Short communication: Presence of G protein-coupled receptor 43 in rumen epithelium but not in the islets of Langerhans in cattle

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ABSTRACT

Volatile fatty acids (VFA) are the major products of microbial fermentation in the rumen. Besides serving as substrates for energy generation, VFA are known to stimulate rumen development, increase serum insulin and glucagon concentrations, and regulate gene expression in cattle and sheep. The mechanisms underlying these regulatory effects of VFA are unknown, but the recent discovery that VFA can bind to G protein-coupled receptor 43 (GPR43) and 41 (GPR41) suggests that the regulatory effects of VFA may be mediated by these receptors. As a step toward testing this possibility, we determined whether GPR43 was expressed in bovine rumen wall and the pancreatic islets of Langerhans. Polyclonal antibody against a bovine GPR43 peptide was generated. The specificity of the antibody for bovine GPR43 was confirmed by Western blot analysis of recombinant bovine GPR43 protein. Immunohistochemical analyses using this antibody revealed the presence of GPR43-immunoreactive cells in the epithelium, but not in the other layers of cattle rumen wall. The same immunohistochemical analyses did not reveal GPR43-immunoreactive cells in the islets of Langerhans or the surrounding exocrine tissue of cattle pancreas. These data support the possibility that the effect of VFA on rumen epithelial growth in cattle is directly mediated by GPR43 in the rumen epithelial cells and that the effect of VFA on pancreatic secretion of insulin and glucagon in cattle is unlikely to be directly mediated by GPR43.

Key words: volatile fatty acid, rumen, pancreas, cattle

Short Communication

Volatile fatty acids are a group of short-chain fatty acids, containing 1 to 7 carbons, produced by microbial fermentation in the gastrointestinal tract (Bergman,

1990). The major VFA are acetate, propionate, and butyrate. Volatile fatty acids are a major source of energy for the animal, in particular, the ruminant (Bergman, 1990). In ruminants, microbial fermentation occurs mainly in the rumen, and rumen VFA are estimated to provide 60 to 70% of the energy requirement of a steer (Siciliano-Jones and Murphy, 1989). In addition to serving as substrates for energy production, VFA have a multitude of regulatory effects (Bergman, 1990). Intraruminal administration or dietary supplementation of VFA stimulates the growth of rumen epithelium in young ruminants (Sakata and Tamate, 1978; Lane and Jesse, 1997; Gorka et al., 2009), and increases blood insulin and glucagon concentrations (Bergman, 1990; Harmon, 1992; Kristensen and Harmon, 2004). Volatile fatty acids regulate the expression of hundreds of genes in various types of cells (Li and Elsasser, 2005; Li and Li, 2006; Alvaro et al., 2008). The mechanisms underlying these regulatory effects of VFA are unclear. It was recently discovered that VFA can bind to G protein-coupled receptors 43 (**GPR43**) and 41 (**GPR41**), which are also named free fatty acid receptor 2 and 3, respectively (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003). This suggests that the regulatory effects of VFA, such as those on rumen epithelium growth and pancreatic secretion of insulin and glucagon, may be directly mediated by GPR43 and GPR41. As a step toward testing this possibility, we produced and validated a polyclonal antibody specific for the bovine GPR43 protein and used this antibody to determine if and where GPR43 protein was present in the rumen and the pancreas of cattle.

A commercially available GPR43 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which was raised against a human GPR43 peptide, did not cross-react with bovine GPR43 (bGPR43) by Western blot analysis (data not shown). We therefore aimed to generate antibody using a bGPR43 peptide as antigen. The sequence of the peptide is EDPGVSQAEGAPSS, which corresponds to the 311 to 324 region of the bGPR43 protein (GenBank accession number NP_001157256.1). This peptide was chosen as antigen based on its predicted high antigenicity. The peptide was conjugated

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to keyhole limpet hemocyanin through an added N-terminal cysteine and injected into rabbits following a standard polyclonal antibody production procedure (Diano et al., 1987). The antibody was affinity-purified using protein A columns. The titer of the purified antibody was determined by ELISA to be 1:512,000. The steps from peptide synthesis to ELISA were contracted to GenScript (Piscataway, NJ).

To determine if the generated bGPR43 antibody reacted with bovine GPR43 protein, we amplified the entire coding region of bGPR43 cDNA by PCR, using forward primer 5'-tgcaagcttCAGC-CATGCCAGACTGGGATAG-3' and reverse primer 5'-GATGGATCC-TCCAAGCCTGCTCAACTCCT-3'. The PCR product was cloned into the enhanced green fluorescent protein (**EGFP**) tagging vector pEGFP-C1 (Clontech, Mountain View, CA). The resulting plasmid pEGFP-bGPR43 was confirmed by DNA sequencing. We transfected pEGFP-bGPR43 or pEGFP-C1 into Chinese hamster ovary (**CHO**) cell line cells using FuGene 6 as transfection reagent (Roche, Indianapolis, IN). The cells were cultured for 48 h at 37°C under 5% CO₂ in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma, St. Louis, MO), and then lysed as described previously (Zhou et al., 2008). Thirty micrograms of total protein lysate was separated by SDS-PAGE, and subsequently transferred to a nitrocellulose membrane as described previously (Wang et al., 2009). The membranes were incubated with the bGPR43 antibody at a dilution of 1:2,500 or a green fluorescent protein (**GFP**) antibody (Sigma-

Aldrich, St. Louis, MO) at dilution of 1:1,000, and then a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody at dilution of 1:1000 (Santa Cruz Biotechnology). We also performed an absorption test to validate the specificity of the bGPR43 antibody. In this test, the bGPR43 antibody was incubated with a 100-fold molar excess of the bGPR43 peptide for 1 h at room temperature before being incubated with the membrane.

Representative images of Western blot analyses are shown in Figure 1. The bGPR43 antibody detected a protein of approximately 65 kDa from CHO cells transfected with the pEGFP-bGPR43 plasmid but not from CHO cells without transfection or CHO cells transfected with pEGFP-C1 (Figure 1A). The approximate size of this protein matched the calculated molecular weight of the bGPR43-EGFP fusion protein (36 kDa for bGPR43 + 27 kDa for EGFP). The same protein was also detected by a GFP antibody (Figure 1B). These findings suggest that the protein detected by the bGPR43 antibody was the bGPR43-EGFP fusion protein, and this in turn suggests that the bGPR43 antibody cross-reacts with bovine GPR43 protein. Pre-incubation of the bGPR43 antibody with an excess of its antigen blocked the detection of the bGPR43-EGFP fusion protein (Figure 1C). This data validated the specificity of the bGPR43 antibody for the bGPR43 peptide.

We next used this antibody in immunohistochemistry (**IHC**) to localize GPR43 in bovine rumen and pancreas tissues. Rumen (ventral sac) and pancreas tissues from adult cattle were collected at slaughter, fixed with 10%

Figure 1. Confirmation of the specificity of the bovine G protein-coupled receptor 43 (bGPR43) antibody for bovine GPR43 protein. Equal amounts of whole cell lysates from Chinese hamster ovary (CHO) cells transfected with plasmids expressing bGPR43-enhanced green fluorescent protein (EGFP) fusion protein or EGFP, or from nontransfected CHO cells (NC) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was analyzed with (A) bGPR43 antibody; (B) anti-GFP antibody; and (C) bGPR43 antibody preincubated with an excess of the bGPR43 peptide. Color version available in the online PDF.

Figure 2. Immunohistochemistry of G protein-coupled receptor 43 (GPR43) in cattle rumen. Rumen sections were incubated first with the bovine GPR43 antibody (bGPR43; A and B) or preimmune rabbit serum (C and D) and then with Alexa Fluor 488-tagged secondary antibody. The sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Immunoreactive signals were visualized with a fluorescence microscope (A and C) in adjacent sections of a partial papilla (B and D show higher magnifications of the boxed regions in A and C, respectively). GPR43 staining is labeled in green; DAPI staining is labeled in blue. SC = stratum corneum; SG = stratum granulosum; SS = stratum spinosum; SB = stratum basale; LP = lamina propria. Bar = 100 μ m.

Figure 3. Immunohistochemistry of G protein-coupled receptor 43 (GPR43) and insulin in cattle pancreas. Pancreas sections were double-incubated with bovine GPR43 antibody (bGPR43) or preimmune rabbit serum and anti-insulin antibody, and then secondary antibodies labeled with Alexa Fluor 488 and Alexa Fluor 594, respectively. The GPR43-immunoreactive cells, if present, would appear in green and the insulin-immunoreactive cells in red, and the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). (A) GPR43 staining in a section; (B) insulin staining in the same section; (C) merger of A and B; (D), (E), and (F) show analysis of an adjacent section using preimmune rabbit serum instead of bGPR43 antibody. Bar = 100 μ m.

neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), and embedded in paraffin. Sections of 5 μ m were cut with a microtome. The sections were deparaffinized in xylene and rehydrated in a graded series of ethanol and PBS as described previously (Capuco et al., 2002; Berry et al., 2003). Antigen retrieval was performed by steaming the sections in 10 mM citrate buffer (pH 6.0) for 35 min and cooling at room temperature (25°C) for 10 min. The sections were incubated with 10% non-immune goat serum for 30 min to block nonspecific binding of antibody.

For IHC of the rumen tissue, the sections were incubated with the bGPR43 antibody at a dilution of 1:1,000 in a humidifying chamber at 4°C overnight. Following rinsing in PBS, the sections were incubated with Alexa 488-tagged goat-anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) at a dilution of 1:200 in the dark for 60 min. For IHC of the pancreatic tissue, the sections were doubly incubated with the bGPR43 antiserum at 1:1,000 and mouse anti-insulin antibody at 1:2,000 (Sigma-Aldrich) at 4°C overnight. These 2 primary antibodies were detected, respectively, by Alexa Fluor 488-tagged goat anti-rabbit antibody at 1:200 and Alexa Fluor 594-tagged goat anti-mouse secondary antibody at 1:200 (Invitrogen). Following rinsing in PBS, the sections were mounted with ProLong Gold antifade reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) to stain the nuclei. The sections were examined with a fluorescence microscope. Adjacent sections with the bGPR43 antibody replaced by rabbit preimmune serum or the bGPR43 antibody omitted served as negative controls.

Representative images of the IHC analyses are presented in Figure 2 and Figure 3. The IHC of rumen sections revealed GPR43 immunostaining in the epithelium layer (labeled in green in Figure 2A, 2B), but not in the lamina propria (Figure 2A, 2B), submucosa, or muscular layers (images not shown). Within the epithelium, GPR43 immunostaining was much stronger in the stratum basale, stratum granulosum, and stratum spinosum than in the stratum corneum (Figure 2B). The specificity of GPR43 immunostaining is supported by the lack of the staining in sections incubated with rabbit preimmune serum (Figure 2C, 2D) or in sections incubated without the GPR43 antibody (images not shown). The IHC of the pancreas sections did not reveal GPR43 immunostaining in either the islets of Langerhans or their surrounding tissue (Figure 3). As expected, insulin-immunoreactive cells (i.e., the β cells) were detected in the islets of Langerhans (labeled in red in Figure 3), but not in the exocrine part of the pancreas (Figure 3).

Given the fact that GPR43 is present in the epithelium of human and rat colon (Karaki et al., 2006, 2008;

Kaji et al., 2011; Tang et al., 2011) and that GPR43 mRNA is expressed in the epithelium of bovine rumen (Wang et al., 2009), it is not surprising that GPR43 is also expressed in the epithelium of bovine rumen, because the rumen in cattle and colon in humans and rats are the primary sites where short-chain fatty acids are produced and absorbed (Bergman, 1990). However, it is surprising that GPR43 protein was not detected in bovine pancreas, as GPR43 mRNA was expressed in this tissue (Wang et al., 2009). Either GPR43 mRNA is not translated efficiently to protein in bovine pancreas, or expression of GPR43 protein in this tissue is insufficient to be detectable by IHC.

The stimulatory effects of VFA on growth of rumen epithelium and pancreatic secretion of insulin and glucagon are well established (Sakata and Tamate, 1978; Bergman, 1990; Harmon, 1992; Lane and Jesse, 1997; Kristensen and Harmon, 2004; Gorka et al., 2009). The presence of GPR43, the receptor for VFA (Brown et al., 2003; Le Poul et al., 2003; Nilsson et al., 2003; Wang et al., 2009), in the epithelium of cattle rumen supports a potential role of GPR43 in directly mediating the effects of VFA on rumen epithelial growth. The mechanisms by which VFA stimulates growth of rumen epithelium through GPR43 remain to be studied. We speculate that binding of VFA to GPR43 might change the expression or activity of cell cycle regulators, enhance the responsiveness to endocrine or paracrine growth factors, or increase the uptake of VFA (and hence increased energy production) in rumen epithelial cells, among many other possibilities. On the contrary, the absence of GPR43 immunoreactive cells in the pancreas does not support a role of GPR43 in directly mediating the stimulatory effects of VFA on pancreatic secretion of insulin and glucagon in cattle.

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