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Short communication

The localization and differential expression of Serum Amyloid A in bovine liver and adipose tissue depots

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ABSTRACT

In this article the localization of the acute phase protein Serum Amyloid A (SAA) in different depots of bovine adipose tissue (AT) and liver is reported. Quantitative (Real Time) PCR was paired to immunohistochemistry after the production of a specific polyclonal antibody. SAA's mRNA was found in all analyzed AT depots included in the present study, the AT located in the withers being the major source of SAA mRNA. A polyclonal antibody was raised against bovine SAA and was used to validate gene expression analyses. Western Blotting confirmed that SAA is present in all the seven adipose tissue depots include in the present experiment. Anti-SAA polyclonal antibody also stained diffusely adipocytes. In liver, intracytoplasmic immunolabeling was observed in hepatocytes. Staining was generally mild and not diffuse: negative hepatocytes were intermixed with positive ones. A positive intracytoplasmic immunostaining was occasionally observed in endothelial cells lining small blood vessels within AT septa and liver parenchyma. Our data confirm that bovine AT may provide an important source of SAA in healthy subjects. It remains to be determined which is the contribution of AT in the serum concentration of SAA.

1. Introduction

Serum Amyloid A (SAA) belongs to a family of structurally related proteins that are constitutively expressed (SAA4) or upregulated due to inflammatory stimuli (SAA1 to SAA3) (Uhlar and Whitehead, 1999). Serum amyloid A (SAA) is regarded as the major APP in most species including cow, pig, cat, dog, horse (Eckersall and Bell, 2010), Sheep (Miglio et al., 2013), goat and wild ruminants (González et al., 2008; Rahman et al., 2010a,b), and poultry (O'Reilly and Eckersall, 2014). Plasma concentration of SAA may rise up to 100–1000-folds in response to inflammation or infection (Eckersall and Bell, 2010). SAA1 and SAA2 are produced by liver and represent the main circulating isoforms (Molenaar et al., 2009) whereas the inflammatory isoform, namely SAA3, is produced by extrahepatic tissues (Jacobsen et al., 2005, 2006; Kjelgaard-Hansen et al., 2007).

The main biological functions of SAA remain still elusive. Being an apolipoprotein, SAA increases the export of cholesterol of phagocytosed cell membranes from cholesterol-laden macrophages via scavenger receptor B1. SAA fulfills also important roles in inflammatory reaction, by acting on one side as an opsonin, and on the other side as chemoattractant molecule for monocytes and neutrophils (Ceciliani et al., 2012).

In cattle, SAA3 has been found to be expressed, beside liver, also in mammary gland (Weber et al., 2006), where it is upregulated after inflammatory challenge and plays a role in the mammary gland defense (Molenaar et al., 2009). It must also be said that SAA3 expression is not necessarily legated to an inflammatory status, since its presence has also been detected in tissues from heathy animals, such as forestomachs (Dilda et al., 2012) and many others (Berg et al., 2011; Lecchi et al., 2012). The serum concentration of SAA increases around peripartum, peaking in the week following parturition (Humblet et al., 2006), which is probably related to the upregulation of SAA due to delivery-related inflammatory status, caused by stress or lesions at the genital apparatus (Murata, 2007; Humblet et al., 2006). A recent investigation identified bovine SAA as an adipokine (Mukesh et al., 2010), and bovine AT was found to be also a major source of SAA, its gene expression being upregulated around peripartum (Saremi et al., 2013). The present study aims to precisely locate the SAA in bovine AT depots and liver by pairing quantitative gene expression studies and immunohistochemistry (IHC), as preliminary step to understand the precise physiological and inflammatory role of this protein in these tissues. In order to gather this information, and not being commercially available any

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antibody that can label SAA with IHC, A polyclonal antibody capable to immunolabel SAA in tissues was also raised.

2. Material and methods

2.1. Sample collection

Samples from bovine AT were collected during the routine slaughtering procedures from six multiparous non-pregnant Holstein cows in their late lactation period. The clinical status of the animals was assessed by ante mortem inspection. The animals enrolled in the study were clinically healthy and no gross lesions were recorded during common slaughterhouse inspection procedure. Samples from seven different fat depots, belonging to subcutaneous adipose tissue (from withers, tail head, and sternum) and visceral adipose tissue (omental, mesenteric, pericardial and perirenal), were collected, washed with sterile Phosphate Buffered Saline (PBS) and (a) snap frozen in liquid nitrogen and then stored at −80 °C for Western Blotting, immunohistochemistry (IHC) cryosections and gene expression analyses or (b) fixed in formalin and then embedded in paraffin for immunohistochemistry (IHC). Liver samples were collected as well, washed with sterile PBS, stored in mRNA later (Sigma-Aldrich) at +4 °C for 12 h and then frozen at −80 °C for gene expression analyses, or fixed in formalin and then embedded in paraffin for IHC.

2.2. mRNA expression studies

Total RNA was extracted using TriZol (Invitrogen, Monza, Italia), treated with DNase I (Invitrogen, Monza, Italia) and quantified using a NanoDrop ND-1000 UV–vis spectrophotometer. The purity of RNA (A_{260}/A_{280}) was ~2. Reverse transcription (RT) was carried out with 1 μg RNA using the iSCRIPT cDNA Synthesis Kit (BioRad, Segrate, Italy). The cDNA was used as the template for qualitative PCR, which was performed in 10 μL final volume containing 1 μL cDNA, 1× buffer (Vivantis, Oceanside, CA, USA.), 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), 1 μM each primer and 0.025 U Taq polymerase (Vivantis, Oceanside, CA, USA.). Qualitative PCR was performed at the following conditions: 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s (Eppendorf Mastercycler, Eppendorf, Germany). PCR products were visualized on 1.6% agarose gels stained with ethidium bromide. Primers are listed in Table 1 of Supplementary material and were used in both qualitative and quantitative PCR. Primers for SAA mRNA PCR were designed on SAA3 nucleotide sequence (NM_181016 | Bos taurus serum amyloid A 3 (SAA3)).

Quantitative reactions were performed in 12 μL of Eva Green mix (BioRad, Segrate, Italy) and 400 nM of GAPDH and LRP10, 500 nM of HPCAL1 and 350 nM of SAA primers (Table 1 Supplemental material) on Eco Real Time PCR detection System (Illumina, San Diego, Ca, USA). Each sample was tested in duplicate. To evaluate PCR efficiency, fourfold serial dilutions were prepared from reference samples (liver). The thermal profile for each target gene was 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 followed by 80 cycles starting at 55 °C and increasing 0.5 °C each 10 s. No-RT controls and no template controls were performed. The relative quantification of genes of interest was carried out after normalization of the sample using the geometric mean of the reference genes. The mRNA abundance data were analyzed by ANOVA using the General Linear Model of SAS (SAS/STAT, Version V8, 1999, SAS Inst). Significance was declared for $p \leq 0.05$.

2.3. Production of polyclonal anti-bovineSAA antibody

Aiming to localize SAA protein in bovine AT tissue by IHC and to detect the presence of specific protein species by Western Blotting, a polyclonal anti-bovine SAA antibody was raised in rabbits following standard immunization procedures, using 2 mg of immunoreactive peptide synthesized as multiple antigenic peptide (MAP; Tam, 1988). In this MAP system, multiple copies of antigenic peptides are simultaneously bound to the α- and ε-amino groups of a non-immunogenic Lys-based dendritic scaffold. The polyclonal antibodies were raised against the peptide $_{86}$TDPLFKGTTSGQGQ$_{99}$, which is not present in the SAA sequence of rabbit. A simulation of hydropathy of SAA (Kyte and Doolittle, 1982) identified the peptide within a hydrophilic domain.

2.4. Western Bloting analysis

The immunoreactivity of the antibody and the cross-reactivity with other bovine proteins was tested by Western Blotting against bovine serum: 100 ng of total protein were separated in 12% acrylamide SDS–PAGE gels and blotted onto nitrocellulose membranes (BioRad, Segrate, Italy). Nitrocellulose blots were blocked for 20 min with 1% (v/v) Roti-Block® (Carl Roth GmbH, Karlsruhe, Germany) in PBS, 0.1% Tween at room temperature. The membranes were then incubated with different concentrations of polyclonal anti-SAA primary antibody (1:1000, 1:2000, 1:5000 and 1:10,000) diluted in 1% (v/v) Roti-Block® in PBS for 1 h at room temperature. After three washings with 1% (v/v) Roti-Block® in PBS (10 min each), the nitrocellulose membranes were further incubated with an anti-rabbit IgG polyclonal antibody (dilution 1:2000), conjugated to horseradish peroxidase (Sigma, Milano, Italy). Following three washes with PBS, 0.1% Tween, the immunoreactive bands were finally visualized by using enhanced chemiluminescence assay (Millipore, Vimodrone, Italy) and were exposed to X-ray film. To rule out unspecific cross-reactivity of the primary antibody. The specificity of antibody was further tested on nitrocellulose membrane after pre-incubation of the primary antibody with the immunoreactive peptide (Rahman et al., 2010a,b).

In a second part of the experiment, the presence of SAA in adipose tissue from different fat depots was validated by means of Western blotting analysis. Aliquots of 100 mg of adipose tissue were prepared as previously described (Rahman et al., 2014) and brought to a final concentration of 1% protease inhibitor cocktail (Sigma-Aldrich, Milano, Italy). Aliquots of 50 μg (total protein) were loaded onto each lane of a 12% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel and Western Blotting was carried out as previously described onto nitrocellulose membranes and immunolabeled with a primary anti-SAA dilution of 1:4000, which was previously shown to be effective for Western blotting experiments. Positive control was the MAP peptide which was used as immunogen for the preparation of the polyclonal antibody anti-SAA. The immunoreactive bands were visualized as previously described. To confirm that an equal amount of protein was loaded in each lane, the membrane was immunolabeled with mouse anti β-actin antibody (Calbiochem, Darmstadt, Germany) at a dilution of 1:10,000 as previously described (Lecchi et al., 2008).

2.5. Immunohistochemistry

Immunohistochemistry was performed on both frozen and formalin-fixed paraffin embedded (FFPE) tissue sections to identify the presence of the SAA proteins in the different cell types present in AT and in liver. Serial sections of 5 μm were obtained from frozen samples, mounted on polylysine-coated slides and fixed in cold acetone (−20 °C) for 2 min. The slides were then immersed in 100 mM Tris buffer saline pH 7.5 (TBS) for 5 min and endogenous