Seasonal effects of central leptin infusion and prolactin treatment on pituitary *SOCS-3* gene expression in ewes

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Abstract

Suppressors of cytokine signalling (SOCS) negatively regulate cytokine-induced signalling pathways and may be involved in leptin and prolactin (PRL) interactions. Herein, we examined the effect of PRL on *SOCS-3* mRNA expression in pituitary explants and investigated whether leptin could modify the expression of *SOCS-3* mRNA in pituitary explants. In the first experiment, we used pituitaries isolated from 16 ewes decapitated in March, May, July and October (four per month). Tissues were cut into 50 mg explants, which were treated with control or medium containing PRL (100 or 300 ng/ml). Incubation was maintained for different time intervals: 0, 60, 120, 180, 240 or 300 min. Real-time PCR was used to measure *SOCS-3* mRNA levels. In the second study, we used 24 ewes surgically fitted with third ventricle cannulas (12 were used during the long-day period, and 12 were used during

the short-day (SD) period). Each ewe was administered an i.c.v. injection of Ringer–Locke buffer or leptin (0.5 or 1.0 µg/kg body weight). Explants of anterior pituitaries were collected and snap frozen 1 h after injection. Semi-quantitative expression of *SOCS-3* mRNA was performed using reverse transcription-PCR. PRL stimulated *SOCS-3* expression in the pituitaries collected in March ($P < 0.05$) and May ($P < 0.01$ and $P < 0.05$ for lower and higher doses respectively), inhibited *SOCS-3* expression in pituitaries collected in July ($P < 0.01$) and had no effect in pituitaries collected in October. Treatment with leptin increased *SOCS-3* expression during the SDs in a dose-dependent manner ($P < 0.01$). The results demonstrated that photoperiod may be involved in leptin and PRL effects on *SOCS-3* expression in sheep.

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Introduction

Leptin and prolactin (PRL) have a wide spectrum of actions; both hormones are involved in the regulation of energy balance and reproduction, and their secretions change in a seasonal manner. They also induce similar intracellular pathways of signal transduction. The functional variability of leptin and PRL is enabled by different intracellular factors that suppress or enhance their activity, the existence of which serves as a counterbalance to the wide spectrum of their action. Recent experiments have proven that auto-suppression by induction of negative feedback is crucial in the regulation of the interactions of these peptide hormones.

Both the PRL receptor and the leptin receptor lack intrinsic enzymatic activity and mediate signals by activation of receptor-associated intracellular Janus kinases (JAKs), which belong to the tyrosine kinase family. The receptors homodimerise upon ligand binding and activate the JAK/signal transducer and activator of transcription (STAT) pathway (White *et al.* 1997). Phosphorylated

STATs dimerise and translocate to the nucleus, where they bind to DNA and affect target gene transcription (White *et al.* 1997, Banks *et al.* 2000). This signalling pathway can be modulated by a wide variety of cellular factors. Recently identified negative inhibitors of cytokine signalling transduction, i.e. suppressors of cytokine signalling (SOCS), can suppress the actions of both PRL (Bole-Feysot *et al.* 1998, Ling & Billig 2001) and leptin (Bjorbaek *et al.* 2000). Except in the brain, genes in the majority of tissues that encode SOCS proteins are not constitutively expressed, and they need specific factors (e.g. cytokines, growth factors and hormones) to induce their transcription. Induction of the different SOCS genes depends on the type of factor involved and the tissue being investigated (Davey *et al.* 1999).

A high leptin-induced *SOCS-3* level in periventricular areas and in the hypothalamus of *ob/ob* mice has been reported to be involved in leptin resistance (Bjorbaek *et al.* 1998, 1999). Increased hypothalamic *SOCS-3* expression was observed in the lethal yellow (Ay/a) mouse, which is subject to both

hyperleptinaemia and leptin insensitivity (Bjorbaek *et al.* 1998). Localisation of SOCS-3, along with a wide variety of factors that are able to induce its expression, in the arcuate nucleus (ARC), ventromedial nucleus, dorsomedial nucleus, paraventricular nucleus and suprachiasmatic nucleus (Tups *et al.* 2004) suggests that SOCS-3 may play a pivotal role in the modulation of neuroendocrine interactions. There is some evidence to suggest that SOCS-3 has important functions within the pituitary. In adrenocorticotrophs, SOCS-3 has been shown to be a potent feedback inhibitor of leukaemia inhibitory factor and interleukin 11 as well as a suppressor of proopiomelanocortin expression and ACTH secretion (Auernhammer & Melmed 1999).

The biological effectiveness of PRL is also regulated by SOCS. *In vivo* studies have shown that PRL stimulated SOCS-3 expression in murine liver (Pezet *et al.* 1999) and adipose tissue (Ling & Billig 2001) as well as in rat ovaries, mammary glands and adrenal glands (Tam *et al.* 2001). In addition, PRL stimulated SOCS-3 expression in murine adipose tissue *in vitro* (Ling & Billig 2001).

Recent experiments have indicated that the SOCS-3 expression level is also dependent on environmental factors, such as the photoperiodic condition and nutritional status. Studies in the Siberian hamster (*Phodopus sungorus*, Tups *et al.* 2004) have demonstrated that SOCS-3 expression is higher during long-days (LDs) and reduced during short-days (SDs). Moreover, leptin was only able to induce SOCS-3 expression on SDs, which indicated that this interaction was dependent on the season (Adam & Mercer 2004, Tups *et al.* 2004). Effects of PRL on SOCS-3 mRNA and potential involvement of SOCS-3 in interactions between leptin and PRL in seasonal mammals were reviewed in detail in a paper by Tups (2009). The influence of the length of day on SOCS-3 transcription has also been observed in bovine mammary glands (Wall *et al.* 2005). Moreover, diurnal changes in the synthesis of SOCS have been observed in the rat brain (Denis *et al.* 2004).

Some reports have indicated a relationship between SOCS-3 expression and light conditions (Tups *et al.* 2004, Wall *et al.* 2005) or nutritional status (Baskin *et al.* 2000, Tups *et al.* 2004), which suggests that SOCS may be responsible for the phenomenal plasticity of an organism to adapt to variable environmental conditions and to maintain energetic homeostasis.

The first experiment reported herein was designed to determine the effects of PRL on SOCS-3 mRNA expression in pituitary explants. The second experiment of the study was designed to examine how leptin modulated SOCS-3 expression in the ovine pituitary during SDs and LDs to better understand the seasonal variability of leptin's actions. The results of these experiments may give a new insight into the molecular interactions between these two hormones and create a successful therapeutic target for the treatment of obesity, reproductive abnormalities and/or other hormonal disturbances.

Materials and Methods

All animal-related procedures were approved by the Local Agricultural Animal Care and Use Committee of Krakow (protocol no. 25/OP/2005).

This study used Polish Longwool ewes, which have been shown to exhibit strong seasonal reproductive activity (Zieba *et al.* 2000). Animals were 2–3 years of age and had a mean body weight (BW) of 60 ± 5 kg. All animals were housed in individual pens under natural photoperiodic and thermo-periodic conditions (longitude: $19^{\circ}57'E$, latitude: $50^{\circ}04'N$) and were in a good body condition (body condition score = 3) on a 1–5 scale (0 = emaciated and 5 = obese; Russel *et al.* 1969). Ewes were fed twice daily at 0700 and 1600 h with a diet formulated to supply 100% of the nutritional requirements according to the recommendations of the National Research Institute of Animal Production for maintenance (Strzelecki 1993). Water was made available *ad libitum*.

Experiment 1: *in vitro* study

Animals and treatment In this experiment, 16 ewes were used. Before the animals were killed, the blood samples were collected from each animal to determine the PRL concentrations. Animals were killed by exsanguination following captive bolt stunning. The pituitary glands were aseptically isolated from the ewes 10–15 min post mortem. The tissues were collected in March, May, July and October (four animals per month). The pituitaries were placed on ice and transported to the laboratory where all subsequent procedures were performed under sterile conditions.

Pituitary tissue cultures Before incubation, the pituitaries were washed three times in M-199 medium (Laboratory of Vaccines, Lublin, Poland). Diencephalons were removed after disconnection of infundibula from adenohypophyses (APs). APs were removed from the sella turcica from each sheep and kept on ice until tissue processing. To unify explants, APs were dissected and sliced sagittally into ~ 2 –4 mm strips (50 mg each). We obtained a total of 54 AP strips from those four APs (each month), and then one strip per well was selected randomly, placed on a stainless steel grid covered with lens paper and incubated in a gas–liquid interface in 2.5 ml medium. Incubations were carried out in a six-well Corning tissue culture dish (Corning Glass Works, New York, NY, USA). Cultures were incubated in 95% humidified air and 5% CO₂ at 37 °C for a maximal period of 5 h. Explants were treated with control medium or medium containing ovine PRL (100 or 300 ng/ml; Sigma). Each treatment group consisted of three replicates in each timepoint. From each experimental group, 1 ml culture medium was harvested every hour and replaced with fresh medium. Explants were incubated for different time intervals: 0, 60, 120, 180, 240 and 300 min. When the incubation was complete, explants were rinsed in PBS solution (Laboratory of Vaccines), frozen in liquid nitrogen and stored at -80 °C. Samples of media were frozen at -20 °C until assay.

Molecular analysis Real-time PCR was used to measure SOCS-3 mRNA levels. Total RNA was prepared using TRIzol reagent (Invitrogen Corporation) according to the manufacturer's instructions. The RNA (0.5 µg) was reverse transcribed to cDNA using Quantiscript reverse transcriptase and reverse transcription (RT) primer mix (QuantiTect RT kit, Qiagen) by incubating the samples at 42 °C for 15 min. The reaction was terminated by heating to 94 °C for 3 min. Genomic DNA was eliminated by adding gDNA wipeout buffer (QuantiTect RT kit) and incubating the samples at 42 °C for 2 min. Amplification of cDNA was performed using TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 7300 Real-Time PCR System. Primers and probes were designed using Primer Express software v.2.0 (Applied Biosystems). Products were amplified using the following primers at 250 nM: 5'-CCTCAAGACCTT-CAGCTCCAA-3' and 5'-CTTGCGCACTGCGTTCAC-3' (corresponding to the bovine SOCS-3 gene; GenBank accession number NM_174466; Sequence Detection Primers, Applied Biosystems) or 5'-CGGCTCCCAGTTCTT-CATCA-3' and 5'-ACTACGTGCTTCCCATCCAAA-3' (corresponding to the bovine *cyclophilin* gene; GenBank accession number D14074; Sequence Detection Primers, Applied Biosystems). The probe sequences were FAM-AGCGAGTACCAGCTGG-MGB (corresponding to the SOCS-3 gene; TaqMan MGB Probes; Applied Biosystems) and FAM-CGTTCCGACTCCGC-MGB (corresponding to the *cyclophilin* gene; TaqMan MGB Probes; Applied Biosystems). Each gene assay was run in a singleplex reaction in triplicate for each cDNA sample. Amplification was carried out under the following conditions: 1) initial incubation at 50 °C for 2 min, 2) polymerase activation at 95 °C for 10 min and 3) 40 cycles with denaturation (95 °C for 15 s) and annealing/elongation (60 °C for 60 s). Data were collected and recorded by Applied Biosystems 7300 Real-Time PCR System SDS software and expressed as a function of the threshold cycle (C_t). Using diluted samples, the amplification efficacies for the gene of interest and the reference gene were found to be identical. There was no significant variation in the C_t values for cyclophilin among the treatment group.

RIA for PRL. Plasma concentrations of PRL were assayed by the double-antibody method using anti-ovine PRL and anti-rabbit-gammaglobulin antisera according to Wolinska *et al.* (1977). Intra-assay coefficient of variation (CV) was 5%. PRL concentrations in media were determined by a method described by Kokot & Stupnicki (1985). Inter-assay CV was 6.2%.

Statistical analysis Concentrations of PRL in culture media were analysed by ANOVA using the general linear models procedure (PROC GLM) of the Statistical Analysis System (SAS 9.1; SAS Institute and Academic Program, Cary NC, USA). Sources of variation for the overall model

included ewe, treatment, month, culture replicate (treatment) and the treatment×month interaction. After a treatment×month interaction was detected ($P<0.01$), treatment effects were evaluated within months. Replicates within each treatment were used as the error term to test for the effects of appropriate hormone treatment. Values were considered to be statistically significant at $P<0.05$. Values reported are least squares means ($\pm$ S.E.M.).

Expression levels were calculated by relative quantification (RQ) analysis. In brief, the amplification plot consisted of the plot of fluorescence versus PCR cycle number. The C_t value was the fractional PCR cycle number at which the fluorescent signal reached the detection threshold. Therefore, the input cDNA copy number and C_t were inversely related. Data were analysed by the $2^{-\Delta\Delta C_t}$ method, and the C_t values were converted to fold change RQ values using the equation $RQ = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t \text{ sample} - \Delta C_t \text{ calibrator} = ((C_t \text{ target gene} - C_t \text{ reference gene}) \text{ sample} - (C_t \text{ target gene} - C_t \text{ reference gene}) \text{ calibrator})$. The RQ values from each gene were then used to compare the target gene expression across all the groups.

The mean mRNA expression for SOCS-3 in each sample was normalised against the expression of a reference gene (*cyclophilin*) and expressed relative to the indicated calibrator sample. We used the mean ΔC_t value for the control group as a calibrator (at the indicated time of incubation) to compare changes in gene expression levels during incubation. Comparisons of basal expression of SOCS-3 depended on the month of the experiment, and we took into consideration ΔC_t values for samples that were not incubated and used the mean ΔC_t value for non-incubated samples isolated in March as a calibrator. Following determination of a significant F value, means were contrasted using Duncan's multiple range test. Differences with $P<0.05$ were considered statistically significant.

Experiment II: in vivo study

Animals and treatment We examined the expression of SOCS-3 mRNA after leptin infusion into the third ventricle of the brain (IIIV). According to the methodology of Traczyk & Przekop (1963), 24 ewes were surgically fitted with IIIV cannulas. The location and function of the cannulas were verified by the continuous flow of cerebrospinal fluid. A period of at least 3 weeks was allowed for the sheep to recover from the surgery. The experiment was carried out in both a LD period (May) and a SD period (November; 12 ewes per period). Recombinant ovine leptin was purchased from Ray Biotech, Inc. (Norcross, GA, USA). The ewes were divided into three groups: 1) control, which received Ringer-Locke buffer (pH 7.4), 2) leptin 1, which received a low dose of leptin (0.5 µg/kg BW) and 3) leptin 2, which received a high dose of leptin (1.0 µg/kg BW). The dose of leptin for i.c.v. administration was determined from our previous experience (Zieba *et al.* 2003a,b, 2004), theoretical calculations and a published study of i.c.v. injection in sheep (Adam *et al.* 2006).

Ewes were killed by captive bolt stunning 1 h after the end of the infusions. Pituitaries were removed and immediately snap frozen in liquid nitrogen and stored at -80°C .

Molecular analysis Semi-quantitative evaluation of SOCS-3 mRNA expression in the pituitary was performed by RT-PCR. Total RNA was prepared using TRIzol reagent (Invitrogen Corporation) according to the manufacturer's instructions. Total RNA ($1\text{ }\mu\text{g}$) was reverse transcribed to cDNA using moloney murine leukaemia virus reverse transcriptase and Oligo(dT) Primer (Advantage RT-for-PCR kit; Clontech) using an UNO II thermocycler (Biomtra, Gottingen, Germany). An initial denaturation step was performed for 5 min at 95°C before first-strand synthesis at 42°C for 60 min. The reaction was terminated by heating to 94°C for 5 min. Each PCR was performed using the Titanium Taq PCR kit (Clontech). The primers were 5'-CCAGCCTGCGCCTCAA-3' and 5'-CTTGCGCACTGCGTTCAC-3' (corresponding to the bovine SOCS-3 gene; GenBank accession number NM_174466) for amplification of a 68 bp fragment. Primers for the reference gene were 5'-CCAACGGCTCCCAGTTCTT-3' and 5'-ACTACGTGCTTCCCATCCAAA-3' (corresponding to the bovine *cyclophilin* gene; GenBank accession number D14074) for amplification of a 64 bp fragment. Amplification was performed in a T3 thermocycler (Biomtra) under the following conditions: 94°C for 5 min followed by 36 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s. Reactions were terminated at 72°C for 10 min. The PCR products were separated (time: 30 min, voltage: 5 V/cm of gel) on a 3% agarose gel, stained with ethidium bromide, in TBE ($0.5\times$), in the presence of a loading dye and a DNA marker (pUC19DNA/MspI,23; Fermentas; Vilnius, Lithuania) and were analysed using the Dscan Ex. v.3.1.0 program (Scanalytics, Inc., La Jolla, CA, USA).

Statistical analysis Tissue mRNA content was evaluated by ANOVA with treatment and season as the independent variables. Following the determination of a significant *F* value, means were contrasted using Duncan's multiple range test. Differences with $P<0.05$ were considered statistically significant.

Results

Experiment I: in vitro PRL study

During March and May, overall mean concentrations of PRL in plasma were much greater ($P<0.05$) than during October – SD (142.58 ± 12.65 vs 47.27 ± 7.16 ng/ml).

There were no statistical differences between mean baseline concentrations (ng/ml) of PRL in control wells during different months ($P>0.01$; Fig. 1A and B). However, PRL 1 and 2 treatment affected PRL secretions (Fig. 2) differentially during March and May, compared with July and

Figure 1 (A) Mean concentrations ($\pm$ S.E.M.) of PRL in control media collected during 5-h experiments. Means represent pooled values ($n=3$ replicates) from adenohypophysis explant cultures performed during March, May, July and October respectively. (B) Mean concentrations ($\pm$ S.E.M.) of PRL in control adenohypophysis explant cultures performed during March, May, July and October. No statistical differences were observed.

October (treatment $\times$ month; $P<0.01$). During March and May, exogenous PRL 1 and 2 treatment increased ($P<0.05$ and $P<0.01$ respectively) PRL secretion from AP explants compared with concentrations of PRL observed in control wells. No effects were observed during July and October ($P>0.01$).

The mean levels of SOCS-3 mRNA expression in pituitary explants after PRL incubation are shown in Fig. 3. Exogenous PRL stimulated the expression of SOCS-3 in the pituitary during the experiments carried out in March ($P<0.05$) and May ($P<0.01$ and $P<0.05$ for the lower and higher concentrations respectively), and it inhibited the expression in July ($P<0.01$) and had no effect in October. In March (data not shown), PRL initially decreased the expression of SOCS-3; however, at 240 min of incubation, a strong stimulatory effect on SOCS-3 expression was observed. In May, PRL stimulated the expression of SOCS-3 at most of the PRL incubation times. In July, PRL decreased more than twice the SOCS-3 expression level in three of the five incubation periods. In October, the lower concentration of PRL did not affect the expression of SOCS-3 in any of the investigated incubation periods, and the effect of PRL at the concentration of 300 ng/ml was dependent on the incubation time (data not shown).

Figure 2 Mean concentrations ($\pm$ S.E.M.) of PRL in control and PRL-treated (PRL 1 – 100 ng/ml and PRL 2 – 300 ng/ml) adenohypophysis explant cultures performed during March, May, July and October. Both doses of PRL increased endogenous prolactin concentrations in March and May compared with controls ($P < 0.05$ for PRL 1 and $P < 0.01$ for PRL 2). No effect of PRL 1 and 2 was observed in July and October compared with controls. Means represent pooled values from 5-h cultures performed during March, May, July and October. * $P < 0.05$ and ** $P < 0.01$ compared with control.

Analysis of the expression of SOCS-3 in the pituitaries frozen directly after isolation showed that the basal expression differed relative to the time of year (Fig. 4). The highest level of SOCS-3 mRNA expression was observed in March, whereas the levels were slightly lower in May and were the lowest in July ($P < 0.01$) and October ($P < 0.01$).

Experiment II: in vivo leptin study

I.c.v. infusions of leptin changed SOCS-3 mRNA expression in the sheep pituitary (Fig. 5). Treatment with leptin increased SOCS-3 expression during SDs in a dose-dependent manner compared with controls ($P < 0.01$). In addition, the relative SOCS-3 mRNA level was higher in sheep that received the dose of 1.0 μ g/kg leptin compared with sheep that received the dose of 0.5 μ g/kg leptin ($P < 0.05$). Interestingly, leptin infusion had no effect on SOCS-3 expression during LDs.

Figure 3 The mean expression of SOCS-3 mRNA ($\pm$ S.E.M.) in the ovine pituitary explants in the presence of PRL at a concentration of 100 ng/ml (PRL 1) or 300 ng/ml (PRL 2) for tests carried out in the indicated months. Expression of SOCS-3 mRNA was reported in arbitrary units relative to *cyclophilin* mRNA expression. The mean value calculated for the control group was used as a calibrator. * $P < 0.05$ and ** $P < 0.01$ compared with control.

Discussion

SOCS-3 are potent inhibitors of the JAK/STAT signalling pathway, which negatively regulates signal transduction of a variety of factors, including leptin and PRL. Although proteins currently classified as SOCS were identified and characterised as negative regulators of cytokine signalling 13 years ago (Endo *et al.* 1997, Naka *et al.* 1997, Starr *et al.* 1997), their role in the coordination of hormonal interactions is still poorly understood. In physiological conditions, the expression of SOCS mRNA is low in the majority of tissues. It is known, however, that some hormones can rapidly change the level of SOCS expression.

Evidence of the relationship between exogenous PRL and the increase in SOCS-3 mRNA levels has been provided by research on cell lines derived from human breast cancers and on the livers of mice treated with PRL (Pezet *et al.* 1999), indicating that SOCS-3 participated in the regulation of cell responsiveness to PRL.

The present results indicate that PRL may inhibit the expression of SOCS-3. These findings suggest an important role for SOCS-3 in the modulation of PRL activity. The ability of PRL to both stimulate and inhibit SOCS-3 transcription may be the result of the pleiotropic characteristic of this hormone, which often involves opposing effects in different organs. The results also suggest that the effect of PRL on the expression of SOCS-3 was dependent upon the time of year. This is not surprising given the strong correlation between the endogenous rhythm of PRL secretion and the photoperiod. In this study, exogenous PRL stimulated the expression of SOCS-3 in March and May (the period of LDs) without changing the mRNA levels of SOCS-3 in October, when the length of the days became shorter. Surprisingly, in pituitary explants collected and incubated in the presence of PRL in July, the transcription of SOCS-3 was significantly reduced. Interestingly, the lowest basal expression level of SOCS-3 was observed in July, which coincided with the peak of the endogenous PRL concentration denoted in the ovine annual cycle (Misztal *et al.* 1999) and in sheep plasma in this study. Thus, we concluded that PRL-induced expression of SOCS-3 was blocked during this period. It is possible that a high concentration of PRL and its intensified effect are needed to facilitate sheep a switch from one physiological state to another during the transition from LDs to SDs.

It should be noted that seasonal variations of pituitary sensitivity to changes in the expression of SOCS-3 varied depending on the hormone used in the experiments. Indeed, the stimulating effect of PRL was evident during LDs, when the high concentration of PRL is observed in sheep plasma and during *in vitro* culture (March and May). One can suspect that, *in vitro*, the pituitary gland is isolated from dopaminergic inhibition and lactotroph cells secrete a large amount of PRL into media; however, this was not observed in this study and in others (Gregory *et al.* 2004). Concentration of PRL and other hormones secreted from pituitary cells into media is

Figure 4 The mean basal expression of SOCS-3 mRNA ($\pm$ S.E.M.) in ovine pituitaries ($n=4$ /month) collected in different months (March, May, July and October). Expression of SOCS-3 mRNA was reported in arbitrary units relative to *cyclophilin* mRNA expression. The mean value for the pituitary isolated in March was used as the calibrator. ** $P<0.01$ compared with control.

highly dependent on the physiological status of the donor animal. Data of Amstalden *et al.* (2003) demonstrated that the amount of LH released from AP explants was dependent on the nutritional history of the animal (fasted versus normal-fed). A paper by Molik *et al.* (2008) showed that pituitaries collected from lactating sheep released much more PRL than explants derived from non-lactating females (the present experiments), and that the time of year influenced the concentration of that hormone in culture media (Molik *et al.* 2008) as we observed in the present experiments – response of AP to exogenous PRL was higher during March and May than during July and October. In this study, leptin affected ovine pituitary only during SDs, in contrast to data showing a strong expression of SOCS-3 mainly during LDs in hypothalamus after i.c.v. leptin infusion (Zieba *et al.* 2008). It is possible that the response of tissue (hypothalamus or pituitary) resulted from direct (hypothalamus) versus indirect (pituitary) leptin effects and that the effect of leptin on pituitary could be mediated through other factors. These results indicate a seasonal/photoperiodic modulation of leptin and PRL actions. The photoperiodic component underlying alterations in this mechanism likely operates through the pineal hormone melatonin. Melatonin receptors are present within the *pars tuberalis* and the *zona tuberalis* of the ovine pituitary gland (De Reviers *et al.* 1989). Therefore, melatonin is able to transfer photoperiodic information directly to the cells of the pituitary; furthermore, leptin–melatonin interactions have been reported (Zieba *et al.* 2007). Whether melatonin can affect leptin action on the pituitary SOCS-3 expression in sheep needs to be further investigated. It may be presumed that SOCS-3 induced by one hormone may influence the actions of other hormones.

The observation that an i.c.v. infusion of leptin influenced SOCS-3 expression in the ovine pituitary is in agreement with results obtained in other species. Leptin administered i.p. (100 μ g) or i.v. (1 μ g/g BW) was responsible for a significant increase in SOCS-3 expression in hypothalamic nuclei of male *ob/ob* mice (Bjorbaek *et al.* 1998). Moreover, it was demonstrated on hamster ovary cell lines that the

increased SOCS-3 level caused by preincubation with leptin was linked with leptin resistance during subsequent incubation (Bjorbaek *et al.* 1999). This information suggests that the leptin-induced increase in SOCS-3 mRNA expression in this study may result in leptin resistance to subsequent administration.

Seasonally dependent changes in the responsiveness of the ovine hypothalamus to leptin have been reported in previous experiments (Miller *et al.* 2002). These findings may have resulted from the increased level of SOCS-3 expression and could explain the lack of sensitivity to the anorexic effects of leptin in the hypothalamus during the LD period. Even with a high concentration of endogenous leptin, the adiposity and appetite of ewes during LDs have been shown to increase. In autumn and winter, however, regulation of appetite is consistent with the nutritional status, and the leptin concentration is low, which corresponds to the physiological state of sensitivity to the hormone. Such a strategy allows animals to take full advantage of widely available food in the months of spring and summer to build up a stock of energy that is needed during periods of reduced food availability. The involvement of SOCS-3 molecules in the modification of seasonal hypothalamic sensitivity to leptin seems to explain the paradox described above.

The dependence of SOCS-3 expression on annual rhythms in the environment was confirmed by studies conducted in other animal species. One of the key publications was a study by Tups *et al.* (2004) that showed changes in SOCS-3 expression in response to varying lengths of day. Using Siberian hamsters, Tups *et al.* (2004) suggested that changes in SOCS-3 mRNA levels occurred in response to short-term fasting, long-term dietary restrictions and the effects of exogenous leptin relative to short (8 h light:16 h darkness) and long (16 h light:8 h darkness) days. In addition, SOCS-3 expression in the ARC was significantly higher during LDs than during SDs in all experimental systems studied (Tups *et al.* 2004).

Interestingly, i.p. administration of leptin (2 mg/kg BW) significantly increased the expression of SOCS-3 factors in

Figure 5 The expression of SOCS-3 mRNA (% of control) in pituitaries of sheep administered an i.c.v. infusion of Ringer-Locke buffer (Control) or leptin at a dose of 0.5 mg/kg BW (Leptin 1) or 1.0 mg/kg BW (Leptin 2) during long-day (LD) and short-day (SD) photoperiods. Denoted differences * $P<0.05$ between leptin-treated animals and differences ** $P<0.01$ and *** $P<0.001$ compared with controls.

the ARC of animals kept in SD conditions, but it did not change the expression in animals during LDs (Tups *et al.* 2004). The lack of effect of leptin during LDs could have resulted from a high endogenous photoperiod-induced SOCS-3 level, which could lead to leptin resistance.

Studies carried out on field vole (*Microtus agrestis*), a seasonal animal, provide very interesting information. Field voles, similar to Siberian hamsters, exhibit annual fluctuations in BW during the year and reduction in leptin resistance during LD (Krol *et al.* 2007). This paper raised the very important question of whether the increased level of expression of SOCS-3 is the result or the cause of concomitant weight gain. Furthermore, authors suggested that melatonin, by way of direct or indirect interaction, may be responsible for seasonal changes in the expression of SOCS-3 in the hypothalamus (Krol *et al.* 2007). Since melatonin is the main molecule responsible for signalling the changes in the length of the day, this pineal hormone and/or other hormones that have concentrations in the bloodstream that are highly dependent on the concentration of melatonin (e.g. PRL) may be involved in the seasonally dependent modulation of the expression of SOCS-3. Moreover, it is possible that seasonal differences in the ability of exogenous leptin to influence SOCS-3 expression in the hypothalamus of hamsters (Tups *et al.* 2004) and sheep (Zieba *et al.* 2008) resulted from the actions of other hormones (steroids). This hypothesis seems to be confirmed by observations of Siberian hamsters by Tups (2009), who showed that increased expression of SOCS-3 in animals kept in LD is maintained, even in the case of weight loss and decreased leptin levels (induced by starvation).

The results obtained in this study suggest that SOCS-3 protein may be involved in interactions between leptin and PRL. In agreement with these results, we observed a convergence between the leptin-induced expression of SOCS-3 in the pituitary and the inhibition of PRL secretion in SDs. It was demonstrated that SOCS-3 protein may be involved in leptin and PRL interactions influenced by photoperiod/melatonin in seasonal animals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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