Journal of Animal Physiology and Animal Nutrition

DOI: 10.1111/j.1439-0396.2010.01005.x

ORIGINAL ARTICLE

Gene expression of tumour necrosis factor and insulin signalling-related factors in subcutaneous adipose tissue during the dry period and in early lactation in dairy cows

H. Sadri[1,2], R. M. Bruckmaier[1], H. R. Rahmani[3], G. R. Ghorbani[3], I. Morel[4] and H. A. van Dorland[1]

1 Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bern, Switzerland,
2 Department of Clinical Science, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran,
3 Department of Animal Science, Isfahan University of Technology, Isfahan, Iran, and
4 Agroscope Liebefeld-Posieux, Posieux, Switzerland

Keywords
adipose tissue, insulin sensitivity, gene expression, cow

Correspondence
H. A. van Dorland, Veterinary Physiology, Vetsuisse Faculty, University of Bern, Bremgartenstr. 109a, 3001 Bern, Switzerland.
Tel: +41 26 40 77 294; Fax: +41 26 40 77 297;
E-mail: anette.vandorland@physio.unibe.ch

Received: 21 August 2009;
accepted: 26 January 2010

Summary

Gene expression of adipose factors, which may be part of the mechanisms that underlie insulin sensitivity, were studied in dairy cows around parturition. Subcutaneous fat biopsies and blood samples were taken from 27 dairy cows in week 8 antepartum (a.p.), on day 1 postpartum (p.p.) and in week 5 p.p. In the adipose tissue samples, mRNA was quantified by real-time reverse transcription polymerase chain reaction for tumour necrosis factor alpha (TNFα), insulin-independent glucose transporter (GLUT1), insulin-responsive glucose transporter (GLUT4), insulin receptor, insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), regulatory subunit of phosphatidylinositol-3 kinase (p85) and catalytic subunit of phosphatidylinositol-3 kinase. Blood plasma was assayed for concentrations of glucose, β-hydroxybutyric acid, non-esterified fatty acids (NEFA) and insulin. Plasma parameters followed a pattern typically observed in dairy cows. Gene expression changes were observed, but there were no changes in TNFα concentrations, which may indicate its local involvement in catabolic adaptation of adipose tissue. Changes in GLUT4 and GLUT1 mRNA abundance may reflect their involvement in reduced insulin sensitivity and in sparing glucose for milk synthesis in early lactation. Unchanged gene expression of IRS1, IRS2 and p85 over time may imply a lack of their involvement in terms of insulin sensitivity dynamics. Alternatively, it may indicate that post-transcriptional modifications of these factors came into play and may have concealed an involvement.

Introduction

In the dairy cow, late gestation and early lactation are periods marked by profound changes in the plasma concentrations of homeorhetic hormones such as placental lactogen, prolactin and growth hormone. In addition, changes also occur in sensitivity and responses of tissue to homeostatic hormones including insulin and catecholamines (Bauman and Currie, 1980; Ingvartsen and Andersen, 2000). Insulin is one of the key factors regulating systemic energy homeostasis through the storage, mobilization and utilization of free fatty acid and glucose in adipose tissue, liver and skeletal muscle (Ruan and Lodish, 2003). However, during late gestation and in early lactation, maternal insulin sensitivity is reduced to ensure that an adequate supply of nutrients is provided to the foetus and to mammary tissues (McDowell et al., 1987; Sano et al., 1993). Reduced insulin sensitivity, as part of

the homeorhetic adaptations in the periparturient cow, has been ascribed to multiple factors, including an important role of growth hormone (Smith et al., 1997). However, several new potential mediators have recently been reported, including tumour necrosis factor (TNFα), adiponectin, resistin and non-esterified fatty acids (NEFA; Havel, 2002; Kirwan et al., 2002; Pires et al., 2007). Notably, many of these factors are secreted by adipose tissue.

Adipose tissue provides the energy that allows most tissues to overcome the negative energy balance occurring during early lactation. Free fatty acids are released into the circulation, in response to this negative energy balance, may reduce peripheral tissue insulin sensitivity (Van Epps-Fung et al., 1997; Pires et al., 2007). The adverse effect of elevated NEFA concentration on peripheral tissue insulin sensitivity has been observed to be more pronounced in obese rats than in normal rats (Noshiro et al., 1997), as well as in p.p. cows that were overfed prepartum and calved overconditioned [body condition score (BCS)>4] compared with thinner cows (Holtenius et al., 2003). These studies additionally indicate that overweight is related to tissue insulin sensitivity, not only because it may determine the rate of lipolysis in cows at calving (Busato et al., 2002), but also because the mass of adipose tissue is acknowledged to be a metabolically active tissue, with endocrine, paracrine and autocrine functions. Hence, adipose tissue itself is an important determinant of whole body insulin sensitivity.

In this regard, alterations in concentrations of a number of adipokines, such as TNFα, resistin and adiponectin, have been implicated as contributing causes of decreased insulin sensitivity, both locally and systematically (Ruan and Lodish, 2003). Although TNFα is synthesized and secreted by a number of tissues, including the placenta (Chen et al., 1991), adipose tissue, and cells of the immune system, the main source of circulating TNFα during pregnancy remains incompletely understood. However, adipocyte-derived TNFα is thought to act mainly in an autocrine or paracrine manner (Ofei et al., 1996; Ronti et al., 2006). In this respect, adipose tissue mass seems, again, to be a relevant factor, as observed by Daniel et al. (2003), who found higher circulating concentrations of TNFα in obese in relation to normal sheep.

To our knowledge, changes in gene expression of TNFα, insulin receptor (INSR) and INSR signalling proteins in adipose tissue of late gestation and early lactation dairy cows are largely unknown. Therefore, in this explorative study, we investigated the changes in expression of TNFα, INSR, INSR signalling proteins and glucose transporters in subcutaneous adipose tissue, and their relationship with blood parameters in late gestation, around parturition and in early lactation in dairy cows. Our objective was to study changes in gene expression of dairy cow adipose factors that have recently emerged as being part of the potential explanation for reducing peripheral tissue insulin sensitivity in human and laboratory animals.

Materials and methods

Animals

Twenty-seven multiparous dairy cows entering their third or greater lactation were used in the present study. Breed types were Holstein ($n = 9$), Red Holstein ($n = 9$) and Swiss Fleckvieh (Simmental×Red Holstein, $n = 9$). The study covered the period from week 8 (drying off) before the anticipated calving through week 7 after calving. All experimental procedures followed the Swiss Law on Animal Protection and were approved by the Committee of Animal Experiments of the Canton Fribourg, Switzerland. All cows were kept in a free stall barn at the Swiss Federal Research Station, Agroscope Liebefeld-Posieux (ALP) (Posieux, Switzerland) during the entire study period. The mean body weight (BW) of the experimental cows was 694 ± 15.2 kg at the start of the study. After that, BW was measured on day 1 p.p. and in week 5 p.p. Body condition score was evaluated in week 8 a.p., on day 1 p.p. and in week 3 p.p., and daily milk yield of each cow was recorded up to week 7 p.p.

The animals in the present study were used simultaneously in a feeding trial to assess the fatty acid profile of milk fat. This feeding trial involved treatments in a 2×2 factorial arrangement, in which two sources of fat in the concentrate mix of the a.p. diet (animal fat vs. sunflower seed) were fed, and a p.p. feeding treatment (control vs. feed restriction of 10 MJ/day/cow less than predicted energy intake up to week 5 after parturition) was applied. Ingredients and nutrient composition of the diets fed during the experimental period were similar, as described in a previous study (Van Dorland et al., 2009).

Adipose tissue collection, RNA extraction and quantitative real-time reverse transcription polymerase chain reaction *(RT-PCR)*

Subcutaneous adipose tissue was collected in week 8 a.p., on day 1 p.p. and in week 5 p.p. from alternate

sides of the tail head from each cow, under local anaesthesia with lidocaine hydrochloride (2%). Immediately after collection, adipose tissue samples (approximately 1–2 g) were cut into smaller pieces (<0.5 cm in any single dimension) and placed into an RNA stabilization reagent (RNAlater® from Ambion, Applied Biosystems, Austin, TX, USA), to allow effective penetration of the solution into the tissue. Within 1–1.5 h after collection in RNAlater, the samples were stored at +4 °C for one day, followed by storage at –20 °C until extraction of RNA.

Total RNA was extracted from adipose tissue samples with RNeasy Lipid Tissue Mini Kit (QIAGEN Sciences, Germantown, MD, USA) according to the manufacturer's instructions, and quantified by spectrophotometry with a BioPhotometer (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). The purity of the RNA was assessed by the ratio of the reading at 260 and 280 nm (A260/A280) in 10 mmol Tris–HCl, pH 7.5 (the value was high, with a ratio of 1.9 on average for all samples).

The reverse transcription reaction was carried out with 1 μg of extracted total RNA using 200 U Moloney Murine Leukemia Virus Reverse Transcriptase RNAase H Minus, Point Mutant (Promega Corporation, Madison, WI, USA) and 100 pmol random hexamer primers (Invitrogen, Leek, The Netherlands) according to the manufacturers' instructions.

The mRNA abundance in subcutaneous adipose tissue was quantified for TNFα, insulin independent (GLUT1) and responsive (GLUT4) glucose transporters, INSR, insulin receptor substrate 1 (IRS1), insulin receptor substrate 2 (IRS2), regulatory subunit of phosphatidylinositol-3 kinase (p85), catalytic subunit of phosphatidylinositol-3 kinase (p110). Exon spanning primers were designed wherever the limitations of the primer design allowed (i.e. TNFα, GLUT1, p85). For primer pairs that were not exon spanning (i.e. INSR, IRS1, IRS2, p110), we performed a DNA control (without a RT step). In addition, a gel was made for each parameter to test the pool, composed of all the samples, to verify that no genomic DNA was present in the samples.

The PCR quantification was performed with a Roter-Gene™ 6000 (Corbett Research, Sydney, Australia), using the software version 1.7.40. Fluorescence take off was calculated with the 'second derivative maximum' program option. A master mix of the following reaction components was prepared: 1.8 μl water, 1.0 μl forward primer (5 pmol), 1.0 μl reverse primer (5 pmol), 0.2 μl 50 × SYBR-Green (20 pmol) and 5.0 μl 2 × SensiMix (1 mM $MgCl_2$) (2 × SensiMix NoRef DNA Kit). Nine microlitres of master mix and 1 μl sample, containing 20 ng of cDNA, were used. The temperature profile of real-time PCR was as follows: denaturation step for 10 min at 95 °C, followed by 40 amplification cycles (each consisting of 15 s at 95 °C, the primer-specific annealing temperature for 30 s (see Table 1), and extension at 72 °C for 20 s with fluorescence

Table 1 PCR primer information, the annealing temperature and the PCR product length

Gene	Sequences 5′–3′	GenBank accession no.	Annealing temperature (°C)	Length
TNFα for	CCACGTTGTAGCCGACATC	AF011926	60	155
TNFα rev	CCCTGAAGAGGACCTGTGAG			
GLUT1 for	GCTTCTCCAACTGGACTTCG	NM_174602	60	225
GLUT1 rev	ACAGCTCCTCAGGTGTCTTG			
GLUT4 for	GACTGGTACCCATGTACGTG	D63150	60	242
GLUT4 rev	CCGGATGATGTAGAGGTAGC			
INSR for	TCCTCAAGGAGCTGGAGGAGT	XM_590552	60	127
INSR rev	GCTGCTGTCACATTCCCCA			
IRS1 for	GCAAGACCATCAGCTTCGTG	XM_581382	60	229
IRS1 rev	CGGAACTCATCGCTCATGGC			
IRS2 for	GTTCCAAGCTGTCCATGGAG	NM_003749	60	238
IRS2 rev	CTCATGAGCACGTACTGGTC			
p85 for	GCAACAAGCTTCCACTCTCC	M61745	63	190
p85 rev	CAAGGAGGCGGTATCACAAT			
p110 for	CAGGAGATGTGTTACAAGGC	NM_006218	60	173
p110 rev	TACTCCAAAGCCTCTTGCTC			

TNFα, tumour necrosis factor α; GLUT1, insulin-independent glucose transporter; GLUT4, insulin-responsive glucose transporter; INSR, insulin receptor; IRS1, insulin receptor substrate 1; INSR2, insulin receptor substrate 2; p85, regulatory subunit of phosphatidylinositol-3 kinase; p110, catalytic subunit of phosphatidylinositol-3 kinase.

measurement), and finally a melting curve program from 60 to 95 °C. The mRNA abundance was presented as delta cycle threshold (CT) values ($\sim\log_2$). The mRNA abundance was calculated relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene; i.e. the calculation of the ratio of the linear expression levels was based on the difference between the CT values of the target gene and the housekeeping gene, which was observed to be stable across time-points (16.0 ± 0.2, 16.4 ± 0.2 and 16.5 ± 0.1 delta CT $\log_2$ in week 8 a.p., on day 1 p.p. and in week 5 p.p. ($p < 0.05$) respectively). In addition, for the calculation of the real-time PCR efficiency (E) of one cycle, dilutions with PCR water were carried out from 100%, to 50%, to 25% and to 12.5%. The real-time PCR efficiency (E) rates of the investigated transcripts ranged from 1.87 to 1.97.

Blood sampling and analysis

Blood samples were collected between 8:00 and 9:00 hours via the jugular vein, using evacuated tubes containing tripotassium ethylenediaminetetraacetic acid. Sampling was carried out in weeks 8 and 3 before the expected calving date, on day 1 after calving, and in week 3, 5 and 7 after calving. All blood tubes were immediately placed in wet ice, and were centrifuged within 1 h at 1500 *g* for 15 min. The plasma was recovered and frozen (–20 °C) until analysis.

Plasma concentrations of metabolites were measured enzymatically using kits, as described by Vicari et al. (2008). Plasma concentrations of glucose were determined by kit no. 61270 (BioMérieux, Marcy l'Etoile, France). Concentrations of NEFA were measured with kit no. 994-75409 (Wako Chemicals, Neuss, Germany), and plasma concentrations of β-hydroxybutyric acid (BHBA) were measured using a commercial kit no. RB1007 (Randox Laboratories, Ibach, Switzerland). The plasma insulin and TNFα concentrations were determined by radioimmunoassay, according to the method described by Vicari et al. (2008) and Blum et al. (2000) respectively. Concentrations of TNFα were only measured on day 1 p.p. and in week 7 p.p. The limit of detection for the TNFα assay was set at 50 pg/ml.

Statistical analysis

Preliminary data analysis involved the evaluation of the effects from the original feeding trial and breed type on the variables measured in this study. This evaluation was performed with the PROC MIXED procedure of SAS (2001), using repeated measures with first-order autoregressive covariance structure in time. The model included biopsy time-point, concentrate type (animal fat or vegetable oil), feeding management (feed restriction or no feed restriction) and breed type as fixed effects. 'Cow' was used as the repeated subject. The outcome of this preliminary statistical evaluation did not show any significant effect of concentrate type, feeding management and breed type on the tested variables in this study. Therefore, concentrate type, feeding management and breed type were disregarded as effects in the model for the final statistical analysis of the collected data on blood parameters, and on mRNA abundance (delta CT $\log_2$) of adipose genes in this study.

Spearman rank correlation coefficients were derived in order to identify possible (numerical) correlations between the measured variables. A threshold of significance was set at $p < 0.05$; trends were declared at $0.05 < p < 0.15$.

Results

Bodyweight, BCS and milk yield of the cows

Cows had a BW of 694 ± 15, 648 ± 15 and 644 ± 13 kg in week 8 a.p., on day 1 p.p. and in week 5 p.p. respectively. The BW of the cows was significantly lower after parturition than before parturition ($p < 0.05$). The BCS was also higher ($p < 0.001$) before parturition (3.16 ± 0.08 in week 8 a.p.) compared with after parturition (2.84 ± 0.06 on day 1 p.p. and 2.83 ± 0.06 in week 3 p.p.).

Mean milk production per cow was 41.5 ± 1.4, 43.0 ± 1.5 and 42.2 ± 1.6 kg/day in week 3, 5 and 7 p.p. respectively. A tendency for a higher milk yield was observed in week 5 p.p., followed by the milk yield in week 7 p.p., compared with week 3 p.p. ($p = 0.08$).

mRNA abundance of genes in the adipose tissue

The mRNA abundance of TNFα changed during the course of the study, being higher on day 1 p.p. and in week 5 p.p., compared with week 8 a.p. ($p < 0.05$; Table 2). The mRNA encoding GLUT1 was highest in abundance in week 8 a.p., followed by week 5 p.p. and lowest on day 1 p.p. ($p < 0.05$). The mRNA abundance of GLUT4 was lower on day 1 p.p. than in week 8 a.p. and week 5 p.p. ($p < 0.05$). There was a trend ($p = 0.13$) for higher mRNA abundance of INSR on day 1 p.p. and in week 5 p.p., compared with week 8 a.p. There were no significant differ-

Table 2 Mean ± SEM of mRNA abundance (log_2) of TNFα, glucose transporters and insulin signalling-related genes in the adipose tissue before and after parturition in dairy cows

Adipose gene	Time relative to parturition*			p value
	−8 week	+1 day	+5 week	
TNFα	10.5 ± 0.27^b	11.2 ± 0.20^a	11.4 ± 0.22^a	0.006
GLUT1	15.9 ± 0.35^a	13.9 ± 0.15^c	15.0 ± 0.29^b	<0.001
GLUT4	15.8 ± 0.20^a	15.3 ± 0.15^b	16.1 ± 0.14^a	0.006
INSR	13.6 ± 0.27	14.0 ± 0.15	14.1 ± 0.14	0.13
IRS1	16.0 ± 0.19	16.0 ± 0.09	16.3 ± 0.12	0.28
IRS2	13.3 ± 0.28	13.5 ± 0.21	13.6 ± 0.17	0.60
p85	14.8 ± 0.33	15.3 ± 0.15	15.2 ± 0.17	0.28
p110	15.6 ± 0.23	15.9 ± 0.12	16.1 ± 0.12	0.06
p85/p110	0.95 ± 0.01	0.96 ± 0.01	0.94 ± 0.008	0.41

TNFα, tumour necrosis factor α; GLUT1, insulin-independent glucose transporter; GLUT4, insulin-responsive glucose transporter; INSR, insulin receptor; IRS1, insulin receptor substrate 1; INSR2, insulin receptor substrate 2; p85, regulatory subunit of phosphatidylinositol-3 kinase; p110, catalytic subunit of phosphatidylinositol-3 kinase.
*mRNA abundance was calculated relative to the expression of the GAPDH gene as reference gene, means within a row with different superscripts (a, b) differ ($p < 0.05$).

ences observed for mRNA abundance of IRS1, IRS2 and p85 during the course of the study. A tendency ($p = 0.06$) was observed for higher p110 mRNA abundance in week 5 p.p. compared with 8 week a.p. No significant effect of time was observed for the ratio of mRNA abundance of p85 to p110.

Plasma metabolites, hormones and TNFα

In week 8 a.p., glucose concentrations were higher than at other biopsy time-points ($p < 0.05$; Fig. 1).

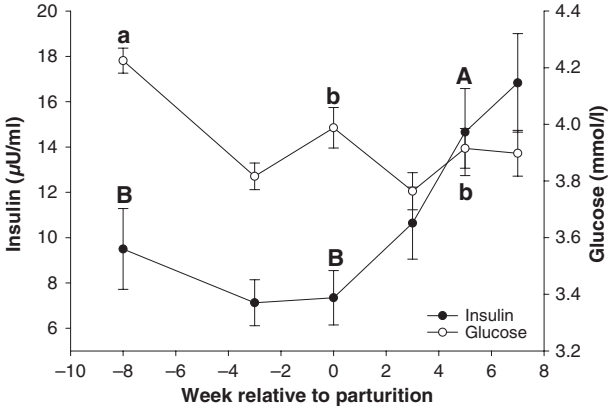

Fig. 1 Patterns of plasma insulin (means ± SEM, μU/ml) and glucose (means ± SEM, mmol/l) concentrations. Different letters indicate differences ($p < 0.05$) between measured biopsy time-points.

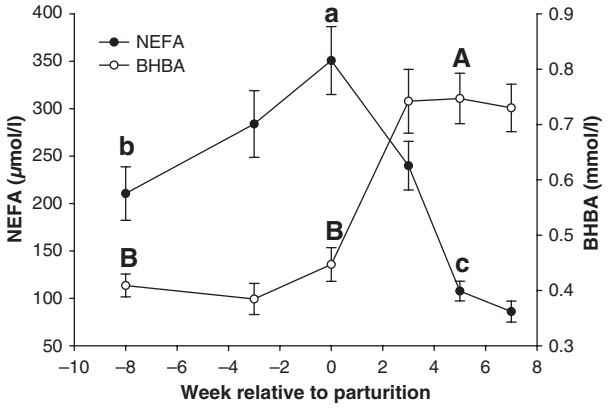

Fig. 2 Patterns of plasma NEFA (means ± SEM, μmol/l) and BHBA (means ± SEM, mmol/l) concentrations. Different letters indicate differences ($p < 0.05$) between measured biopsy time-points.

Mean plasma concentrations of insulin were highest in week 5 p.p. compared with the other biopsy time-points ($p < 0.05$; Fig. 1). On day 1 p.p., highest concentrations of NEFA were measured compared with the other biopsy time-points ($p < 0.05$; Fig. 2). Concentrations of BHBA in week 5 p.p. were significantly higher compared with the other biopsy time-points. Plasma TNFα concentrations were 92 ± 13 pg/ml on day 1 p.p. and 85 ± 12 pg/ml in week 7 p.p., but these were not significantly different.

Correlations between concentrations of plasma variables and mRNA abundance of genes in the adipose tissue

Correlation analysis revealed a tendency for a negative correlation between GLUT4 mRNA abundance and plasma NEFA concentration (r=–0.32, $p = 0.10$) in week 8 a.p. A negative correlation between plasma BHBA concentration and mRNA abundance of GLUT1 (r=–0.42, $p = 0.03$) was observed on day 1 p.p.

Discussion

In dairy cows, late gestation and early lactation periods are characterized by a moderate degree of reduced peripheral tissue insulin sensitivity. This promotes the mobilization of NEFA and amino acids and facilitates the preferential utilization of nutrients by the foetus and mammary tissue (Bell, 1995; Bell and Bauman, 1997). Decreased insulin sensitivity may be a consequence of adjustments at the pre-receptor, receptor or post-receptor level; however, the underlying molecular mechanisms responsible

for reducing insulin sensitivity in adipocytes and skeletal muscles during late gestation and early lactation in dairy cows are not yet well understood.

Recent studies in human and laboratory animals have been focused on unravelling the molecular mechanisms of reduced insulin sensitivity observed during pregnancy and type II diabetes. These studies have now identified a variety of factors that may either be independent of, or co-exist with, defects in insulin signal transduction. We based our choices of adipose factors for evaluation on these findings; thus, the present study focused on TNFα, INSR, glucose transporters (GLUT1, GLUT4), proteins of the insulin signalling pathway (IRS1, IRS2, p85 and p110).

Studying these factors by gene expression measurements allowed a large number of genes to be studied from only a small sample of tissue. However, even though gene expression data can provide reliable information about up- and downregulation of genes under different physiological situations, it is important to note that mRNA abundance may not always correlate with protein concentrations and protein activity. Our evaluation was performed using tail head subcutaneous fat, which is very active and readily mobilized during the early lactation. Therefore, it should also be noted that findings from this study apply to subcutaneous fat, and may not be representative of other body fat deposits, such as abdominal fat.

Tumour necrosis factor is a multifunctional regulatory cytokine that is involved in inflammation, cell apoptosis and production of other cytokines, as well as reduction of insulin sensitivity in animal models of obesity and insulin resistance (Uysal et al., 1997). Kirwan et al. (2002) showed that reduced insulin sensitivity during late gestation is significantly correlated with changes in circulating TNFα, irrespective of fat mass in women with normal glucose tolerance and gestational diabetes mellitus. The main source of TNFα during pregnancy remains unclear, although it is synthesized and secreted from the placenta (Chen et al., 1991), adipose tissue and cells of the immune system. Substantially decreased plasma TNFα concentrations are observed after delivery of placenta, indicating that the placenta may be the major source of circulating TNFα during late pregnancy (Kirwan et al., 2002). However, as there are significant differences between human and ruminant placenta types (discoid vs. cotyledonary), the major source of circulating TNFα during pregnancy may differ in ruminants compared with humans.

Adipocyte-derived TNFα is thought to act primarily in an autocrine or paracrine manner in adipose tissue (Ofei et al., 1996; Ronti et al., 2006). In the present study, upregulation of TNFα mRNA abundance was observed in adipose tissue during the p.p. period. Simultaneously, very low concentrations of plasma TNFα were observed in p.p. cows, as previously reported by Winkelman et al. (2008). Therefore, we assume that adipocyte-derived TNFα may act locally in adipose tissue during early lactation, as has been reported previously (Ofei et al., 1996; Ronti et al., 2006).

A number of studies with murine 3T3-L1 preadipocytes have described direct and indirect roles for TNFα in the pathophysiology of reduced insulin sensitivity. Tumour necrosis factor is thought to act by interfering with insulin signalling via repression of IRS1 gene expression, by slightly decreasing the amount of INSRs, by repressing of GLUT4 gene transcription, and by decreasing GLUT4 mRNA stability (e.g. Stephens et al., 1997). It blocks insulin signalling by increasing serine phosphorylation of IRS1 and decreasing IRS1-associated phosphatidylinositol-3 kinase (PI3-K) activity (Hotamisligil et al., 1996). In addition, TNFα is reported to induce lipolysis in adipocytes and to increase levels of circulating NEFA (Ruan et al., 2002). Whether TNFα acts in the same manner in dairy cows is currently unknown; however, the observed upregulation of TNFα expression during the p.p. period in the present study may reflect a contribution of this cytokine in the shifting of adipose tissue metabolism towards catabolism.

Although a negligible amount of glucose is used for lipogenesis in ruminants (Ingle et al., 1972), generation of $NADPH_2$ and α-glycerophosphate from direct oxidation of glucose, via the hexose monophosphate shunt, is indispensible for lipogenesis (Smith, 1983). Therefore, reduced abundance of GLUT4 and GLUT1 mRNA on day 1 p.p. may reflect a physiological adaptation in adipose tissue towards mobilization of NEFA in place of triglyceride accumulation. Komatsu et al. (2005) reported no significant differences in the abundance of GLUT4 mRNA in the adipose tissue among the peak-, late- and non-lactating cows, and suggested that the change in insulin-dependent glucose uptake in lactation is not regulated by GLUT4 expression. Our observations in the present study are in agreement, as there were no significant changes in the expression of GLUT4 between week 8 a.p. and week 5 p.p. We did observe a downregulation of GLUT4 expression on day 1 p.p., but the previous study of Komatsu et al. (2005) did not include cows around calving.

This downregulation of GLUT4 expression in subcutaneous adipose tissue may indicate that the change in insulin-dependent glucose uptake around parturition is regulated in part by GLUT4.

Interestingly, the mRNA encoding for GLUT1, which is an insulin-independent glucose transporter, followed a similar pattern to that observed for GLUT4. A negative correlation between blood BHBA and mRNA abundance of GLUT1 was observed on day 1 p.p. Thus, the decrease in GLUT1 expression in adipose tissue around parturition may act to supply extra glucose for vital organ function and to support milk production. Whether or not similar mechanisms control the downregulation of GLUT4 and GLUT1 expression on day 1 p.p. is not known; however, suppression of GLUT4 expression by TNFα (Stephens et al., 1997) and impairment of GLUT4 transporter activity by elevated NEFA concentration (Van Epps-Fung et al., 1997) appear to be likely mechanisms for the observed downregulation of GLUT4. In the present study, the decrease in mRNA abundance of GLUT4 coincided with the increase in mRNA abundance of TNFα and the highest concentration of plasma NEFA. Therefore, on the basis of these previous studies and on our observations, TNFα and elevated NEFA quite possibly contribute to downregulation of GLUT4 expression around parturition.

In the present study, a tendency for an increased mRNA abundance of INSR was observed p.p., compared with 8 week a.p. This shows that INSR is not strongly involved in reduction of insulin sensitivity in subcutaneous adipose tissue around parturition. Upon insulin stimulation, the phosphorylated IRS proteins interact and activate PI3-K. PI3-K, in turn, initiates its dependent pathway, resulting in translocation of GLUT4 from intracellular sites to the plasma membrane (Saltiel and Kahn, 2001; Saltiel and Pessin, 2002). Mice lacking IRS1 (Tamemoto et al., 1994) and IRS2 (Withers et al., 1998) exhibit a reduction in peripheral tissue insulin sensitivity. Our study showed no changes in mRNA abundance of IRS1 over time; however, it has also been reported that serine phosphorylation of IRS1 can reduce its ability to bind to the INSR and initiate downstream signalling (Aguirre et al., 2002). Therefore, further studies that include measurement of tyrosine and serine phosphorylation of IRS1 are needed to confirm this hypothesis.

PI3-K consists of a p85 regulatory subunit and a p110 catalytic subunit, with a p85–p110 heterodimer being responsible for PI3-K activity. Results from recent human and mice studies have led to the proposal that increases in p85 can cause a competition between the p85 monomer and the p85–p110 heterodimer for the same binding sites on tyrosine phosphorylated IRS1. This would effectively prevent further insulin signalling downstream (Barbour et al., 2004, 2005). We found no changes in the expression of p85 or in the ratio of mRNA abundance of p85 to p110 over time, implying that changes in adipose tissue insulin sensitivity in late gestation and early lactation are not mediated by p85 expression. The reason for a tendency for p110 mRNA abundance to increase in week 5 p.p. compared with week 8 a.p. is not known.

Gradual increases in the plasma NEFA concentration during the last weeks of gestation were confirmed by the observed changes in BCS, with a peak concentration on day 1 p.p., in agreement with previous reports (Grummer, 1993). The increases in the plasma NEFA concentration during late gestation and early lactation are the result of the orientation of adipose tissue towards mobilization of NEFA in place of lipid deposition (McNamara, 1991). This change is mediated by alterations in the concentrations of placental derived hormones, cortisol, insulin, growth hormone, catecholamines, and by changes in tissue responsiveness to these hormones (Bell and Bauman, 1997). Alterations in concentrations of adipose tissue-derived endocrine and autocrine/paracrine factors, such as adiponectin and TNFα are also involved, but the identity of the initiating factor(s) that triggers the observed increases in NEFA concentrations is unclear. Van Epps-Fung et al. (1997) reported that elevated NEFA concentrations were associated with substantial decreases in insulin-induced glucose transport in adipose tissue cell lines. In the present study, downregulation of GLUT4 and upregulation of TNFα expression, which coincided with increased in plasma NEFA concentration, may confirm previous reports concerning the orientation of adipose tissue towards mobilization of NEFA rather than lipid deposition at the onset of lactation.

In conclusion, understanding the regulation of insulin responsiveness of adipose tissue, a major insulin-responsive tissue and of the molecular mechanisms involved in the reduction of insulin sensitivity during late gestation and early lactation, becomes increasingly important, given the therapeutic potential of improving insulin sensitivity and reducing lipid-related metabolic disorders. In dairy cows, the cause of reduced insulin sensitivity during late gestation and early lactation seems to be multifactorial. This study provides the first analysis of expression of

genes encoding INSR signalling proteins in adipose tissue of dry and early lactation dairy cows. Further studies that include protein expression and activity of selected factors may provide additional clues to the involvement of factors in, and the mechanisms underlying, the reduced insulin sensitivity during late gestation and in early lactation in dairy cows.

Acknowledgements

The excellent laboratory assistance performed by Claudine Morel and Yolande Zbinden from the Veterinary Physiology group is gratefully appreciated. Andreas Gutzwiller from the ALP is thanked for obtaining the adipose tissue samples. In addition, appreciation is extended to the Iranian Government for financial support of Hassan Sadri.

References

Aguirre, V.; Werner, E. D.; Giraud, J.; Lee, Y. H.; Shoelson, S. E.; White, M. F., 2002: Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *Journal of Biological Chemistry* **277**, 1531–1537.

Barbour, L. A.; Shao, J.; Qiao, L.; Leitner, W.; Anderson, M.; Freidman, J. E.; Drazin, B., 2004: Human placental growth hormone increases expression of p85 regulatory unit of phosphatidylinositol 3-kinase and triggers severe insulin resistance in skeletal muscle. *Endocrinology* **145**, 1144–1150.

Barbour, L.; Rahman, S. M.; Gurevich, I.; Leitner, J. W.; Fisher, S.; Roper, M.; Knotts, T.; Vo, Y.; Yakar, S.; LeRoith, D.; Kahn, C. R.; Cantley, L.; Freidman, J.; Draznin, B., 2005: Increased p85 alpha is a potent negative regulator of skeletal muscle insulin signaling and induces *in vivo* insulin resistance associated with growth hormone excess. *Journal of Biological Chemistry* **280**, 37489–37494.

Bauman, D. E.; Currie, W. B., 1980: Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. *Journal of Dairy Science* **63**, 1514–1529.

Bell, A. W., 1995: Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *Journal of Animal Science* **73**, 2804–2819.

Bell, A. W.; Bauman, D. E., 1997: Adaptations of glucose metabolism during pregnancy and lactation. *Journal Mammary Gland Biology Neoplasia* **2**, 265–278.

Blum, J. W.; Dosogne, H.; Hoeben, D.; Vangroenweghe, F.; Hammon, H. M.; Bruckmaier, R. M.; Burvenich, C., 2000: Tumor necrosis factor-α and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domestic Animal Endocrinology* **19**, 223–235.

Busato, A.; Faissler, D.; Küpfer, U.; Blum, J. W., 2002: Body condition scores in dairy cows: associations with metabolic and endocrine changes in healthy dairy cows. *Journal of Veterinary Medicine Series A* **49**, 455–460.

Chen, H.; Yang, Y.; Hu, X.; Yelavarthi, K.; Fishback, J.; Hunt, J., 1991: Tumor necrosis factor alpha mRNA and protein are present in human placental and uterine cells at early and late stages of gestation. *American Journal of Pathology* **139**, 327–335.

Daniel, J. A.; Elsasser, T. H.; Morrison, C. D.; Keisler, D. H.; Whitlock, B. K.; Steele, B.; Pugh, D.; Sartin, J. L., 2003: Leptin, tumor necrosis factor-α (TNF), and CD14 in ovine adipose tissue and changes in circulating TNF in lean and fat sheep. *Journal of Animal Science* **81**, 2590–2599.

Grummer, R. R., 1993: Etiology of lipid-related metabolic disorders in periparturient dairy cows. *Journal of Dairy Science* **76**, 3882–3896.

Havel, P. J., 2002: Control of energy homeostasis and insulin action by adipocyte hormones: leptin, acylation stimulating protein, and adiponectin. *Current Opinion Lipidology* **13**, 51–59.

Holtenius, K.; Agenas, S.; Delavaud, C.; Chilliard, Y., 2003: Effects of feeding intensity during the dry period. 2. Metabolic and hormonal responses. *Journal of Dairy Science* **86**, 883–891.

Hotamisligil, G. S.; Peraldi, P.; Budavari, A.; Ellis, R.; White, M. F.; Spiegelman, B. M., 1996: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha and obesity-induced insulin resistance. *Science* **271**, 665–668.

Ingle, D. L.; Bauman, D. E.; Garrigus, U. S., 1972: Lipogenesis in the ruminant: *in vitro* study of tissue sites, carbon source and reducing equivalent generation for fatty acid synthesis. *Journal of Nutrition* **102**, 609–616.

Ingvartsen, K. L.; Andersen, J. B., 2000: Integration of metabolism and intake regulation: a review focusing on periparturient animals. *Journal of Dairy Science* **83**, 1573–1597.

Kirwan, J. P.; Hauguel-De Mouzon, S.; Lepercq, J.; Challier, J. C.; Huston-Presley, L.; Friedman, J. E.; Kalhan, S. C.; Catalano, P. M., 2002: TNF-alpha is a predictor of insulin resistance in human pregnancy. *Diabetes* **51**, 2207–2213.

Komatsu, T.; Itoh, F.; Kushibiki, S.; Hodate, K., 2005: Changes in gene expression of glucose transporters in lactating and nonlactating cows. *Journal of Animal Science* **83**, 557–564.

McDowell, G. H.; Gooden, J. M.; Leenanuruksa, D.; Jois, M.; English, A. W., 1987: Effects of exogenous growth hormone on milk production and nutrient uptake by

muscle and mammary tissues of dairy cows in mid-lactation. *Australian Journal of Biological Sciences* **40**, 295–306.

McNamara, J. P., 1991: Regulation of adipose tissue metabolism in support of lactation. *Journal of Dairy Science* **74**, 706–719.

Noshiro, O.; Hirayama, R.; Shimaya, A.; Yoneta, T.; Niigata, K.; Shikama, H., 1997: Role of plasma insulin concentration in regulating glucose and lipid metabolism in lean and obese Zucker rats. *International Journal of Obesity* **21**, 115–121.

Ofei, F.; Hurel, S.; Newkirk, J.; Sopwith, M.; Taylor, R., 1996: Effects of an engineered human anti-TNF-α antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM. *Diabetes* **45**, 881–885.

Pires, J. A. A.; Souza, A. H.; Grummer, R. R., 2007: Induction of hyperlipidemia by intravenous infusion of tallow emulsion causes insulin resistance in Holstein cows. *Journal of Dairy Science* **90**, 2735–2744.

Ronti, T.; Lupattelli, G.; Mannarino, E., 2006: The endocrine function of adipose tissue: an update. *Clinical Endocrinology* **64**, 355–365.

Ruan, H.; Lodish, H.F., 2003: Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-α. *Cytokine and Growth Factor Reviews* **14**, 447–455.

Ruan, H.; Miles, P. D. G.; Christine, M. L.; Kenneth, R.; Golub, T. R.; Olefsky, J. M.; Lodish, H. F., 2002: Profiling gene transcription *in vivo* reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. *Diabetes* **51**, 3176–3188.

Saltiel, A. R.; Kahn, C.R., 2001: Insulin signaling and the regulation of glucose and lipid metabolism. *Nature* **414**, 799–806.

Saltiel, A. R.; Pessin, J.E., 2002: Insulin signaling pathways in time and space. *Trends in Cell Biology* **12**, 65–71.

Sano, H.; Narahara, S.; Kondo, T.; Takahashi, A.; Terashima, Y., 1993: Insulin responsiveness to glucose and tissue responsiveness to insulin during lactation in dairy cows. *Domestic Animal Endocrinology* **10**, 191–197.

SAS, 2001: *SAS User's Guide: Statistics, Version 8 Edition*. SAS Institute, Cary, NC.

Smith, S. B., 1983: Contribution of the pentose cycle to lipogenesis in bovine adipose tissue. *Archives of Biochemistry and Biophysics* **221**, 46–56.

Smith, T. R.; Elmendorf, J. S.; David, T. S.; Turinsky, J., 1997: Growth hormone-induced insulin resistance: role of the insulin receptor, IRS-1, GLUT-1, and GLUT-4. *American Journal of Physiology: Endocrinology and Metabolism* **272**, E1071–E1079.

Stephens, J.; Lee, J.; Pilch, P., 1997: Tumor necrosis factor-α induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor mediated signal transduction. *Journal of Biological Chemistry* **272**, 971–976.

Tamemoto, H.; Kadowaki, T.; Tobe, K.; Yagi, T.; Sakura, H.; Hayakawa, T.; Terauchi, Y.; Ueki, K.; Kaburagi, Y.; Satoh, S.; Sekihara, H.; Yoshioka, S; Horikoshi, H.; Furuta, Y.; Ikawa, Y.; Kasuga, M.; Yazaki, Y.; Aizawa, S., 1994: Insulin resistance and growth retardation in mice lacking insulin receptor substarte-1. *Nature* **372**, 182–186.

Uysal, K. T.; Wiesbrock, S. M.; Marino, M.; Hotamisligil, G.S., 1997: Protection from obesity-induced insulin resistance in mice lacking TNFα function. *Nature* **389**, 610–614.

Van Dorland, H. A.; Richter, S.; Morel, I.; Doherr, M. G.; Castro, N.; Bruckmaier, R. M., 2009: Variation in hepatic regulation of metabolism during the dry period and in early lacation in dairy cows. *Journal of Dairy Science* **92**, 1924–1940.

Van Epps-Fung, M.; Williford, J.; Wells, A.; Hardy, R. W., 1997: Fatty-acid induced insulin resistance adipocytes. *Endocrinology* **138**, 4338–4345.

Vicari, T.; van den Borne, J. J. G. C.; Gerrits, W. J. J.; Zbinden, Y.; Blum, J. W., 2008: Postprandial blood hormone and metabolite concentrations influenced by feeding frequency and feeding level in veal calves. *Domestic Animal Endocrinology* **34**, 74–88.

Winkelman, L. A.; Elsasser, T. H.; Reynolds, C.K., 2008: Limit-feeding a high-energy diet to meet energy requirements in the dry period alters plasma metabolite concentrations but does not affect intake or milk production in early lactation. *Journal of Dairy Science* **91**, 1067–1079.

Withers, D. J.; Gutierrez, J. S.; Towery, H.; Burks, D. J.; Ren, J.; Previs, S.; Zhang, Y.; Bernal, D.; Pons, S.; Shulman, G. I.; Bonner-Weir, S.; White, M.F., 1998: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391**, 900–904.