Changes in lipid metabolism during last month of pregnancy and first two months of lactation in primiparous cows – analysis of apolipoprotein expression pattern and changes in concentration of total cholesterol, HDL, LDL, triglycerides

A.K. Kurpińska, A. Jarosz, M. Ożgo, W.F. Skrzypczak

Department of Physiology, Cytobiology and Proteomics, Faculty of Biotechnology and Animal Husbandry, West Pomeranian University of Technology Szczecin, Doktora Judyma 6, 71-466 Szczecin, Poland

Abstract

The final weeks of pregnancy and period of increasing lactation abound with adaptive changes in the intensity of metabolic processes. Maintaining the homeostasis of an organism in prepartum and postpartum periods is the key condition in maintaining the health of the mother and the fetus/calf. The aim of the study was to analyze physiological changes in lipid metabolism in cows during the last month of first pregnancy and in the first two months of lactation, based on the expression of identified apolipoproteins and changes in selected parameters of the lipid metabolism in peripheral blood plasma. Statistically significant changes in the expression of identified apolipoproteins were observed for apolipoprotein A-1 precursor, apolipoprotein A-IV precursor, apolipoprotein E precursor and apolipoprotein J precursor. The lowest expression of the apolipoproteins was noted around parturition and higher expression was observed during the final weeks of pregnancy and during lactation. Tendencies of changes in the concentration of total cholesterol, HDL and LDL were similar in blood plasma from analyzed cows – in the last month of pregnancy a decrease was observed and subsequently an increase in the first two months of lactation was noted. In contrast to abrupt changes observed for total cholesterol, HDL and LDL, changes in concentration of triglycerides were not that extensive and during lactation this parameter was rather stable. Evaluation of changes in the analyzed parameters may contribute to a better understanding of the changes in lipid metabolism occurring in the body of pregnant and lactating young cows.

Key words: proteomics, lipid metabolism, blood plasma, cows, pregnancy, lactation

Correspondence to: W.F. Skrzypczak, e-mail: wieslaw.skrzypczak@zut.edu.pl
Introduction

The final weeks of pregnancy and increasing lactation abound with adaptive changes (Kindahl et al. 2004). Maintaining the homeostasis of an organism in prepartum and postpartum periods is the key condition in maintaining the health of the mother and the fetus/calf. It is particularly important in primiparous cows in the context of their reproductive and milk performance (Knight 2001). Thus, it is highly recommended that cow health, especially during the periparturient period, should be monitored. Blood analysis might be a valuable, easily accessible tool to control and predict health disturbances. Changes in the analyzed parameters may broaden the knowledge about physiological changes, and may also be useful in early diagnosis of health disturbances (Kupczyński and Chudoba-Drozdowska 2002, Szeląg-Gruszka and Skrzypczak 2009).

Proteomics is a dynamic branch of science. It enables in-depth analysis of an organism function. Complex analysis of the proteins in a particular biological material and analysis of changes in the protein expression, supported by biochemical analysis is a high potential diagnostic and therapeutic tool (Lippolis and Reinhardt 2008).

Hence, the aim of this study was to analyze the physiological changes occurring in the lipid metabolism in cows during the last month of first pregnancy and in the first two months of lactation, based on changes in the expression of identified apolipoproteins and changes in selected parameters of the lipid metabolism in the peripheral blood plasma of cows. Their evaluation may contribute to a better understanding of changes in lipid metabolism occurring in the body of pregnant and lactating young cows.

Materials and Methods

Animals

The study was performed on 10 primiparous cows of Polish Holstein-Friesian Black-and-White variety, aligned genetically (95-100% HF; half-sisters) in the last month of pregnancy and the first two months of lactation. The Animals were kept on a large farm, in a free-stall housing system and were fed according to INRA dietary standards (2001), in conformity with a TMR system, with free access to water. All animals were clinically healthy, without any periparturient health disturbances.

The studies were performed on blood drawn from the external jugular vein, each sample taken at the same time (at approximately 11.00), on the following days: 30, 14, 7 days before parturition, and 1, 7, 14, 30 and 60 days after calving. Preselection of 14 animals for analysis – with final selection of 10, as well as calculation of date of parturition – meant length of pregnancy at selected farm was ≈277 days (data from farm) with experimental length of pregnancy 276.7 days, and intensity of sampling enabled samples on selected days before parturition to be obtained.

Blood samples were collected using sterile needles and tubes coated with anticoagulant (EDTA K3) (proteomic analysis) and heparin, and were then centrifuged (3000 rpm; temp. 4°C). The resulting plasma samples were stored at (-80)°C until analysis. The study design was approved by the Local Ethics Committee for Experiments on Animals in Szczecin (resolution No. 22/2009, dated 10.07.2009).

Two-Dimensional electrophoresis and image analysis

Plasma samples were proceeded with ProteoMiner Protein Enrichment Kit (Bio-Rad) according to the manufacturer’s instructions. The resulting samples, depending on the purpose of further studies, were treated differentially: (a) Samples for statistical analysis (afterwards 2DE gels stained with silver stains). Each sample (70 μl of the ProteoMiner kit eluate) was diluted in chilled (-20°C) acetone (1:4 ratio), precipitated (2h) and centrifuged (14 000 rpm, temp. 0°C, time: 0.5h). The resulting pellet was dissolved in lysis buffer (500 μl). (b) Samples for identification of proteins (afterwards 2DE gels stained with coomassie brilliant blue). Each sample (300 μl of the ProteoMiner kit eluate) was treated with ReadyPro-rep™ 2-D Cleanup Kit (Bio-Rad) according to the manufacturer’s instructions. Composition of lysis buffer for procedures „a” and „b”: 5M urea, 2M thiourea, 2mM TBP, 40mM Tris, ampholyte (pH 3-10) (0.2% w/v), CHAPS (4% w/v). Protein concentration was determined with the aid of a modified Bradford method (2D-QuantKit; Amersham, BioSciences), according to the manufacturer’s instructions. Samples containing, either 140 μg of total proteins (samples for statistical analysis) or 1.5 mg of total proteins (samples for protein identification) were mixed with rehydration buffer containing 9M urea, CHAPS – 4% (w/v),100 mM DTT, ampholyte (pH 3-10) – 0.2% (w/v), bromophenol blue traces. The samples were loaded on IPG strips (24 cm, pH 4-7, Bio-Rad). The strips were covered with mineral oil and rehydrated actively (17h, 20°C, 50 V). Rehydration and then isoelectric focusing were performed with the aid of Protean IEF (Bio-Rad). Depending on protein load, samples were processed differentially: samples containing 140 μg of
total proteins – with total voltage of 92 550 Vh and 1.5 mg – 93 650 Vh. In order to reduce proteins each IPG strip was incubated for 15 minutes in DTT – 1% (w/v) in 10 ml of equilibration buffer: 0.5 M Tris/HCl pH 6.8; 6 M urea, SDS – 2% (w/v), 30% glycerol (w/v). Alkylation of IPG strips was done for 20 minutes in 2.5% (w/v) iodoacetamide in 10 ml of equilibration buffer with bromophenol blue traces. A second dimension of electrophoresis was performed in 12% polyacrylamide gels (1.5 M Tris-HCl pH 8.8, 30% acrylamid/bis 37, 5:1 (2.6% C), 10% (w/v) SDS, 10% (w/v) ammonium persulfate, 1% TEMED, deionised water) using PROTEAN® plus Dodeca™ Cell (Bio-Rad). IPG strips were embeded with 0.5% molten agarose. Gels were run in migration buffer (25 mM Trizma Base; 192 mM glycine; SDS – 0.1%, w/v) at 10°C, at stable parameter 15 mA/gel, at 40V for 1h and at 120V for 19 hours. Molecular mass marker was used (10 μl/gel – Precision Plus Protein™ Kaleidoscope™ Standard; Bio-Rad). If the gels were prepared for statistical analysis they were stained with silver stains in Dodeca™ Stainer large (Bio-Rad). If the gels were prepared for identification they were stained with Coomassie Brilliant Blue G-250.

For image analysis, stained gels were immediately scanned using a Bio-Rad GS 800 calibrated densitometer. PDQuest™ 8.0 Advanced (Bio-Rad) was used for image analysis; however, statistical analysis was performed on gels stained with silver stains. Defined landmarks were used to match all gels. The smallest spot, the most faint spot and the size of the largest spot were also defined. Normalization was done using a local regression model. Analysis was performed on gels from cows at selected experimental stages (30 and 14 days before calving, on first day after calving, 7, 30 and 60 days after parturition). Statistical analysis was carried out with the use of t-test integrated with PDQuest software.

Mass spectrometry analysis

All protein spots present in the gel were manually excised from the gel (each spot was identified twice) by using pipette tips. Spots were destained with 40 mM NH₄HCO₃ (95%) and acetonitrile – ACN (5% v/v) (20min.) and washed using a mixture of 40 mM NH₄HCO₃ (50%) and ACN (50% v/v) (2x20 min). Dehydration was performed in 100% ACN (10 min.). The spots were dried in a concentrator (Concentrator 5301; Eppendorf). The protein spots were digested with trypsin (12.5 μg/ml in 40 mM NH₄HCO₃; Sigma-Aldrich) and incubated for 16h at 37°C. The resulting peptides were extracted in 100% ACN (15 min.). The dried droplet technique was used for sample/target preparation. Peptide solution (1 μl) was applied to MALDI-MSP AnchorChip™ 600/96 (Bruker Daltonics) target and mixed with 1 μl of matrix solution (5 mg/ml CHCA, trifluoroacetic acid – 0.1% (v/v), ACN- 50% (v/v), deionised water). For calibration Peptide Mass standard II, 700-4000 Da (Bruker Daltonics) was used. Mass spectra were obtained in a Microflex™ MALDI TOF (Bruker Daltonics) mass spectrometer. The following search parameters were used: trypsin as cleavage enzyme, one missed cleavage, fixed modification – carbamidomethylation of cysteine residues, variable modification – methionine oxidation, mass tolerance <150 ppm.

Biochemical analysis

Selected lipid metabolism parameters were determined in blood plasma using a Marcel Media Plus spectrophotometer. Using commercially available reagents, concentrations of triglycerides (TG), total cholesterol and HDL were determined, according to the manufacturer’s instructions. The concentration of TG was determined by the colorimetric-enzymatic method with glycrophosphate oxidase (BioMaxima). The colorimetric-enzymatic method with esterase and cholesterol oxidase was used in order to determine the concentration of total cholesterol (BioMaxima). The concentration of HDL was determined with the use of an indirect enzymatic method with precipitaton (BioMaxima). Additionally, the concentration of LDL was calculated using the Friedwald (1972) equation. Mean values and standard deviations were calculated. Statistical analysis was performed using Statistica 10.0 software with the aid of analysis of variance with repeated measurements with posthoc Tukey’s test.

Results

The excision and process of identification of all protein spots resolved in the gel enabled identification of 8 protein spots representing apolipoproteins: apolipoprotein A-1 precursor (spots 3001, 2002, 5005, 5009), apolipoprotein A-IV precursor (spot 4206), apolipoprotein E precursor (spot 4203), apolipoprotein J precursor (spot 0205, 0206). The identified apolipoproteins showed statistically significant changes in expression (Fig. 1). The expression of apolipoprotein A-1 precursor decreased until the 1st day after calving, and subsequently increased until the end of the experimental period. Statistically significant differences were observed between the 30th day before...
calving and the 1st day after calving (p<0.01), and between day 1 after calving and lactation days – 30 (p<0.05), 60 (p<0.01) (Fig. 1). The lowest expression of apolipoprotein A-IV was noted on the 1st day after calving. A statistically significantly higher expression of this protein was observed 30 days before calving (p<0.05). Its high expression was also noted on day 60 after calving, however these changes were not confirmed statistically (Fig. 1). The expression of apolipoprotein E precursor was lower during prepartum and increased during the postpartum period. Statistically significant differences were noted between the 14th day before calving and the 60th day of lactation (p<0.05) (Fig. 1). The expression of apolipoprotein J precursor was low before parturition (the lowest expression was noted on day 1 after calving) and increased after calving. Statistically significant changes were observed between day 30 before calving and lactation days – 30 (p<0.05), 60 (p<0.05), as well as between day 1 after calving and lactation days – 30 (p<0.01) and 60 (p<0.01) (Fig. 1).

The concentration of total cholesterol in cow blood plasma ranged from 2.73 to 6.03 mmol/l (Fig. 2, Table 1). During the last month of pregnancy this par-
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Fig. 2. Changes in total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides concentration in blood plasma from cows (mean values presented in the figure are in mmol/l); abbreviations used in figure: D – Day, * – before parturition.

Table1. Statistically significant changes in analyzed biochemical parameters (total cholesterol, LDL, HDL, triglycerides); x – mean values (mmol/l) and standard deviations (SD) are presented in the table; values between which a statistically significant difference was observed are marked with the same latter in line: capital letter denotes $p<0.01$, small letter – $p<0.05$; D – Day, * – before parturition; abbreviations used in figures, are in brackets.

<table>
<thead>
<tr>
<th></th>
<th>30 days prepartum (14D*)</th>
<th>14 days prepartum (14D*)</th>
<th>7 days prepartum (7D*)</th>
<th>1st day postpartum (P)</th>
<th>7 days postpartum (7D)</th>
<th>14 days postpartum (14D)</th>
<th>30 days postpartum (30D)</th>
<th>60 days postpartum (60D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>3.98 ABCDE</td>
<td>3.35 FG</td>
<td>3.05 HI</td>
<td>2.73 BK</td>
<td>2.96 CDMN</td>
<td>3.68 BOP</td>
<td>4.80 FHKMOR</td>
<td>6.03 FGHIKMN</td>
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<tr>
<td>SD</td>
<td>0.54</td>
<td>0.57</td>
<td>0.58</td>
<td>0.43</td>
<td>0.81</td>
<td>0.86</td>
<td>0.85</td>
<td>0.98</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.73 ABCDE</td>
<td>0.52 EF</td>
<td>0.44 FG</td>
<td>0.31 HI</td>
<td>0.34 CI</td>
<td>0.48 LMN</td>
<td>0.77 FHLN</td>
<td>1.27 DEGIKMN</td>
</tr>
<tr>
<td>SD</td>
<td>0.18</td>
<td>0.17</td>
<td>0.18</td>
<td>0.07</td>
<td>0.18</td>
<td>0.20</td>
<td>0.22</td>
<td>0.35</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>3.10 ABCDE</td>
<td>2.65 FG</td>
<td>2.47 HI</td>
<td>2.32 BL</td>
<td>2.49 CNOP</td>
<td>3.08 HKR</td>
<td>3.91 DFLORT</td>
<td>4.64 GIMPST</td>
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<tr>
<td>SD</td>
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<td>0.44</td>
<td>0.44</td>
<td>0.37</td>
<td>0.64</td>
<td>0.69</td>
<td>0.66</td>
<td>0.70</td>
</tr>
<tr>
<td>TG</td>
<td>0.32 ab</td>
<td>0.34 ABCD</td>
<td>0.32 cd</td>
<td>0.27 a</td>
<td>0.29</td>
<td>0.28 b</td>
<td>0.26 c</td>
<td>0.26 De</td>
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<tr>
<td>SD</td>
<td>0.07</td>
<td>0.06</td>
<td>0.05</td>
<td>0.05</td>
<td>0.04</td>
<td>0.05</td>
<td>0.06</td>
<td>0.05</td>
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</table>

Parameters decreased from 3.98 mmol/l (4 weeks before calving) to 2.73 mmol/l (the first day after calving) ($p<0.01$). During the first two months of lactation its concentration statistically significantly increased to 6.03 mmol/l on the 60th day after calving. Concentration of HDL cholesterol in cow blood plasma ranged from 2.32 to 4.64 mmol/l (Fig. 2, Table 1). The pattern of changes was similar to total cholesterol. HDL concentration was 3.10 mmol/l a month before calving and decreased statistically significantly to 2.32 mmol/l on the first day after calving, and subsequently increased until the end of the experimental period to 4.64 mmol/l (on the 60th day after calving). LDL cholesterol concentration ranged from 0.31 to 1.27 mmol/l (Fig. 2, Table 1). A decrease was observed from the 30th day before calving (0.73 mmol/l) to the first day after calving (0.31 mmol/l) ($p<0.01$). Statistically significant increase ($p<0.01$) was noted (1.27 mmol/l on the 60th day of lactation) until the end of the experimental period. The concentration of triglycerides in blood plasma ranged from 0.26 to 0.34 mmol/l (Fig. 2, Table 1). It is worth noting that there is a statistically significant decrease ($p<0.01$) during the last two weeks of pregnancy (from 0.34 to 0.27 mmol/l). During the postpartum period the concentration of triglycerides was relatively stable, but lower compared to prepartum.

**Discussion**

A higher concentration of apo A-I (also HDL) during the last weeks of pregnancy was reported by
Silliman et al. (1993) in women. During lactation a gradual increase in apo A-1 blood serum concentration was demonstrated by Marcos et al. (1990) in cows. Changes in concentration of thyroid hormones and estrogens might influence apo A-1 expression. Strobl et al. (1990) showed that the high concentration of thyroid hormones caused an increase in apo A-1 gene expression in rat liver. It was also reported that increasing concentration of estrogens in cow blood plasma during the last weeks of pregnancy (Convey 1973), decreased the transcription of apo A-1 gene (Taylor et al. 2000). Changes in the expression of apo A-1 in blood plasma might also be affected by changes in zinc concentration. Wu et al. (1999) showed that zinc insufficiency might lead to a decrease in the concentration of apo A-1 in hamster and rat blood plasma. Our previous study revealed similar tendencies for changes in the expression of apo A-1 and changes in the zinc concentration in cow blood plasma during the periparturient period (Kurpińska et al. 2011).

The increased apo A-IV gene expression at the end of pregnancy might be influenced by the high concentration of thyroid hormones and insulin. This correlation was reported by Elshourbagy et al. (1985) in rats. The lowering expression of apo A-IV in cows until parturition noted in our study was also observed by Cairoli et al. (2006). During lactation the concentration of apo A-IV in investigated plasma increased. Takahashi et al. (2004) showed a higher concentration in lactating cows in comparison to non-lactating cows. The slight increase in the expression of apo A-IV during the first two months of lactation observed in our studies might be a result of increased uptake of fodder (fat) (Kalogeris et al. 1997, Takahashi et al. 2004). Worth noting is a correlation between increasing cholesterol concentration and an increase in mRNA expression for apo A-1 and apo A-IV in the small intestine and apo A-IV in the liver reported by Crespo et al. (1992).

The increased expression of apo E in cow blood plasma during lactation was described by Takahashi et al. (2003) and Yang et al. (2012). Kockx et al. (2008) stated that the secretion of apo E may be stimulated by apo A-1, apo A-IV and LDL, HDL. Worth noting is a reverse tendency for two analyzed parameters observed in our studies – an increase in apo E during lactation, and a decrease in triglyceride concentration. This correlation was observed in the human (Blum 1982).

Tissue regeneration and changes in hormonal balance might affect apolipoprotein J (clusterin) expression (Brown et al. 1995, 1996). Brown et al. (1995) observed an increase in apo J expression in the uterine cells from mice and humans in response to lower-concentration of progesterone. The authors observed an increase in the apo J expression during uterus involution before menstruation in women, and during and just after oestrus in mice. Burkey et al. (1992) demonstrated a positive correlation between blood plasma apo J concentration and TG concentration. Our studies did not fully confirm this dependency. It was only observed during the prepartum period.

Tendencies of changes in the concentration of total cholesterol, HDL and LDL were similar in the blood plasma of cows. Other authors have also reported a similar tendency (Turk et al. 2004, Darul and Kruczyńska 2005, Miyamoto et al. 2006). Turk et al. (2005) suggested that the concentration of total cholesterol in cow blood at the end of pregnancy is lower than earlier in pregnancy, due to increased demands of the fetus and increased maternal steroid hormone production. Pösö et al. (2000) pointed out that the lower concentration of cholesterol observed immediately after parturition might result from lower activity of LCAT. Darul and Kruczyńska (2005) showed that immediately after parturition lipoprotein synthesis might be altered, due to lower liver uptake of fatty acids. Quiroz-Rocha et al. (2009) explained that lower cholesterol concentration is connected with a significant decrease in LDL and VLDL synthesis. Mazur and Reyssiquier (1988) clarified that an increase in HDL, LDL and cholesterol concentration during lactation is a result of increased VLDL catabolism, and increased synthesis of cholesterol esters for HDL production. Herdt and Smith (1996) pointed out that diet is a crucial factor in lipoproteinemia in cows, especially fat content in fodder. It is worth noting that in humans, changes in the concentration of lipids and lipoproteins are positively correlated with changes in the concentration of estradiol, progesterone and placental lactogen (Mazurkiewicz et al. 1994).

In contrast to abrupt changes observed for total cholesterol, HDL and LDL, changes in that concentration of triglycerides were not that extensive and, especially during lactation, this parameter was rather stable. According to Turk et al. (2004, 2005) and Uchida et al. (1993) the higher TG concentration in blood before parturition might be connected with a decreased TG catabolism and increased TG synthesis. The authors reported that, at the end of pregnancy, acetate produced in the rumen was not fully utilized by the mammary gland and thus its uptake by the liver for TG synthesis was increased. After parturition a decrease in TG concentration was observed due to TG uptake by the mammary gland for milk fat synthesis. Piccione et al. (2009) explained that decreased TG concentration immediately after parturition might result from increased lipolysis and de-
creased insulin-stimulated lipogenesis. Mazurkiewicz et al. (1994) demonstrated that increasing prolactin concentration might lead to the inhibition of lipoprotein ligase activity in adipose tissue, which causes an increase in TG concentration in late pregnant women. The relative stability of this parameter around parturition was reported by Mohebbi-Fani et al. (2006).

**Conclusion**

During the last month of pregnancy and first two months of lactation abrupt changes in lipid metabolism are observed, as is indicated by changes in expression observed for apolipoproteins (A-I, E, A-IV, J) and associated changes in the concentration of biochemical parameters of lipid metabolism (total cholesterol, HDL, LDL, triglycerides). Worth mentioning is a dependence between the changes in expression of apo A-I and changes in zinc concentration in blood plasma. Despite intense changes in the cow organism at the end of pregnancy and during the first months of lactation, homeostatic mechanisms are effective and the cows ability to adapt is efficient. This study enabled the multidimensional description of physiological changes in lipid metabolism. Our results may be useful for comparative studies both in physiology and health disturbances research in cows during the pre and post-partum period.

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**References**


