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Distribution of acute phase proteins in the bovine forestomachs and abomasum

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ABSTRACT

Acute phase proteins (APPs) are produced mainly by the liver and their concentration is increased during the systemic inflammatory response. Expression of haptoglobin (Hp), serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP) and α-1 acid glycoprotein (AGP) was determined in the mucosa of the normal bovine forestomachs and abomasum by qualitative and quantitative reverse transcriptase-PCR for mRNA and by Western blot analysis and immunohistochemistry for proteins. Although expression of SAA mRNA was evident in the forestomachs and abomasum, SAA protein was identified only in the abomasum. Expression of Hp protein was high in the forestomachs and abomasum, even though expression of Hp mRNA was negligible. The main site of expression of LBP mRNA was the omasum, whereas the highest protein expression was evident in the abomasum. AGP was expressed at low levels in the bovine forestomachs. Western blot analysis revealed a heterogeneous electrophoretic pattern for AGP, LBP and Hp, indicating that different stomach compartments produce isoforms that are different to those expressed by the liver. Expression of APPs by the bovine forestomachs and abomasum may contribute to regulation of the innate immune response against pathogens.

Introduction

The gastrointestinal tract fulfils divergent roles of nutrient absorption and host defence. The mucosal surfaces of the forestomachs and intestines of ruminants are in continuous contact with a vast, diverse and dynamic microbial community. Since microbes can cross the epithelial surface through the intestinal barrier, protective immune responses must be raised against pathogens, whereas there is a need to tolerate innocuous antigens from commensal bacteria. Intestinal defences include epithelial-derived antimicrobial peptides, cytokines and acute phase proteins (APPs) (Dommett et al., 2005).

APPs belong to a large family of structurally unrelated proteins that are produced as part of the systemic inflammatory response and play a role in modulating innate immunity and scavenging inflammatory by-products (Gabay and Kushner, 1999). They are produced mainly by the liver after stimulation by pro-inflammatory cytokines (Baumann and Gauldie, 1994), but extrahepatic expression also has been reported (McDonald et al., 2001; Upragarin et al., 2005; Lecchi et al., 2009; Rahman et al., 2010).

There is little information on the involvement of the ruminant gastric mucosa in innate immunity. The mucosa of the three forestomachs (rumen, reticulum and omasum) is lined by stratified, squamous, keratinised epithelium and the mucosa of the abomasum is lined by simple, glandular, columnar epithelium (Scala et al., 2011). The rumen contains >10^{10} commensal microbiota/g contents and the mucosal surface represents an important line of defence against the penetration of microorganisms (Krause and Russell, 1996).

There is no organised lymphoid tissue in the mucosa of the forestomachs and abomasum. Scattered lymphocytes and Langerhans' cells are present in the forestomachs (Josefsen and Landsverk, 1996) and there are scattered mast cells and lymphocytes in the abomasum (Balic et al., 2000).

The four major bovine APPs are haptoglobin (Hp), serum amyloid A (SAA), lipopolysaccharide-binding protein (LBP) and α-1 acid glycoprotein (AGP). Previous studies have identified expression of LBP (Rahman et al., 2010) and AGP (Lecchi et al., 2009) in the stomachs and intestinal tract of ruminants. In the present study, we performed a more detailed examination of expression of Hp, SAA, LBP and AGP in the normal bovine forestomachs and abomasum.

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Table 1
Sequences of oligonucleotide primers for acute phase proteins.

Name	Fragment size (base pairs)	Sense	Antisense
SAA	121	5′-CCTGCTGGCCTGCCTGAC-3′	5′-GCTGCCTTCTGAGGACAGAG-3′
LBP	130	5′-CCTGATTCTAGCATTCGACAG-3′	5′-GGCTGAAGTTCAGGCACG-3′
Hp	337	5′-GCAGCTTTCCCTGGCAGG-3′	5′-CCAGACACATAACCCACACG-3′
Hp_RT	67	5′-CCAAGATGGTCTCCCAGCAT-3′	5′-GTGAGGAGCCATCGTTCAATTG-3′
AGP	226	5′-GCATAGGCATCCAGGAATCA-3′	5′-GCACCGAAACAAACTTTATTGATGC-3′
AGP_RT	112	5′-GCATAGGCATCCAGGAATCA-3′	5′-TAGGACGCTTCTGTCTCC-3′
β-actin	133	5′-CCAAAGCCAACCGTGAGA-3′	5′-CCAGAGTCCATGACAATGC-3′

SAA, serum amyloid A; LBP, lipopolysaccharide-binding protein; Hp, haptoglobin; AGP, α-1 acid glycoprotein. SAA, LBP, Hp, AGP and β-actin primers were designed on the basis of GenBank sequences (Accession Numbers NM181016.3, NM001038674.1, NM_001040470.1, AM403243 and BC142413.1, respectively). Hp_RT primers sequences are from Eckersall et al. (2006).

Materials and methods

Tissue collection and preservation

Bovine gastric tissues were collected from four clinically healthy animals at a local abattoir. The tunica mucosa was mechanically separated from the submucosa with a scalpel and portions of tissue were preserved in RNAlater (Sigma–Aldrich) and stored at −80 °C. Samples for Western blot analysis were collected immediately into liquid nitrogen and stored at −80 °C. Samples for immunohistochemistry were embedded in Killik (Bio-Optica), frozen in liquid nitrogen-cooled isopentane (Sigma–Aldrich) and stored at −80 °C. Unless otherwise stated, all subsequent procedures were conducted at room temperature.

Qualitative and quantitative mRNA expression

Total RNA was extracted using TriZol (Invitrogen) and treated with DNase I (Invitrogen). Total RNA was quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. Reverse transcription (RT) was carried out with 1 μg RNA using the iSCRIPT cDNA Synthesis Kit (BioRad). The cDNA was used as the template for PCRs, which were performed in 10 μL final volumes containing 1× buffer (Vivantis), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 μM each primer and 0.025 U *Taq* polymerase (Vivantis). The same primers were used in qualitative and quantitative PCR for SAA and LBP, whereas different primers were used for Hp and AGP (Table 1). PCR conditions were 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s (Eppendorf Mastercycler). PCR products were visualised on 1.9% agarose gels stained with ethidium bromide.

Quantitative reactions were performed using 20 μL Eva Green mix (BioRad) and 450 nM each primer (SAA, LBP, Hp_RT, AGP_RT and β-actin; Table 1). Each sample was tested in duplicate. To evaluate PCR efficiency, fourfold serial dilutions were prepared from reference samples. The thermal profiles for each target gene were 95 °C for 90 s, 50 cycles of 95 °C for 5 s and 60 °C for 10 s; conditions for melting curve construction were 55 °C for 60 s then 80 cycles starting at 55 °C and increasing 0.5 °C each 10 s. Results were compared using the Δ–Δ Cq method (Giulietti et al., 2001).

Western blot analysis

Antibodies validated in cattle for detection of APPs by Western blot analysis and immunohistochemistry are listed in Table 2. Samples for Western blot analysis were prepared from aliquots of 50–100 mg tissues using protease inhibitors (Sigma–Aldrich), as previously described (Rahman et al., 2008). The protein content of the supernatant was quantified at A_{280} nm. Aliquots of 25–50 μg/mL were separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotted onto nitrocellulose membranes. The membranes were immunolabelled for the presence of APPs using specific antibodies (Table 2) and immunoreactive bands were visualised by enhanced chemiluminescence (ECL) using Immobilon Western Chemiluminescence substrate (Millipore). To confirm that an equal amount of protein was loaded in each lane, membranes were stripped and immunolabelled with mouse anti β-actin antibody (1:10,000). Liver lysates were used as positive controls.

Immunohistochemistry

Immunohistochemistry for AGP and Hp was performed on cryostat sections incubated in 0.3% H_2O_2 in methanol (Sigma–Aldrich) for 30 min to block endogenous peroxidase activity. Non-specific binding sites were blocked with 10% normal goat serum (Sigma–Aldrich) for 30 min and sections were incubated overnight at 4 °C in the presence of specific antibodies (Table 2). Staining of specific proteins was carried out with diaminobenzidine (DAB, Vector Laboratories) after incubation with peroxidase-conjugated secondary antibodies (Sigma–Aldrich). Nuclei were counterstained with Mayer's haematoxylin (DDK Italia). Sections were mounted in Poly-mount (Polysciences) and examined using a Nikon Eclipse E600 microscope. Bovine liver was used as a positive control. Primary antibodies were omitted for negative controls.

Results

Expression of acute phase protein mRNA in the bovine forestomachs and abomasum

Expression of Hp, SAA, LBP and AGP RNA was detected by qualitative RT-PCR in the mucosa of the bovine forestomachs and abomasum (Supplementary Fig. 1). Quantitative RT-PCR was used to determine the relative expression of Hp, SAA, LBP and AGP RNA after normalisation against β-actin (Fig. 1). Increased amounts of SAA and LBP RNA relative to the liver were expressed in the mucosa of the forestomachs and abomasum; the highest levels of SAA were detected in the omasum and abomasum, whereas the highest levels of LBP were detected in the omasum. Expression of AGP and Hp in the mucosa of the forestomachs and abomasum was negligible compared to the liver.

Expression of acute phase proteins in the bovine forestomachs and abomasum

Hp, LBP and AGP were detected by Western blot analysis in all samples of mucosa from the forestomachs and abomasum (Fig. 2). AGP was detected as a low molecular weight (MW) immunoreactive band of 21 kDa expressed mainly in the rumen, reticulum

Table 2
Primary antibodies for Western blotting and immunohistochemistry.

Name	Western blot analysis			Immunohistochemistry	
	Primary antibody	Incubation time	Reference	Primary antibody	Incubation time
Mouse anti-bovine SAA (C100–8)	1:100 (3.9 μg/mL)	60 min	McDonald et al. (1991)	–	–
Mouse anti-human LBP (biG42)	1:200 (5 μg/mL)	45 min	Rahman et al. (2010)	–	–
Rabbit anti-bovine Hp	1:2,000 (1.37 μg/mL)	45 min	–	1:200 (13.75 μg/mL)	Overnight
Rabbit anti-bovine AGP	1:2,000 (1.65 μg/mL)	45 min	Ceciliani et al. (2007b)	1:200 (16.5 μg/mL)	Overnight
Mouse anti-β-actin (CP01)	1:10,000	45 min	Lecchi et al. (2008)	–	–

SAA, serum amyloid A; LBP, lipopolysaccharide-binding protein; Hp, haptoglobin; AGP, α-1 acid glycoprotein.