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Characterisation of adiponectin and its receptors in the bovine mammary gland and in milk

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ABSTRACT

Adiponectin is an adipocyte-derived hormone, which circulates in the form of homo-multimers. The individual oligomers have a distinct profile of activity, playing crucial roles in several biological processes, including metabolism and inflammation. Adiponectin exerts many of its effects by interacting with the receptors, AdipoR1 and AdipoR2. In the present study, mRNA expression of adiponectin, AdipoR1 and AdipoR2 was evaluated by quantitative PCR in different areas of the mammary gland in healthy lactating cows. The adiponectin isoforms in milk and blood were investigated by Western blotting and 2D-electrophoresis, and the presence of adiponectin protein was determined by immunohistochemistry.

Low level expression of adiponectin mRNA was found in all areas of bovine mammary gland tissues examined. AdipoR1 and AdipoR2 mRNAs were also detected in mammary tissues and their expression was particularly prominent in the parenchyma and cistern. Western blotting revealed a heterogeneous electrophoretic pattern, indicating that different adiponectin isoforms exist in milk, compared with blood. In particular, milk shows a low molecular weight isoform of adiponectin, corresponding to the globular domain. Adiponectin in milk is characterised by a more complex 2D electrophoretic pattern, compared with blood, as illustrated by the presence of proteins of different molecular weights and isoelectric points. Adiponectin protein was detected by immunohistochemistry in epithelial cells lining the secretory alveoli, in secretum within the alveolar lumen and in small peripheral nerves. The study findings support a role for adiponectin in regulating metabolism and immunity of the bovine mammary gland and potentially the calf intestine, following ingestion of milk.

Introduction

Adiponectin (AdipoQ) is a hormone, produced almost exclusively by adipocytes, which is present in serum at concentrations that are inversely related to the degree of hypertrophy of adipose tissue. In addition, circulating AdipoQ usually shows an inverse relationship with insulin, triglycerides, very low density lipoproteins (VLDL) and pro-inflammatory cytokines (Matsubara et al., 2002). AdipoQ has anti-inflammatory effects and demonstrates other properties, including insulin-sensitisation of tissues and vascular protection (Yamauchi et al., 2002; Fantuzzi, 2013).

AdipoQ belongs to the complement 1q (C1q) family of proteins (Scherer et al., 1995). Its primary structure is highly conserved, with over 80% amino acid sequence identity comparing mammalian species (Wang et al., 2002). Bovine AdipoQ is a 240 amino acid protein of approximately 28–30 kDa and circulates as homo-multimers, which vary from low molecular weight trimers to high molecular weight (12- to 18-mer) oligomers (Wang et al., 2004; Tsao, 2014). The protein undergoes extensive post-translational modification, such as hydroxylation and hydroxyl-glycosylation (Wang et al., 2002; Richards et al., 2006, 2010).

AdipoQ exerts many of its biological effects by binding to the receptors, adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2), which have distinct distribution patterns in different tissues. AdipoR1 is abundantly expressed in skeletal muscle and is linked to activation of cAMP-mediated kinase pathways, whereas AdipoR2 is abundantly expressed in the liver and is associated with activation of PPARα pathways (Kadowaki and Yamauchi, 2005). Both AdipoR1 and AdipoR2 are involved in regulating energy metabolism in these tissues.

Expression of AdipoQ has been studied in the adipose tissue of cattle during the transition period (Lemor et al., 2009; Ohtani et al., 2012), particularly in the mammary gland (Ohtani et al., 2011) and ovaries, including follicles, oocytes (Tabandeh et al., 2012) and

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granulosa cells (Maillard et al., 2010). AdipoQ has also been quantified in bovine plasma (mean ± SD of 32 ± 1.0 μg/mL) and in milk (0.61 ± 0.03 μg/mL) (Singh et al., 2014). The aim of the present study was to investigate the distribution pattern of AdipoQ and AdipoRs in the mammary gland of lactating cows to elucidate the possible role of adiponectin on mammary gland health.

Materials and methods

Sample collection

Blood and milk samples were collected from clinically healthy multiparous Holstein-Friesian cows, aged between 3 and 8 years, obtained 4–10 weeks after calving as part of routine health monitoring. Samples were made available for research following completion of diagnostic testing. Whole blood was collected via coccygeal venepuncture using tubes coated with clot activator and silicone. Serum was obtained by centrifugation of clotted blood at 1200 *g* for 10 min at 4 °C, which was stored in aliquots at –80 °C. Milk whey was prepared from milk with a somatic cell count (SCC) < 250,000 cells/mL. Ten millilitres of milk were centrifuged at 1200 *g* for 15 min at 4 °C to remove fat and cells, then the whey was further centrifuged at 13,000 *g* at room temperature and stored in aliquots at –80 °C.

Mammary tissue samples were obtained from six cows immediately after routine slaughtering procedures. Representative tissues of each area of the mammary gland were sampled and stored in RNAlater (Sigma-Aldrich) at –80 °C before RNA extraction. For the immunohistochemical study, samples of mammary tissues of approximately 1 cm × 1 cm were collected and fixed in 10% buffered formalin. Samples of subcutaneous adipose tissue, to be used as a positive control, were similarly collected and formalin fixed.

Real-time quantitative PCR

Total RNA was extracted from tissue lysates in TRIzol reagent (Invitrogen), according to the manufacturer's recommendations. RNA concentrations were quantified by use of the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA (A_{260}/A_{280}) was ~2. Genomic DNA was eliminated using DNase I (Invitrogen) and reverse transcription was performed with 1 μg RNA as the template, using the iSCRIPT cDNA Synthesis Kit (BioRad).

Quantitative PCR was performed in 12 μL reactions, using the Eco Real Time PCR detection System (Illumina). Each reaction contained Eva Green mix (BioRad) and 400 nM of primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), AdipoR1 and AdipoR2 or 300 nM primers specific for AdipoQ (see Appendix: Supplementary material). The thermocycler conditions consisted of 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 8 s and 60 °C for 18 s. For assessment of melting curves, PCR products were incubated at 55 °C for 60 s then the temperature was increased to 95 °C at 0.5 °C increments for 10 s.

The MIQE guidelines (Bustin et al., 2009) were followed. The PCR efficiencies were determined using four-fold serial dilutions of cDNA, prepared from adipose tissue, performed in triplicate. cDNA samples from mammary tissue were assessed in duplicate. Non-reverse transcribed controls and no template controls were included in the assays. Three reference genes (GAPDH, ACTB, YWHAZ) (Lecchi et al., 2012) were selected and the geometric mean of reference gene abundance was used for normalisation purposes. Relative quantification of genes of interest was carried out with adipose tissue used as the reference sample for mammary gland tissues.

Western blotting

A rabbit anti-human AdipoQ polyclonal antibody (PAI-84881, Pierce Biotechnology), raised against a peptide located near the carboxy-terminal globular domain, was used for experiments. Cross-reactivity of this antibody with bovine AdipoQ was assessed by testing bovine serum and defatted milk in Western blotting. AdipoQ multimers were converted to dimers and monomers by reducing the samples with 200 mM DTT and denaturing for 5 min at 95 °C (Mielenz et al., 2013). Following SDS-PAGE and transfer of protein to nitrocellulose membranes, these were incubated with the anti-adiponectin primary antibody for 60 min at room temperature. The optimal antibody concentration was determined to be 0.5 μg/mL (1:2000 dilution). Immunoreactive bands were visualised by enhanced chemiluminescence (Millipore), using human serum as the positive control.

2D-electrophoresis

Defatted milk and serum were concentrated 10 and 5 times, respectively, by ultrafiltration using Amicon Ultra-15 centrifugal filter devices (Millipore), with Ultracel membranes (molecular weight cut off of 3000), spun at 3800 *g*. 2D isoelectric focusing (IEF) SDS-PAGE was performed as reported by Lecchi et al. (2013). Strips were focused in the first dimension at 8500 Vh, with a maximum of 2500 V, at 20 °C using a Multiphor II Electrophoresis unit (GE Healthcare). Strips were then incubated in equilibration buffer (375 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol) with 10 mg/mL DTT for 15 min, then with 25 mg/mL iodoacetamide in the same buffer without DTT for 10 min. Second dimension separation was performed using a Mini-Protean III cell (Bio-Rad) with a 12% polyacrylamide gel (acrylamide:bis-acrylamide 37.5:1) at 16 mA constant current for 90 min. Electrotransfer onto nitrocellulose membranes (Whatmann Protran BA85) was performed for 75 min at 50 mA with a semi-dry apparatus (GE Healthcare) using 39 mM glycine, 48 mM Tris-HCl (pH 9.0), 1.3 mM SDS and 12% methanol as transfer buffer.

Immunohistochemistry

Formalin-fixed, paraffin-embedded mammary and adipose tissues were routinely processed for histology. Five micrometre sections were obtained from paraffin blocks and mounted on polylysine-coated slides. Immunolabelling was performed by the standard ABC method (Hsu et al., 1981). Primary anti-adiponectin antibody (diluted 1: 2000 in Tris buffer) was incubated overnight at 4 °C. Immunolabelling was revealed using 3-amino, 9 ethyl-carbazole (AEC; Vector Laboratories) as the chromogen and sections were counterstained with Mayer's haematoxylin. As a negative control, primary antibody was replaced with Tris buffer. The presence of immunostaining (positive or negative) with anti-adiponectin antibody and its localisation within the mammary and adipose tissue were assessed by light microscopy.

Statistical analysis

All statistical analyses were performed using IBM SPSS 21.0 for Windows. Data were $\log_{10}$ transformed, to normalise their distribution, conformed by the Shapiro–Wilk test. Analysis of variance (ANOVA) was performed to compare the means of the values of gene expression, followed by post-hoc testing using the Bonferroni method. Statistical significance was accepted at $P < 0.05$.

Results

Evaluation of AdipoQ and AdipoR mRNA expression

Different levels of expression of AdipoQ, AdipoR1 and AdipoR2 mRNA were detected in the different areas of the mammary gland tissue evaluated (duct, parenchyma, Fürstenberg's rosette and cistern; Fig. 1). AdipoQ was expressed in mammary gland parenchyma, cistern, ducts and in Fürstenberg's rosette to a more limited degree, compared with adipose tissue (Fig. 1A). AdipoR1 mRNA was found to be expressed at a relatively high level in all the analysed areas of the mammary gland and particularly in parenchyma and cistern (Fig. 1B). Expression of AdipoR2 mRNA was similar comparing mammary gland parenchyma and adipose tissue and lower in Fürstenberg's rosette, cistern and duct (Fig. 1C).

Validation of anti-adiponectin polyclonal antibody and detection of AdipoQ in bovine milk

Western blotting was performed to determine whether the selected anti-human adiponectin antibody cross-reacted with the corresponding bovine protein. The antibody reacted to a band of approximately 28–30 kDa (Fig. 2) that corresponds to the anticipated molecular weight of the AdipoQ monomer. Larger molecular weight bands of ~52 kDa were also present, corresponding to the expected size of dimers in both bovine and human serum samples with a similar electrophoresis pattern, but different stoichiometry.

A weak band of ~28 kDa, corresponding to the AdipoQ monomer, was detected in bovine milk (Fig. 2). Although the AdipoQ dimer was not identified in milk, additional high molecular weight proteins, ranging from 60 to 100 kDa, corresponding to AdipoQ oligomers were detected. A band with a low molecular weight of 15 kDa, corresponding to the globular domain, was also detected in bovine milk.

Several AdipoQ isoforms can be detected in bovine milk by 2D electrophoresis

A number of different bovine AdipoQ isoforms were detected by 2D electrophoresis. In milk samples, the electrophoretic pattern was more complex than those obtained from serum (Fig. 3).