Expression of α1-acid glycoprotein and lipopolysaccharide binding protein in visceral and subcutaneous adipose tissue of dairy cattle

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Abstract

Adipose tissue is an endocrine compartment that plays an important role in immune defence by producing and releasing a wide range of proteins, including acute phase proteins (APPs). The liver is the main organ of APP synthesis, although extrahepatic production has also been reported. In the present study, expression of two APPs in dairy cattle, lipopolysaccharide binding protein (LBP) and α1-acid glycoprotein (AGP), was determined in four visceral (pericardial, mesenteric, omental and retroperitoneal) and three subcutaneous (withers, tail head and sternum) adipose tissue depots. mRNA expression was evaluated using qualitative and quantitative PCR, protein profiles were assessed by Western blot analysis and cellular localisation was determined by immunohistochemistry. The presence of LBP and AGP was demonstrated at mRNA and protein levels in all seven adipose tissue depots. Expression of AGP and LBP suggests that they may have roles as local and systemic inflammatory adipokines.

Keywords

Bovine

Adipose tissue

Acute phase proteins

α1-Acid glycoprotein

Lipopolysaccharide binding proteins

Introduction

Adipose tissue (AT) stores energy and is involved in metabolic processes by influencing lipid metabolism via lipolysis and lipogenesis. AT is a highly active endocrine compartment that plays a central role in several aspects of the immune system by producing and releasing a wide range of messenger molecules, which are commonly referred to as adipokines (Cao, 2014). The main functions of adipokines are to regulate and coordinate the complex network between lipid metabolism, energy expenditure and insulin sensitivity, and to integrate these with the immune system (Bastard et al., 2006).

The subcutaneous AT (SAT) comprises AT located in the hypodermis, whereas visceral AT (VAT) includes the fat depots in the intrathoracic cavity (including the epicardial fat), the intra-abdominal cavity (including the omental, mesenteric, retroperitoneal and perirenal fat) and the intrapelvic cavity (including the gonadal fat). In humans, the visceral fat depot size is linked to systemic inflammation associated with obesity and related diseases (Van Gaal et al., 2006). Fat storage and allocation within body depots of farm animals has an impact on animal health and meat quality (Wood et al, 2008, Sauerwein et al, 2013). In addition to its involvement in regulating metabolism, bovine AT responds to pro-inflammatory challenges in a depot specific manner by synthesising immune related proteins, such as the acute phase proteins (APPs) (Mukesh et al., 2010).

The APPs belong to a group of proteins that undergo changes in circulating concentrations in response to external or internal challenges (Gabay and Kushner, 1999). They are part of the non-specific innate immune system involved in the restoration of homeostasis before acquired immunity is active. The four most studied APPs in cattle include serum amyloid A (SAA), haptoglobin (Hp), α1-acid glycoprotein (AGP) and lipopolysaccharide binding protein (LBP) (Ceciliani et al., 2012). The liver is the principal organ of APP synthesis, but extrahepatic production has also been reported (Thielen et al, 2007, Lecchi et al, 2009, Rahman et al, 2010, Dilda et al, 2012). The serum concentrations of APP increase towards calving because of increased endometrial and hepatic synthesis (Cairoli et al, 2006, Gabler et al, 2010, Bossaert et al, 2012).

Hp and SAA are expressed in SAT and VAT, with increased mRNA expression at the time of parturition (Saremi et al, 2012, Saremi et al, 2013). Expression of AGP has been reported in porcine AT (Rødgaard et al, 2013a, Rødgaard et al, 2013b). An association between LBP expression, adiposity and insulin sensitivity has been demonstrated (Moreno-Navarrete et al., 2013). The aim of this study was to determine whether different bovine AT depots, including VAT and SAT, express AGP and LBP at both the mRNA and protein level.

Materials and methods

Tissue collection and preservation

Samples from bovine AT were collected from six non-pregnant, late lactation, multiparous Holstein cows during routine slaughter at an abattoir. Samples were obtained from animals that were clinically healthy at antemortem inspection and had no gross pathological findings during routine slaughterhouse inspection. Samples collected from the SAT (withers, tail head and sternum) and VAT (omental, mesenteric, pericardial and perirenal fat) were washed with sterile phosphate buffered saline (PBS), then either frozen in liquid nitrogen and stored at −80 °C or fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE). Samples of liver were washed with sterile PBS, placed in mRNA later (Sigma-Aldrich) at 4 °C for 12 h and stored at −80 °C, or fixed in formalin and embedded in paraffin (FFPE).

mRNA expression

Total RNA was extracted using TriZol (Invitrogen), treated with DNase I (Invitrogen) and quantified using a NanoDrop ND-1000 UV-vis spectrophotometer (Thermo Fisher Scientific);

the absorbance at 260 nm/280 nm was < 2. Reverse transcription (RT) was performed with 1 µg RNA using the iSCRIPT cDNA Synthesis Kit (BioRad). The cDNA was used as the template for PCR, which was performed in a 10 µL final volume containing 1 µL cDNA, 1x 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 primers used in the qualitative and quantitative PCR for AGP and LBP are listed in Table 1. The programme for qualitative PCR was 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s (Mastercycler, Eppendorf). PCR products were visualised on 1.6% agarose gels stained with ethidium bromide.

Table 1. List of primer sequences for the target and reference genes. The relative standard curves were prepared starting from bovine liver cDNA.

Gene	GenBank	Forward (5′–3′)	Reverse (5′–3′)	PCR efficiency (r^2)[a]
LRP10[b]	BC149232	CCAGAGGATGAGGACGATGT	ATAGGGTTGCTGTCCCTGTG	98.8% (0.996)
GAPDH[c]	NM_001034034	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	104% (0.997)
HPCAL1[b]	NM_001098964	CCATCGACTTCAGGGAGTTC	CGTCGAGGTCATACATGCTG	106% (0.997)
AGP[d]	AM403243.2	GCATAGGCATCCAGGAATCA	TAGGACGCTTCTGTCTCC	100.3% (0.998)
LBP[e]	NM_001038674.2	CCTGATTCTAGCATTCGACAG	GCTGAAGTTCAGGCACG	106.9% (0.998)

LRP10, low density lipoprotein receptor-related protein 10; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPCAL1, hippocalcin-like 1; AGP, α1-acid glycoprotein; LBP, lipopolysaccharide binding protein.

a r2, regression coefficient.

b Hosseini et al. (2010).

c Lecchi et al. (2009).

d Designed with Primer3 (http://primer3.ut.ee/).

e Rahman et al. (2010).

Hippocalcin-like 1 (HPCAL1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and low density lipoprotein receptor-related protein 10 (LRP10) were used as the reference genes (Hosseini et al., 2010). Quantitative PCR reactions were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) using 20 μL Eva Green mix (BioRad) and 450 nM each primer (AGP, LBP, HPCAL1, GAPDH and LRP10; Table 1). Each sample was tested in duplicate. To evaluate PCR efficiency, fourfold serial dilutions were prepared from the reference sample (liver). The thermal profile for each target gene was 95 °C for 90 s, then 50 cycles of 95 °C for 5 s and 60 °C for 10 s. The conditions for the melting curve analysis were 55 °C for 60 s, followed by 80 cycles starting at 55 °C and increasing 0.5 °C each 10 s. No-RT controls and no template controls were included. The relative quantification of the genes of interest was performed after normalisation of the sample using the geometric mean of the reference genes. Data were evaluated by analysis of variance (ANOVA) using the General Linear Model of SAS/STAT version 8 (IBM). Significance was set at $P < 0.05$.

Western blot analysis

The samples for Western blot analysis were prepared from aliquots of 100 mg tissue (Rahman et al., 2008) and brought to a final concentration of 1% protease inhibitor cocktail (Sigma-Aldrich). After centrifugation, the protein content of the supernatant was quantified at 280 nm. Aliquots of 50 μg (total protein) were loaded onto each lane of a 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel. After electrophoretic separation, the proteins were electrotransferred to nitrocellulose membranes, which were then immunolabelled with a primary antibody dilution of 1:2000 for anti-AGP (Ceciliani et al., 2007) raised against purified bovine AGP, which was previously shown to be effective for both detection of proteins by Western blot analysis and immunohistochemistry (IHC) (Lecchi et al., 2009). The mouse anti-human LBP monoclonal antibody (biG42, Biometec) was diluted 1:400 (Rahman et al., 2010). Purified AGP (Ceciliani et al., 2005) and recombinant LBP (Biometec) were used as positive controls. The 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, the membrane was immunolabelled with a mouse anti β-actin antibody (Calbiochem) at a dilution of 1:10,000.

Immunohistochemistry

Serial sections (5 μm thickness) were obtained from frozen samples, mounted on poly-lysine-coated slides and fixed in acetone at −20 °C for 5 min. The slides were then immersed in 100 mM Tris buffer pH 7.4 for 5 min. Endogenous peroxidase activity was blocked with 0.3% H2O2 in Tris for 30 min. The slides were then incubated for 20 min at room temperature (RT) with 10% normal horse serum to block nonspecific protein binding. The slides were incubated at 4 °C overnight in a humidified chamber with the same primary antibodies used for Western blot analysis at a dilution of 1:20 for anti-LBP and 1:200 for anti-AGP. The sections were rinsed in Tris-buffered saline (TBS) for 5 min and treated with PolyView mouse/rabbit nanopolymer detection reagents (Enzo Life Sciences) for 20 min at RT. After three washes in TBS, 3-amino-9-

ethylcarbazole (Vector Laboratories) was applied for 10 min. The slides were then rinsed in tap water, counterstained with Mayer's haematoxylin for 1 min and mounted with glycerine jelly (Kaiser's glycerol gelatine, Merck). Negative controls were prepared by replacing the anti-LBP primary antibody with normal mouse immunoglobulins (Mouse isotype control, Invitrogen) and the anti-AGP antibody with normal rabbit serum (non-immune rabbit serum, Dakocymation). To exclude nonspecific cross-reactivity the biG42 anti-LBP and anti-AGP specific sites were blocked by pre-incubation with human recombinant LBP and bovine purified AGP, respectively, prior to IHC (Ceciliani et al, 2005, Rahman et al, 2010).

Sections (5 μm thickness) from FFPE samples were mounted on poly-lysine-coated slides, deparaffinised in xylene and rehydrated through an ascending series of ethanol concentrations. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval was then performed by heating the slides in a pressure cooker for 10 min in citrate buffer solution (pH 6.5), followed by cooling for 40 min and rinsing with Tris. The IHC staining procedure was then performed as described above for the frozen specimens.

Statistical analysis

For all statistical procedures, means and SE were computed using the General Linear Model of SAS/STAT version V8 (SAS Institute). Statistical analysis was performed by using analysis of variance (ANOVA) with non-transported animals as fixed factor and transported animals as the randomised factor. Least significant difference was used to compare mean values. Statistical significance was accepted at $P \leq 0.05$.

Results

Detection of α1-acid glycoprotein and lipopolysaccharide binding protein mRNA in bovine adipose tissue

Expression of RNA from bovine AT was examined using qualitative PCR, then quantified by real time PCR. In AT overall, the mRNA abundance of LBP was 2–6% of the quantity in the liver and the mRNA abundance of AGP was 0.01–0.1% of the quantity in the liver (Fig. 1). The mRNA abundance of AGP in the pericardial fat was relatively higher than in the other fat depots and was 0.3% of that in the liver ($P = 0.035$). No statistically significant differences were detected between VAT and SAT.

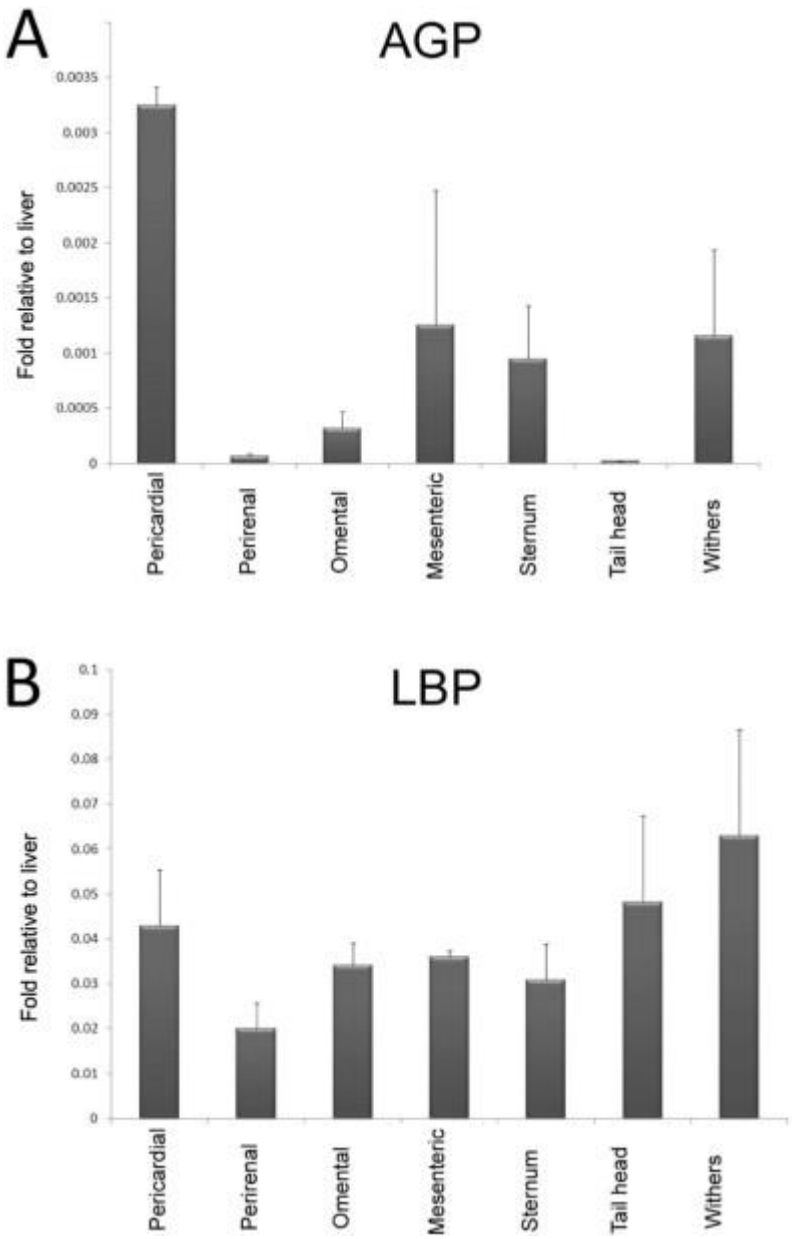

Fig. 1. Relative expression of α-1 acid glycoprotein (AGP) (A) and lipopolysaccharide-binding protein (LBP) (B) in different bovine adipose tissue depots collected from clinically healthy dairy cows (n = 6). The values are expressed as the fold difference relative to the liver and are normalised using glyceraldehyde 3-phosphate dehydrogenase, low density lipoprotein receptor-related protein 10 and hippocalcin-like 1 as reference genes. The data are the means ± SE

Expression of α1-acid glycoprotein and lipopolysaccharide binding proteins in bovine adipose tissue

Using Western blot analysis, both AGP and LBP were identified in all seven AT depots tested. As shown in Fig. 2, the most prominent immunoreactive band for AGP had a molecular weight (MW) of 55–66 kDa. Other bands were visible in the range of 40–42 kDa and at 48 and 20 kDa,

particularly in the SAT. Immunostaining for LBP yielded only one band of 55–60 kDa (Fig. 2), which was consistently observed in all AT depots tested.

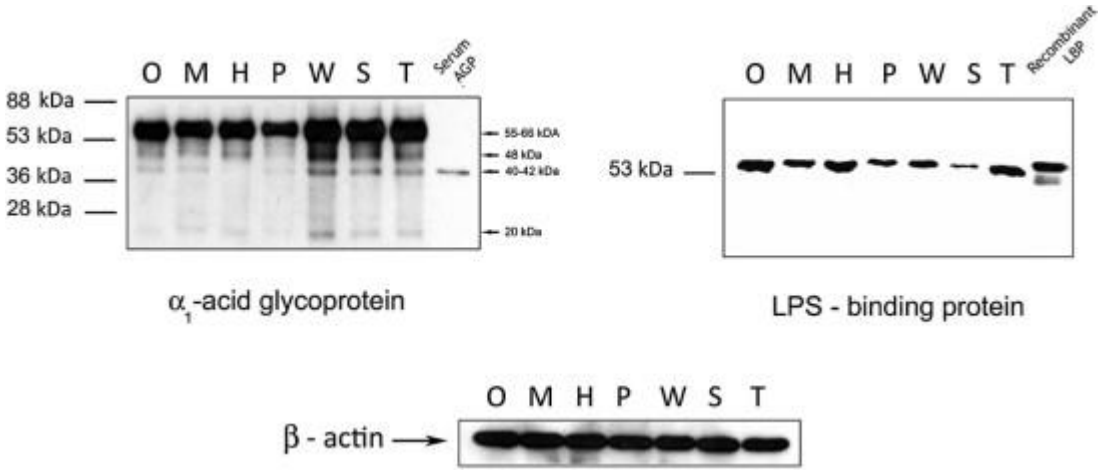

Fig. 2. Western blot analysis of α1-acid glycoprotein (AGP, left panel) and lipopolysaccharide-binding protein (LBP, right panel) in different bovine adipose tissue (AT) depots collected from clinically healthy dairy cows (n = 6). The capital letters designate the AT depots, including visceral AT (O, omental; M, mesenteric; H, heart; P, perirenal) and subcutaneous AT (W, withers; S. sternum; T, tail head). Positive samples were purified AGP (Ceciliani et al., 2007) and recombinant LPS (Biometec). Among AGP bands, the 55–66 kDa band is the AGP glycoform previously reported in bovine neutrophils (Rahman et al., 2008), the 20 kDa protein species is the deglycosylated protein (Rahman et al., 2008) and the 40–42 kDa protein species are the plasma glycoforms (Ceciliani et al., 2005).

Localisation of acute phase proteins in bovine adipose tissue

AGP and LBP were both detected in frozen and FFPE sections prepared from VAT, SAT and liver. Anti-AGP and anti-LBP antibody staining produced similar results, yielding moderate to intense signals in the AT (Figs. 3A, B) without differences between the different depots (SAT or VAT). Immunostaining was mostly diffuse and individual adipocytes with negative staining were only occasionally observed. Positive signals were localised at the periphery of the adipocyte cytoplasm, consistent with membranous staining. No specific difference in staining intensity between the two antibodies was detected. The positive immunolabelling of the liver control and AT disappeared when the tissue sections were stained with the pre-incubated antibodies, confirming the specificity of the anti-AGP and anti-LBP antibodies against their respective antigens in the tissues (see Appendix: Supplementary Fig. S1).

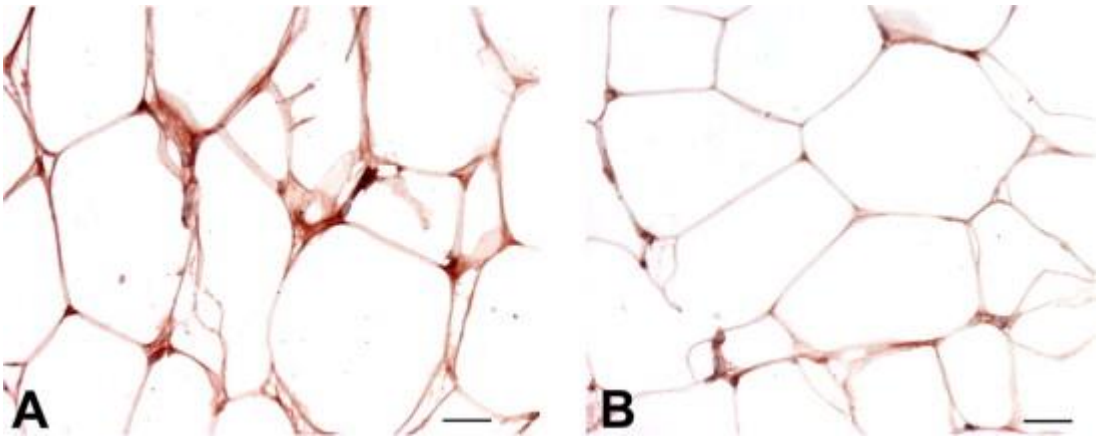

Fig. 3. Immunolocalisation of α1-acid glycoprotein (AGP) and lipopolysaccharide binding protein (LBP) in bovine adipose tissue (AT). (A) AGP: Omental AT; intense positive immunostaining (red staining) defines adipocyte cell borders. (B) LBP: Omental AT; moderate to intense positive immunostaining (red staining) defines adipocyte cell borders. Haematoxylin-blue counterstain. Scale bars = 50 μm.

Discussion

The present study demonstrates that AGP and LBP are adipokines in cattle; mRNA and protein expression were detected in seven AT depots. The identity of the proteins is supported by their immune reactivities and the MWs obtained by Western blot analysis, which are consistent with the expected sizes for these APPs. The 55–66 kDa bands of AGP correspond to the glycoform reported previously in human and bovine neutrophils (Theilgaard-Monch et al, 2005, Rahman et al, 2008), while the low MW band of ~20 kDa detected primarily in the SAT is consistent with the deglycosylated protein (Rahman et al., 2008). The 40–42 kDa glycoforms commonly found in plasma (Ceciliani et al., 2005) were also present in AT. The previously reported MW of LBP for cattle (55–60 kDa; Khemlani et al., 1992) is slightly higher than the size obtained in our present study (53 kDa); this difference may be caused by different post-translational modifications.

LBP has been identified in AT in humans and mice (Moreno-Navarrete et al., 2013). In these species, LBP synthesis and release by adipocytes is related to obesity and pro-inflammatory challenge. The function of LBP in bovine AT in non-pathological conditions remains to be elucidated. The immunomodulatory function of LBP depends on its concentration; at low concentrations, LBP amplifies the immune response against bacteria, whereas at higher concentrations LBP has inhibitory effects (Zweigner et al., 2006). In addition to its inflammation-related activity, LBP is also involved in adiposity and insulin sensitivity. Since LBP synthesis and release are related to adipocyte differentiation (Moreno-Navarrete et al., 2013), LBP gene expression should be evaluated during lipid accretion/adipogenesis and lipid mobilisation.

AGP has an anti-inflammatory function, but little information is available about the role of AGP in AT. In the present study, levels of expression of AGP mRNA in bovine AT were low compared with liver. Alfadda et al. (2012) found no differences between AGP expression in the

VAT and SAT in mice. AT AGP was recently studied in a pig model of adiposity, and differences in AGP expression between breeds were also identified (Rødgaard et al., 2013a). In view of the anti-inflammatory role of AGP (Ceciliani and Pocacqua, 2007), we hypothesise that its expression in fat depots may exert local activity by protecting AT from increased oxidative stress (Lykkesfeldt and Svendsen, 2007).

In humans and mice, expression of APPs in AT is related to inflammatory disorders associated with obesity (Pini et al., 2011). Since obesity is usually not a health issue in farm animals, different physiological roles other than those related to an inflammatory status must be envisaged. In dairy cows, the transition from late pregnancy to lactation is one of the major metabolic challenges during their lifetime. The rapid mobilisation of body fat plays critical roles in the development of peripartum diseases, such as fatty liver and ketosis. Serum concentrations of APP increase during the transition period (Cairoli et al., 2006) and are induced by an inflammatory challenge from uterine damage related to parturition and/or concomitant bacterial infection (Gabler et al., 2010).

In mice (Chiba et al, 2009, Ahlin et al, 2013) and pigs (Rødgaard et al., 2013a), serum concentrations of adipocyte-derived APPs are low. Since SAA and Hp are upregulated during the transition period in cattle (Saremi et al, 2012, Saremi et al, 2013), there would be value in determining whether AT is a source of increased concentrations of circulating APPs during the peripartum period at a time of lipomobilisation.

Conclusions

Bovine ATs produce the APPs, LBP and AGP, but relative levels of AGP and LBP mRNA in AT is low compared with liver. The contribution of AT APPs to modulation of innate immunity during critical periods of the life cycle of the dairy cow remains unclear and requires further investigation.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix. Supplementary material

The following is the supplementary data to this article:

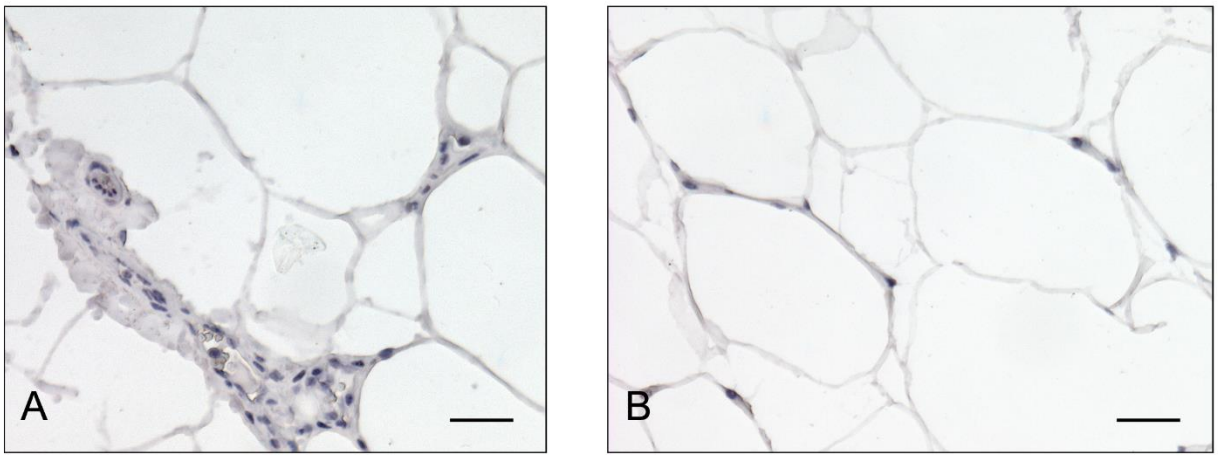

Fig. S1. Section of omental adipose tissue with immunohistochemical staining with (A) anti-lipopolysaccharide binding protein (LBP) or (B) anti-α1-acid glycoprotein (AGP) after pre-incubation of antibodies with human recombinant LBP and bovine purified AGP, respectively. Adipocytes and blood vessel lumina were not immunostained (A). Haematoxylin-blue counterstain. Scale bars = 50 μm.

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