyses where a *P*-value of 0.01 was used.

RESULTS

Quality Controls

Quality of total RNA as assessed by the Agilent RNA Integrity Number (RIN) ranged from 5.0 to 9.7, and averaged 8.1. A total of 3 (2 omasum and 1 rumen) out of 135 samples had a RIN value <6. As a point of reference, a RIN value of ≥ 5 is recommended for downstream applications such as quantitative PCR (Fleige and Pfaffl, 2006). Linearity of the standard curves for the 22 genes in the multiplex ranged from 0.9938 to 0.9998 (Table 1). Negative control reactions for multiplex reverse transcription-PCR resulted in no amplification of target transcripts (data not shown).

GLP-2 Pathway Gene Expression

Relative mRNA expression of GLP-2 pathway members *GCG*, *PCSK1*, *DPP4*, and *GLP2R* varied ($P < 0.001$; Figure 1) across the 9 GIT tissues collected from prepubertal and mature dairy cattle. Expression of transcripts for *GCG* and *PCSK1* was below the reliable detection limit in rumen, reticulum, and omasum. Expression of *DPP4* and *GLP2R* mRNA was either nondetectable or extremely low in these 3 tissues. Expression of *GCG* and *PCSK1* mRNA was greatest in rectum and expression of *DPP4* and *GLP2R* was greatest in small intestine. Expression of *GLP2R* mRNA was moderately high in cecum relative to other tissues despite low expression levels of other GLP-2 pathway members in this tissue.

For mRNA expression of GLP-2 pathway members, an interaction was found between tissue and developmental stage ($P < 0.05$). Within each tissue type, expression of *GCG* mRNA and *PCSK1* mRNA did not differ by developmental stage or stage of lactation ($P \geq 0.15$). However, mRNA expression of *DPP4* and *GLP2R* was affected by developmental stage or lactational state in some tissues (Figure 2). Specifically, in duodenum, *DPP4* mRNA expression was generally lowest in prepubertal heifers ($P < 0.03$), greatest in nonlactating cows ($P < 0.04$), and intermediate among lactating cows. In

Table 2. Stability of normalization gene expression in bovine gastrointestinal tract tissues based on GeNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>)

Tissue	Normalization genes, ¹ n	Normalization genes ²	Respective M values ³
All tissues	3	<i>ATP5B</i> , <i>GAPDH</i> , <i>HPRT1</i>	0.455, 0.518, 0.563
Rumen	2	<i>ATP5B</i> , <i>HPRT1</i>	0.095, 0.095
Reticulum	2	<i>ATP5B</i> , <i>HPRT1</i>	0.180, 0.180
Omasum	2	<i>ATP5B</i> , <i>HPRT1</i>	0.298, 0.298
Abomasum	4	<i>ATP5B</i> , <i>B2M</i> , <i>GAPDH</i> , <i>HPRT1</i>	0.551, 0.757, 0.573, 0.664
Duodenum	2	<i>ATP5B</i> , <i>GAPDH</i>	0.227, 0.227
Jejunum	2	<i>ATP5B</i> , <i>GAPDH</i>	0.277, 0.277
Ileum	2	<i>ATP5B</i> , <i>B2M</i>	0.290, 0.290
Cecum	2	<i>ATP5B</i> , <i>GAPDH</i>	0.099, 0.099
Rectum	2	<i>ATP5B</i> , <i>B2M</i>	0.338, 0.338

¹Optimal number based on GeNorm pairwise variations between 2 sequential normalization factors containing an increasing number of genes. A cut-off value of 0.15 was used, below which inclusion of no additional reference genes was determined.

²*ATP5B* = ATP synthase; *HPRT1* = hypoxanthine guanine phosphoribosyl transferase 1; *B2M* = β -2 microglobulin.

³Stability measure from GeNorm based on average pairwise variation in that gene versus all other normalization genes. A value <1.5 is considered acceptable. The lower the M value for a gene, the more stable its expression.

cecum, *DPP4* mRNA was greatest in nonlactating cows relative to all other stages ($P \leq 0.05$). In rectum, *DPP4* mRNA expression was greatest in late lactation relative to all other stages of lactation or prepubertal heifers ($P \leq 0.02$). Expression of *DPP4* mRNA in rectum was similar between cows in late lactation and nonlactating cows ($P = 0.06$).

Expression of *GLP2R* mRNA varied across developmental or lactational stages in several tissues (Figure 2). In duodenum, jejunum, ileum, and rectum, expression of *GLP2R* mRNA had a similar pattern of expression across developmental stages and was generally lowest in prepubertal heifers relative to all other stages examined.

Correlation of *GCG* and *GLP2R* mRNA Expression with Other Molecular Markers

A significant positive correlation was observed in ileum between *GCG* mRNA expression and mRNA expression of urea transporter (*SLC14A1*; $r = 0.74$, $P = 0.001$), maltase-glucoamylase (*MGAM*; $r = 0.76$, $P = 0.001$), and aminopeptidase (*ANPEP*; $r = 0.65$, $P = 0.009$). In cecum, abundances of *GCG* mRNA and *IGF-I* mRNA were positively correlated ($r = 0.62$, $P = 0.01$). Likewise, in duodenum, a positive correlation was found between *GCG* mRNA and sucrose-isomaltase (*SI*) mRNA ($r = 0.79$, $P < 0.001$). Last, in jejunum, expression of *GCG* transcripts was positively correlated with mRNA expression of proliferative markers transcription factor *SOX9* (*SOX9*; $r = 0.91$, $P < 0.0001$) and proliferating cell nuclear antigen (*PCNA*; $r = 0.71$, $P = 0.003$).

Expression of *GLP2R* mRNA was correlated with expression of both apoptotic and proliferative markers in small intestine and cecum. Specifically, *GLP2R* mRNA was positively correlated with caspase 3 (*CASP3*) mRNA ($r = 0.64$, $P = 0.01$) in cecum and negatively correlated with caspase 6 (*CASP6*) mRNA in jejunum ($r = -0.67$, $P = 0.006$) and cecum ($r = -0.61$, $P = 0.01$). Transcripts for proliferative markers *PCNA*, *IGF1*, and cyclin D1 (*CCND1*) were positively correlated with *GLP2R* mRNA in multiple segments of the small intestine and cecum, with the exception of transcripts of *IGF1*, which was not correlated to *GLP2R* in cecum. This included *PCNA* in duodenum ($r = 0.63$, $P = 0.01$), jejunum ($r = 0.62$, $P = 0.01$), and cecum ($r = 0.78$, $P < 0.001$); *CCND1* in duodenum ($r = 0.73$, $P = 0.003$), jejunum ($r = 0.82$, $P < 0.001$), ileum ($r = 0.89$, $P < 0.0001$), and cecum ($r = 0.71$, $P = 0.003$); and *IGF1* in duodenum ($r = 0.80$, $P = 0.001$) and jejunum ($r = 0.76$, $P < 0.001$). Finally, levels of *GLP2R* mRNA were positively correlated with nitric oxide synthase 3 (*NOS3*) mRNA, a marker of blood flow, in all 3 segments of the small intestine (duodenum: $r = 0.85$, jejunum: $r = 0.79$, and ileum: $r = 0.76$; $P < 0.001$).

GLP2R Protein Expression

Expression of *GLP2R* protein was examined and detected by immunohistochemistry in duodenum, jejunum, ileum, cecum, and rectum of dairy cattle (Figure 3, panels A to E) and duodenum of pig as a positive control (Figure 3, panel G). There was no staining in the negative control samples for either bovine or porcine tissues (Figure 3, panels F and H) incubated without *GLP2R* antibody. In each tissue, expression of

Figure 1. Differential mRNA expression of proglucagon (*GCG*; panel A), prohormone convertase 1 (*PCSK1*; panel B), glucagon-like peptide 2 receptor (*GLP2R*; panel C), and dipeptidyl peptidase IV (*DPP4*; panel D) in gastrointestinal tract segments of dairy cattle ($n = 13$ to 15 per tissue). Developmental stages surveyed include prepubertal heifers and lactating and nonlactating cows. ND = less than the reliable detection level. Bars sharing a common letter are not statistically different ($P > 0.05$) from one another.

the protein was limited almost exclusively to cells lining the crypts.

DISCUSSION

The GLP-2 pathway plays an important role in regulating proliferation and integrity of the intestinal tract of mammals (Estall and Drucker, 2003) and may be involved in intestinal development (Lovshin et al., 2000). Despite the roles of GLP-2 in normal gut function in monogastric species, its functional role in ruminant species has not been well investigated and is poorly understood. The goal of this study was to determine the distribution of the gene expression of the key GLP-2 pathway members, *GCG*, *PCSK1*, *GLP2R*, and *DPP4*, throughout the bovine GIT using a multiplex gene expression profiling assay, and to determine whether expression of these genes varies among different stages of lactation and development. In addition, we sought to evaluate a putative role of GLP-2 in physiological responses in the gut such as cell proliferation, apoptosis,

enzyme activity, nutrient transport, and blood flow by evaluating the relationships between mRNA expression of *GCG* or *GLP2R* in GIT tissues and the expression of molecular markers of these processes. Although this is not a direct measure of GLP-2 action on intestinal cells, it provides a basis for inferring a physiological role of GLP-2 in normal bovine GIT function and further investigation into the use of GLP-2 to improve gut function and nutrient uptake in cattle. Results of this study will further provide the basis for examination of gene expression changes in GIT tissues of cattle treated with GLP-2 in future studies.

In the present study, the distribution of *GCG*, *PCSK1*, *GLP2R*, and *DPP4* mRNA was characterized in 9 segments of the GIT of dairy cattle and expression was found primarily in the small and large intestines, although expression of all 4 transcripts was also detectable in abomasum. In addition, *GLP2R* mRNA was detectable in omasum and *DPP4* was detectable in reticulum and omasum of the ruminant GIT. Similarly, expression of *GCG* mRNA among monogastric species

Figure 2. Differential mRNA expression of dipeptidyl peptidase IV (*DPP4*) and glucagon-like peptide 2 receptor (*GLP2R*) across developmental and lactational stages in specific gastrointestinal tract tissues of dairy cattle ($n = 2$ to 3 per stage). The mRNA expression of *DPP4* in duodenum (panel A), cecum (panel B), and rectum (panel C); *GLP2R* mRNA expression in duodenum (panel D), jejunum (panel E), ileum (panel F), and rectum (panel G). Bars sharing a common letter are not statistically different ($P > 0.05$) from one another. SEM = standard error of the mean as determined by ANOVA.

Figure 3. Immunohistochemical localization of glucagon-like peptide 2 receptor (GLP2R) in bovine gastrointestinal tract tissues where *GLP2R* mRNA was detected by a multiplex gene expression profiling assay. Representative micrographs are shown for (A) duodenum of non-lactating cow; (B) jejunum of mid-lactation cow (d 145); (C) ileum of nonlactating cow; (D) cecum of late-lactation cow (d 250); (E) rectum of early-lactation cow (d 50); (F) negative control [rectum of late-lactation cow (414 d)]; (G) positive control (duodenum of 1-yr-old, first-parity gilt at 110 d of gestation); and (H) negative control (duodenum of gilt). Negative control slides were incubated without GLP2R antibody and showed no staining. Scale bar = 100 μ m.

has been demonstrated in the 3 segments of the small intestine, cecum, and colon of the rat (Lovshin et al., 2000; Zhou et al., 2006), and expression in stomach (equivalent to the ruminant abomasum) has been inferred from an expressed sequence tag from a human stomach library (GenBank accession no. BM836042.1). Furthermore, expression of *PCSK1* mRNA observed in the present study was found to have a similar tissue distribution to that reported for PCSK1 protein in small intestine of the mouse (Gagnon et al., 2009), and *PCSK1* mRNA has been demonstrated in rat stomach (Macro et al., 1996) and colon (Cani et al., 2005), although expression of *PCSK1* mRNA has not been reported specifically in cecum. Likewise, the expression pattern of *GLP2R* mRNA in cattle was consistent with what has been reported in the literature for the rat (Munroe et al., 1999), and protein expression as determined by immunohistochemistry in small intestine, cecum, and rectum of cattle was limited to the cells lining the crypts. This finding agrees with the localization of this G protein-coupled receptor in human intestine (Yusta et al., 2000). Last, expression of *DPP4* mRNA or its protein has been demonstrated in rat intestine (Dunphy et al., 1999) and stomach (Hong et al., 1989) as well as human small intestine (Darmoul et al., 1994), cecum, and colon (Young et al., 1992), which is consistent with its observed distribution in the bovine GIT. Thus, based on the distribution of mRNA expression of the GLP-2 pathway members and GLP2R protein expression in bovine GIT, it can be inferred that GLP-2 has similar functions in ruminants as in monogastric species.

In support of a potential role of GLP-2 in proliferation and maintenance of the ruminant GIT, positive correlations between mRNA expression of the GLP-2 precursor, *GCG*, as well as *GLP2R*, were observed between markers of cell proliferation, including transcripts for *IGF1*, *SOX9*, *PCNA*, and *CCND1*, in multiple segments of the small and large intestines. In addition, a positive correlation was found between *CASP3* and *GLP2R* mRNA in cecum and a negative correlation was observed between *CASP6* mRNA and *GLP2R* mRNA in duodenum. The relationships with these markers of apoptosis suggest that GLP-2 may mediate apoptosis in these tissues. Burrin et al. (2005) found that GLP-2 treatment of neonatal pigs on total parenteral nutrition dose-dependently decreased *CASP6* and *CASP3* activities in intestinal tissue, reducing the apoptotic rate of crypt and villus cells, but increased *CASP3* protein abundance in intestinal cells. This illustrates the potential disconnect between mRNA abundance and the abundance or activity of its encoded enzyme, and the caution one must exercise when interpreting the physiological implications of changes in mRNA

expression. Despite this limitation, the relationships observed between *GCG* or *GLP2R* gene expression in bovine GIT and expression of markers of proliferation and apoptosis suggest that GLP-2 may play a role in these processes in cattle and other ruminants.

As a result of reports of GLP-2 effects on intestinal transcripts for enzymes such as glycoside hydrolases and aminopeptidases in piglets (Petersen et al., 2002), relationships between *GCG* and *GLP2R* mRNA expression and expression of *SI*, *MGAM*, and *ANPEP* mRNA in bovine GIT were evaluated in the present study. Our results supported a potential role of the GLP-2 pathway in nutrient uptake in ruminant GIT, because expression of *GCG* was positively correlated with changes in expression of *MGAM*, *ANPEP*, and *SI* genes in bovine small intestine. However, no apparent association was observed between *GLP2R* mRNA and expression of markers of enzyme activity.

Conversely, a positive correlation between *GLP2R* and *NOS3* mRNA in all 3 segments of the bovine small intestine is consistent with observed effects of GLP-2 infusion in piglets (Burrin et al., 2005). Experimental evidence indicates that GLP-2 stimulates blood flow in GIT through the GLP2R and endothelial nitric oxide synthase activation (Guan et al., 2003, 2006). A preliminary report by Taylor-Edwards et al. (2009a) demonstrated increased splanchnic blood flow in calves infused with GLP-2. It is thought that increased blood flow to gut tissues induced by GLP-2 might be mediated by *NOS3* and may contribute to improved nutrient absorption in the ruminant gut.

Specifically, improved intestinal transport and absorption of nutrients including hexoses, amino acids, and lipids have been reported in response to GLP-2 treatment (Burrin et al., 2003; Estall and Drucker, 2006). The exact mechanisms for these responses are not well understood, but may be mediated by changes in the expression of transporters for these nutrients. For instance, Ramsanahie et al. (2004) observed increased expression of sodium-dependent glucose transporter (*SGLT1*) protein in enterocytes and localized changes in *SGLT1* mRNA expression to the intestinal villus of GLP-2-treated rats, and GLP-2 treatment was found to increase abundance of *SGLT1* mRNA in small intestine of piglets (Sangild et al., 2006). Further, increased expression of the facilitated glucose transporter (*GLUT2*) protein in the brush border membrane has been observed in GLP-2-treated rats (Au et al., 2002). However, no apparent relationship was observed between expression of *GCG* or *GLP2R* mRNA and abundance of *SGLT1* or *GLUT2* mRNA transcripts in any segments of the bovine GIT. A weak association ($r = 0.63$, $P = 0.02$) was observed between *GCG* mRNA and *PEPT1* mRNA in duodenum. It should be noted that expression of

GLUT2 mRNA was below the reliable detection level in most segments of the bovine GIT, excluding duodenum and jejunum, and bovine rectum expressed only *SGLT1* mRNA of the 4 transporters examined. In addition, the current study was based on basal expression levels of these genes, whereas the data in the literature are based on responses to GLP-2 supplementation. The effects of pharmacological doses of GLP-2 on bovine intestinal transporter mRNA expression are unknown and are currently under investigation in our laboratory. Of interest, however, was a strong positive correlation between *GCG* mRNA and expression of urea transporter (*SLC14A1*) in bovine ileum. This is the first known report of this apparent association and is suggestive of GLP-2 effects on nitrogen salvaging via urea transporter expression in the ruminant gut, warranting further investigation.

Finally, evidence suggests that expression and function of GLP-2 pathway members is developmentally regulated. For instance, although *GLP2R* mRNA is expressed in fetal and neonatal piglets, administration of pharmacological doses of GLP-2 does not induce intestinal proliferation and differentially affects intestinal enzyme activity between fetal and neonatal piglets (Petersen et al., 2001). Furthermore, intestinal *GCG* mRNA expression significantly changes with age and weaning in rats (Reimer et al., 1997). To evaluate a potential role of the GLP-2 pathway in ruminant GIT development, we examined the distribution of *GCG*, *PCSK1*, *GLP2R*, and *DPP4* mRNA in prepubertal calves and adult cows during different stages of lactation. Despite a small sample size, differential mRNA expression across the stages examined was observed for *DPP4* and *GLP2R*. The variation in *DPP4* mRNA expression was in duodenum, cecum, and rectum, with expression levels generally greatest in nonlactating cows or cows in late lactation, depending on the tissue examined. Because *DPP4* plays a role in cleavage of multiple peptide targets, these changes may be related to functions other than inactivation of GLP-2; however, the increases in *DPP4* mRNA expression would likely result in reduced GLP-2-mediated responses in these tissues because *DPP4* appears to be regulated at the transcript level (Hong et al., 1989; Darmoul et al., 1991). In other words, one would expect an increase in intestinal *DPP4* mRNA expression to reduce intestinal growth mediated by GLP-2. Darmoul et al. (1994) measured *DPP4* mRNA and its protein expression in human small intestine and colon, as well as *DPP4* enzyme activity in epithelial cells isolated from these tissues and found increasing mRNA and protein levels from duodenum to ileum, and nondetectable levels in colon by Northern and Western blotting. Low-level *DPP4* mRNA expression could only be detected by the more sensitive technique of reverse

transcription-PCR. Expression of bovine *DPP4* mRNA as measured by reverse transcription-PCR in the present study showed a pattern of expression similar to that of human. Interestingly, in a previous study examining changes in intestinal proliferation during 4 stages of lactation in dairy cattle by our laboratory (Baldwin et al., 2004), it was found that mean meter weight of duodenum of lactating dairy cows declined from approximately 275 g at d 90 of lactation, to 240 g at d 120 of lactation, and to 234 g at 240 d of lactation. This would correspond to measures of gene expression at early, mid, and late lactation in our study in which the mean duodenal *DPP4* mRNA values had a correlation of -0.99 with the corresponding mean duodenum weights in the study by Baldwin et al. (2004). Although this is highly circumstantial evidence, it is consistent with the hypothesis that *DPP4* may play a role in intestinal growth regulation in dairy cattle during lactation.

The basic expression pattern of *GLP2R* mRNA measured in prepubertal heifers and cows during different stages of lactation in our study showed relatively stable transcript expression in small intestine and rectum in adult cows regardless of lactational stage. However, expression in adult cows was at a greater level than in prepubertal heifers. This would suggest that basal *GLP2R* expression might only be responsive to or mediate relatively large changes in intestinal growth, such as those involved in the transition from a prepubertal state to a pregnant or lactating state, where energy demands and nutrient intake are substantially greater than in the young animal. Clearly, an experiment specifically designed to examine the ontogeny of *GLP2R* mRNA and *GLP2R* protein expression during intestinal development in ruminants is needed.

CONCLUSIONS

Because of the importance of GLP-2 in gut development, proliferation, and integrity in monogastric species, the GLP-2 pathway may contribute to several economically relevant processes in ruminant livestock species. Results of this study suggest that the GLP-2 pathway is expressed and functional in the bovine GIT. Understanding mechanisms governing the GLP-2 pathway in cattle may ultimately provide novel means to improve GIT development and function in calves, or manipulate proliferation of the GIT, one of the greatest nutrient users among the visceral tissues (Johnson et al., 1990), in adult dairy cows during lactation and the dry period.

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