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Downregulation of LRb in VMH/DMH during the second half of gestation and upregulation of SOCS-3 in ARC in late-pregnant ewes – Implications for leptin resistance

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ABSTRACT

To investigate factors involved in pregnancy-induced regulation of tissue sensitivity to leptin, we determined leptin concentrations and expression levels of the long form of the leptin receptor (LRb) and suppressor of cytokine signalling (SOCS)-3 in the ventro- and dorsomedial nuclei (VMH/DMH), arcuate nucleus (ARC), median eminence (ME) and anterior pituitary (AP) in 15 Polish Longwool ewes euthanized at 30, 60, 90 and 120 days of pregnancy and before gestation (n = 3 per group). Leptin concentrations increased during the first half of pregnancy, peaked on day 60, and then declined. In the VMH/DMH, LRb mRNA levels decreased from day 60 of pregnancy; in the ARC, LRb mRNA levels remained stable before and throughout pregnancy. LRb expression in the ME was lower in the first two months of pregnancy than before pregnancy ($P < 0.01$) and peaked at day 90. In the AP, LRb mRNA levels were higher during mid-pregnancy ($P < 0.05$) than before pregnancy. SOCS-3 expression in the VMH/DMH was higher throughout gestation ($P < 0.05$) than before pregnancy but was undetectable at day 120. SOCS-3 transcript levels were higher in the ARC ($P < 0.05$) in late-pregnancy (at day 120) than in non-pregnant ewes. SOCS-3 mRNA levels in the ME were lower at days 30 and 60 ($P < 0.05$) than at day 120 or before pregnancy. In the AP, SOCS-3 transcription was stable throughout gestation except at day 120, when it increased ($P < 0.05$). The changes in plasma leptin concentrations during pregnancy, hypothalamic LRb downregulation in the VMH/DMH during the second half of gestation and SOCS-3 upregulation in the ARC in late-pregnant ewes identified here may be essential components of the mechanisms driving ovine leptin insensitivity.

1. Introduction

Leptin, which is primarily produced in adipose tissue, is a key hormone involved in maintaining energy homeostasis. It reduces food intake by inhibiting orexigenic neurons and stimulating anorexigenic neurons within the hypothalamus (Halaas et al., 1995), and it increases energy expenditure by promoting energy-absorbing processes such as reproduction. Gestation is a very significant period that regulates leptin physiology and affects leptin synthesis, availability and activity. Pregnancy, which is characterized by hyperphagia and hyperleptinemia (Henson and Castracane, 2006), is also a state of physiological resistance to leptin in relation to its anorexigenic action, but the origin of this phenomenon seems to be species-specific and may be accomplished by different mechanisms. Increased leptin concentrations, which are associated with pregnancy, depend on placenta-derived factors in humans (Henson and Castracane, 2006) and mice (Gavrilova et al., 1997), but placental leptin production occurs on a very small scale in sheep (Thomas et al., 2001). Similarly, different factors have been reported to be the main causes of leptin insensitivity/resistance, including leptin receptor (LR) desensitization (Uotani et al., 1999), a decrease in leptin transport across the blood-brain barrier (El-Haschimi and Lehnert, 2003) and disturbances in intracellular signal transduction, including auto-suppression following the stimulation of suppressor of cytokine signalling (SOCS)-3 expression by leptin (Bjorbaek et al., 2000). The presence of specific pregnancy-induced adjustments in the actions of leptin across different species indicates that leptin plays essential roles

Abbreviations: AP, anterior pituitary; ARC, arcuate nucleus; CPH, cyclophilin; DMH, dorsomedial nuclei; GH, growth hormone; JAK, Janus kinase; LD, long days; LR, leptin receptor; LRb, long form of the leptin receptor; ME, median eminence; PRL, prolactin; PVN, paraventricular nucleus; ShD, short days; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription; VMH, ventromedial nuclei

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in mammalian pregnancy.

The biological function of leptin is mediated by a specific receptor, LRb (a long form of the LR), that induces the activation of several intracellular signal transduction pathways, including the Janus kinases/signal transducer and activator of transcription (JAK/STAT) pathway, the extracellular signal-regulated kinase signalling cascade and the phosphatidylinositol 3-kinase signalling pathway (Villanueva and Myers, 2008). However, the most common and essential effect of leptin on energy homeostasis is via the JAK2/STAT3 pathway (Bates et al., 2003). LRb lacks intrinsic enzymatic activity and mediates signals through the activation of receptor-associated, intracellular JAKs, which are members of the tyrosine kinase family. The receptors homodimerize upon ligand binding and then activate the subsequent factors in this pathway (White et al., 1997). Cytoplasmic STATs, which are activated by phosphorylation, dimerize and are translocated to the nucleus, where they bind to DNA and affect the transcription of target genes (White et al., 1997; Banks et al., 2000). Currently, a number of factors are known to influence this signalling pathway. However, a major negative regulator of the JAK/STAT pathway is the SOCS-3 protein, which suppresses not only leptin activity (Bjorbaek et al., 2000) but also the actions of other hormones, e.g., prolactin (PRL) (Ling and Billig, 2001) and growth hormone (GH) (Adams et al., 1998), thus acting as an important element modifying hormonal interactions.

The experiment reported herein was designed to determine the gestational profiles of LRb and SOCS-3 mRNA expression in sheep to broaden our knowledge of the mechanisms underlying the pregnancy-induced adjustment of the sensitivity of brain tissues to the actions of leptin.

2. Materials and methods

All of the animal-related procedures in these studies were approved by the First Local Ethics Committee on Animal Testing in Krakow (No. 81/2013).

2.1. Animals

The experiments were performed at the Experimental Station of the Department of Animal Biotechnology, University of Agriculture in Krakow (longitude: 19°57′E, latitude: 50°04′N) on a total of fifteen adult 3- to 4-year-old female Polish Longwool sheep rated as 3 on a five-point condition scale (Russel et al., 1969) and weighing 54.3 ± 5.3 (SD; standard deviation) kg before mating and 66.3 ± 5.1 (SD) kg at day 120 of gestation. Ewes were fed twice daily at 07:00 and 16:00 with a diet formulated to provide 100% of the requirements for maintenance according to their physiological status as recommended by the National Research Institute of Animal Production (Norms, 1997). Water was available *ad libitum*, and ewes were group-housed under natural photoperiodic and thermoperiodic conditions. Ewes were placed in individual carts during blood sample collection.

2.2. Procedures and treatments

In a reproductive season, the oestrous cycles of each ewe were synchronized using a 14-day treatment with intravaginal progestogen-impregnated sponges (40 mg fluorogestone acetate, FGA; Chronogest, Intervet International, Boxmeer, the Netherlands). On the day of sponge removal, ewes were intramuscularly injected with a single dose of 500 IU of pregnant mare serum gonadotrophin (PMSG or serogonadotropin; Biowet, Drwalew, Poland). Oestrus detection was performed twice daily (at 08:00 and 20:00) with an adult ram equipped with an apron. Oestrus was defined as mounting acceptance. After oestrus detection, ewes were individually presented to the male and mated naturally. All ewes were mated 36 ± 12 h after PMSG injection at the same time of year (October), and pregnancy was verified 40 days later using an ultrasonographic scanner (Aloka SSD 500 Micrus, Equine Therapy System, Inc., Greenwood Village, CO, USA). All ewes used in this experiment had twin pregnancies.

Animals were randomly divided into five experimental groups to collect blood and tissue samples at 30, 60, 90, 120 days of pregnancy (groups P30, P60, P90 and P120, respectively) and before gestation (3 animals per indicated time-point). In the morning of the experiment, sheep were fitted with jugular catheters, and blood samples (5 mL) were collected at 15-min intervals for 1 h. The blood samples were dispensed into tubes containing 150 µL of a heparin solution (10,000 IU/mL), and the plasma was separated by centrifugation at 3000 × *g* at 4 °C for 10 min and stored at −20 °C for subsequent leptin concentration measurements. After blood sampling, animals were killed by exsanguination following captive bolt stunning. The brains were rapidly removed and frozen on dry ice, and samples of the ventro- and dorsomedial nuclei (VMH/DMH), arcuate nucleus (ARC), median eminence (ME), and anterior pituitary (AP) were aseptically isolated from the ewes 10–15 min post mortem. To eliminate the possibility of contamination by transferring tissue between samples, separate sterile tools were used to dissect each area. Tissue samples were rinsed in phosphate-buffered saline (PBS; Laboratory of Vaccines, Lublin, Poland), snap frozen in liquid nitrogen and then transferred and stored at −80 °C until analysis. The experimental timeline and procedures are shown in Fig. 1.

2.3. Molecular analysis

Real-time PCR was used to measure LRb and SOCS-3 mRNA expression. Samples were homogenized using a rotor-stator tissue homogenizer (Omni TH, Omni International, Inc., Kennesaw, GA, USA) equipped with single-use tips (Soft Tissue Omni Tip Plastic

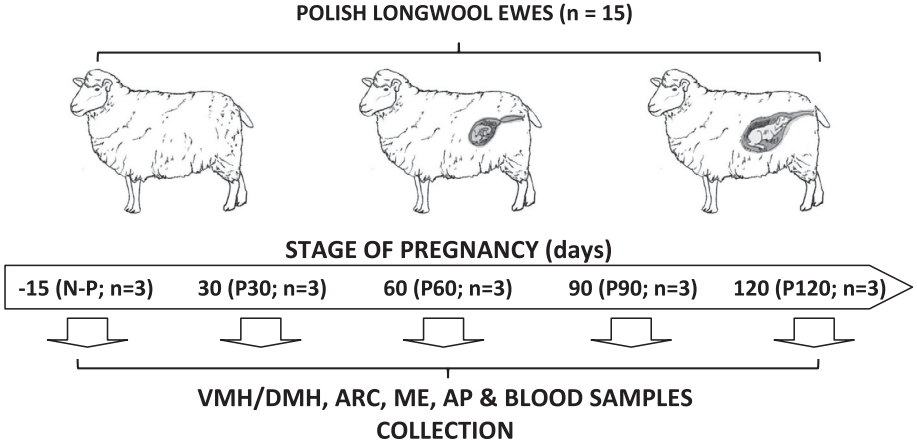

Fig. 1. Timeline for experimental procedures. Fifteen Polish Longwool ewes were randomly assigned to one of five groups (n = 3/group) and euthanatized before gestation (N-P; non-pregnant) or at days 30 (P30), 60 (P60), 90 (P90), and 120 (P120) of pregnancy to collect the ventro- and dorsomedial nuclei (VMH/DMH), arcuate nucleus (ARC), median eminence (ME), anterior pituitary (AP) and blood samples.

Homogenizing Probes, Omni International, Inc., Kennesaw, GA, USA), and total RNA was obtained using TRIzol reagent (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. All the isolated samples were characterized by the adequate integrity and purity of the RNA. A total of 1 μg of RNA was then reverse transcribed to cDNA using Quantiscript reverse transcriptase and RT primer mix (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany) 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 Reverse Transcription Kit; as above) and incubating the samples at 42 °C for 2 min. Each cDNA was amplified using TaqMan Gene Expression Master Mix (Life Technologies; Foster City, CA, USA); specific primers corresponding to the target/reference genes, each at a final concentration of 900 nM (Sequence Detection Primers, Life Technologies, Foster City, USA); specific probes corresponding to the target/reference genes, each at a final concentration of 250 nM (TaqMan MGB Probes; Life Technologies, Foster City, CA, USA); and an Applied Biosystems 7300 Real-Time PCR System. Primers and probes were designed using Primer Express v. 2.0 software (Applied Biosystems; Foster City, CA, USA). The sequences of the primers and probes, amplicon sizes and sequence accession numbers are listed in Table 1.

The gene assay was carried out in triplicate for each cDNA sample, and amplification was performed 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 using the Applied Biosystem 7300 Real-Time PCR System SDS software, and the results were expressed as a function of the threshold cycle (Ct). Using diluted samples, the amplification efficacies for the target genes and the reference gene were found to be identical.

2.4. Molecular data analysis

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

The mean mRNA expression for the target genes in each sample was normalized against the expression of a reference gene, cyclophilin (CPH), and expressed relative to the calibrator sample. There was no significant variation ($P > 0.05$) in the Ct values for CPH among the treatment groups.

We used the mean ΔCt value for each indicated tissue collected from non-pregnant ewes as a calibrator to compare the changes in gene expression levels among tissues isolated at different stages of gestation. Following the determination of a significant F-value, differences in the means were compared using all pairwise multiple comparison procedures (Tukey test) using SigmaPlot statistical software (version 11.0; Systat Software Inc., Richmond, CA, USA). All differences with P values < 0.05 were considered statistically significant.

2.5. Hormone assays

Circulating leptin concentrations were determined using a highly specific ovine leptin radioimmunoassay (RIA) with the double-antibody method that employed a specific, high-affinity rabbit antibody generated against recombinant ovine leptin and anti-rabbit-γ-globulin antisera and a recombinant ovine leptin standard, as described by Delavaud et al. (2000). The intra- and interassay coefficients of variation of the leptin assay were 3.2% and 11.0%, respectively, and the assay sensitivity was 0.3 ng/mL.

2.6. Hormone data analysis

Hormone data were analysed by a series of one-way analyses of variance using SigmaPlot statistical software (version 11.0; Systat Software Inc., Richmond, CA, USA) for repeated measures. The values determined for all samples at the indicated stages of gestation were averaged to calculate the means used for comparisons among groups, and the statistical models included the main effects of the stage of gestation. After determining a significant F-value, the means were contrasted using a procedure for performing all pairwise multiple comparisons (Tukey test). A P-value of < 0.05 was considered statistically significant. All data are expressed as the means ± standard error of the mean (SEM).

3. Results

3.1. Leptin concentrations

The concentrations of endogenous leptin varied depending on the stage of gestation (Fig. 2). During the course of the first half of pregnancy, leptin concentrations increased to a peak on day 60 and then began to decline. Plasma leptin concentrations in ewes from the P60 group were significantly ($P < 0.05$) higher than those in the non-pregnant, early-pregnant (P30) and late-pregnant (P120) ewes, but they were comparable ($P \geq 0.05$) to concentrations in the P90 group. During late pregnancy (P120), leptin concentrations decreased to values similar ($P \geq 0.05$) to those determined before gestation. Lower leptin concentrations were detected in early-pregnant ewes (P30) than in ewes ($P < 0.05$) at all other stages of gestation.

3.2. Leptin receptor expression

LRb transcripts were detected at varying levels in all examined tissues but not at every stage of pregnancy (Fig. 3). We detected LRb expression in the VMH/DMH of non-pregnant and early-pregnant ewes, while at later stages of pregnancy (60, 90 and 120 days), LRb

Table 1
Sequences of oligonucleotides used as primers and probes to analyse the mRNA expression of cyclophilin (CPH; reference gene), long form of the leptin receptor (LRb; target gene), and suppressor of cytokine signalling-3 (SOCS-3; target gene) in sheep.

Gene	Primer sequence (5′–3′)	Probe sequence (5′–3′)	Amplicon size	GenBank accession number
CPH	CGGCTCCCAGTTCTTCATCA ACTACGTGCTTCCCATCCAAA	FAM-CGTTCCGACTCCGC-MGB	64 bp	D14074
LRb	CGACGAGGGTGGCATATTTAA CAGACATAACCTGTGAGGATGGAA	FAM-CAGGAGACAGCCCTC-MGB	63 bp	U62124.1
SOCS-3	CCTCAAGACCTTCAGCTCCAA CTTGCGCACTGCGTTCAC	FAM-AGCGAGTACCAGCTGG-MGB	68 bp	NM_174466

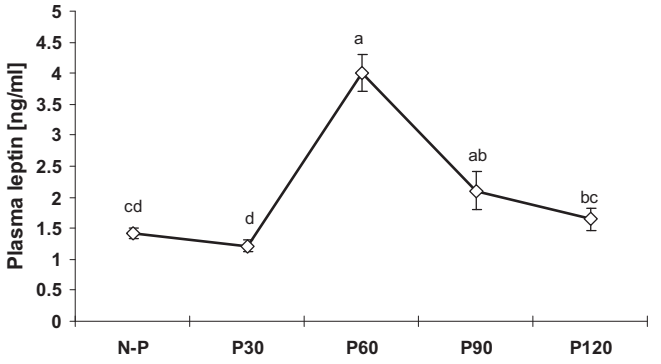

Fig. 2. Plasma leptin concentrations. Mean (± SEM) plasma leptin concentrations in ewes before gestation (N-P; non-pregnant; n = 3) or at days 30 (P30; n = 3), 60 (P60; n = 3), 90 (P90; n = 3), and 120 (P120; n = 3) of pregnancy. Means marked with different letters differ significantly ($P < 0.05$).

transcripts were undetectable in this hypothalamic area. Within the ARC, LRb mRNA levels remained relatively stable throughout gestation and were comparable ($P \geq 0.05$) to the expression levels determined before pregnancy. Expression in the ME decreased in the first two months of pregnancy when compared to expression in the pre-gravid state ($P < 0.01$), reached a peak at 90 d, and then decreased again to non-detectable levels in late-pregnant ewes. In the AP, there were significant changes in LRb transcript levels during pregnancy; LRb mRNA expression was significantly increased on days 60 ($P < 0.05$) and 90 ($P < 0.01$) by 2.0- and 2.2-fold, respectively, compared with levels in the AP from ewes before gestation. There were no significant changes ($P \geq 0.05$) in pituitary LRb expression between non-pregnant, early-pregnant (P30) and late-pregnant (P120) individuals.

3.3. SOCS-3 expression

Expression of SOCS-3 was detectable in all examined tissues, but it occurred at different levels depending on the tissue and stage of pregnancy (Fig. 4). Compared to expression in the pre-gravid state, SOCS-3 was significantly upregulated in VMH/DMH collected from ewes at days 30 ($P < 0.05$), 60 ($P < 0.05$) and 90 ($P < 0.01$) of gestation by 4.5-, 1.9- and 6.8-fold, respectively; however, SOCS-3 mRNA was undetectable at day 120. Despite the fact that SOCS-3 mRNA expression tended to increase during gestation, significant changes in SOCS-3 transcript levels in the ARC were found only in samples collected during late pregnancy (P120) relative to levels in non-pregnant ewes ($P < 0.05$). A variable gestational profile of the SOCS-3 expression level was also observed in the ME. Transcript levels of this suppressor determined at days 30 and 60 were significantly lower ($P < 0.05$) than at later stages of gestation or before pregnancy. In the AP, the level of SOCS-3 mRNA remained relatively stable throughout gestation with the exception of late pregnancy, when it was significantly ($P < 0.05$) up-regulated in relation to expression determined in pituitaries collected at 30 and 90 days of gestation or before pregnancy.

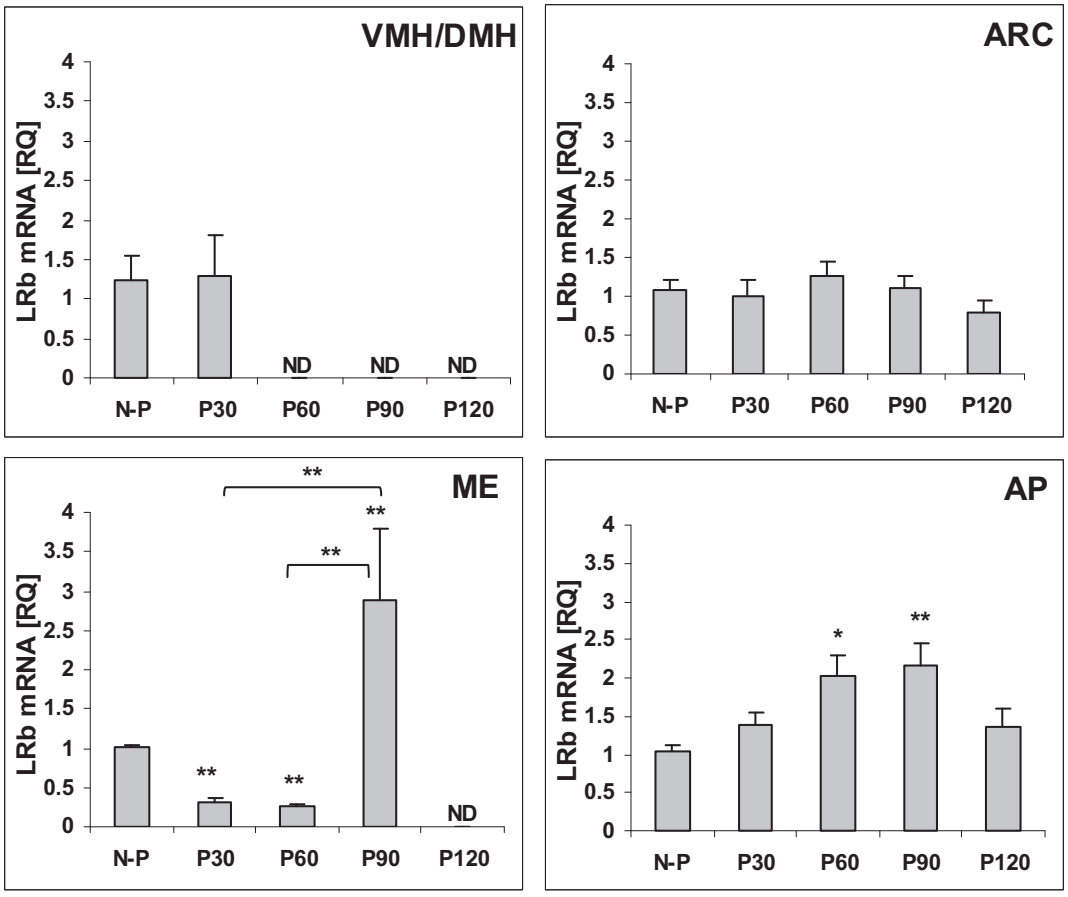

Fig. 3. Leptin receptor expression. The mean expression (± SEM) of the long form of leptin receptor (LRb) mRNA in ovine ventro- and dorsomedial nuclei (VMH/DMH), arcuate nucleus (ARC), median eminence (ME) and anterior pituitary (AP) collected before gestation (N-P; non-pregnant) or at days 30 (P30), 60 (P60), 90 (P90), and 120 (P120) of pregnancy. The expression of LRb mRNA is reported in arbitrary units (RQ) relative to cyclophilin mRNA expression, and the mean value calculated for the indicated tissue collected from non-pregnant ewes (N-P) was used as a calibrator. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote differences relative to the control or between the indicated group. Samples in which the expression of the target gene was undetectable are denoted with ND.

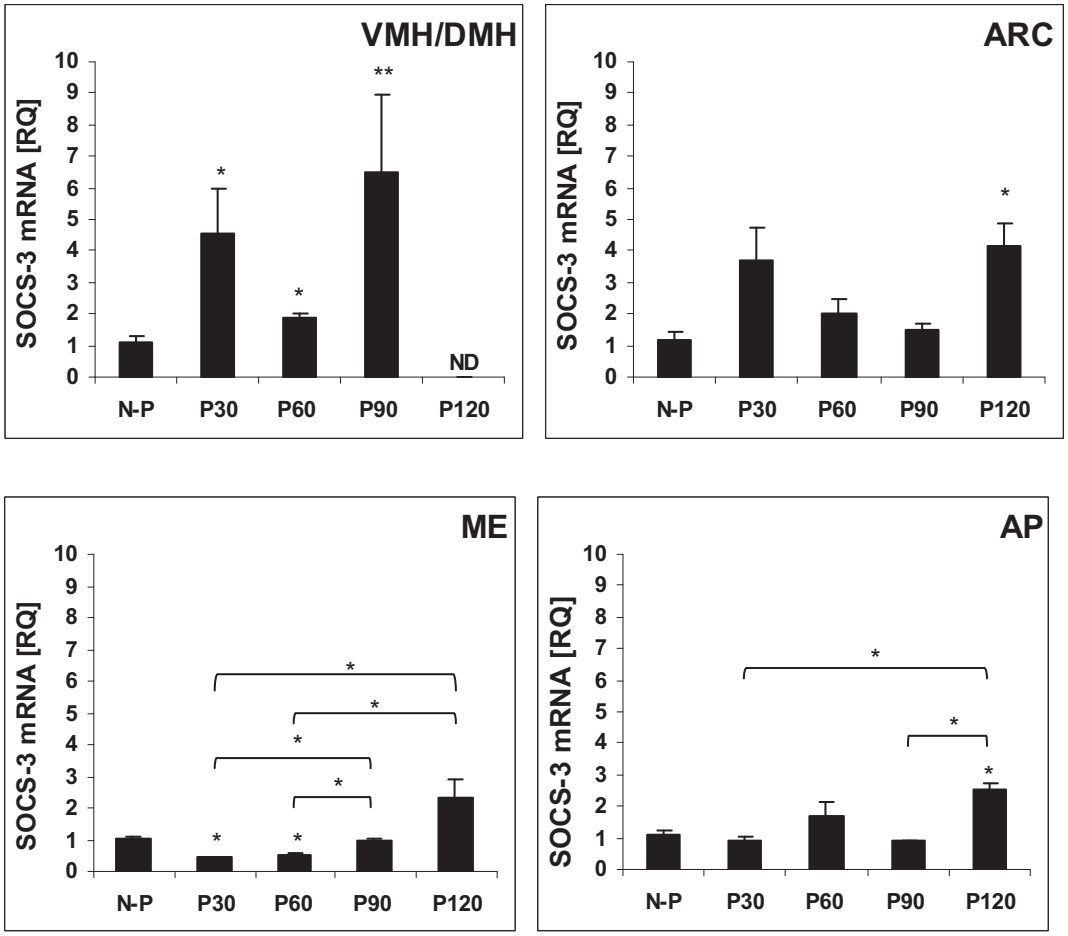

Fig. 4. Expression of SOCS-3. The mean expression (± SEM) of suppressor of cytokine signalling-3 (SOCS-3) mRNA in the ovine ventro- and dorsomedial nuclei (VMH/DMH), arcuate nucleus (ARC), median eminence (ME) and anterior pituitary (AP) collected before gestation (N-P; non-pregnant) or at days 30 (P30), 60 (P60), 90 (P90), and 120 (P120) of pregnancy. The expression of SOCS-3 mRNA is reported in arbitrary units (RQ) relative to cyclophiline mRNA expression, and the mean value calculated for the indicated tissue collected from non-pregnant ewes (N-P) was used as a calibrator. $^{*}P < 0.05$ and $^{**}P < 0.01$ denote differences relative to the control or between indicated group. Samples in which the expression of the target gene was undetectable are denoted with ND.

4. Discussion

Two possible mechanisms that can modify the sensitivity of tissues to leptin and, under specific circumstances, lead to a state of physiological pregnancy-induced leptin resistance in sheep were investigated in the present study, i.e., changes in the expression of LRb and the suppressor SOCS-3 in selected regions of the ovine brain and AP. We demonstrated the expression of both LRb and SOCS-3 in all examined tissues, and we proved, for the first time, that the levels of these transcripts in the hypothalamus and AP depend on the stage of pregnancy in ewes. Moreover, the gestation-related changes that we measured in plasma leptin concentrations and the expression of important components of the leptin signalling pathway mentioned above indicate that the sensitivity of brain tissues to leptin may be regulated in different manners. For example, insensitivity to leptin in pregnant sheep may occur due to hypothalamic downregulation of the LRb receptor within the VMH/DMH after 60 days of pregnancy, while it may be due to upregulation of SOCS-3 factors at 120 days of gestation in the ARC.

Pregnancy is a state of intensified energetic demand characterized by increases in food intake and body mass. The gestational rise in leptin concentrations observed in ewes in the present study is a well-known phenomenon that has also been observed in different animal species and humans (Chien et al., 1997; Hardie et al., 1997), but it is not connected to the anorexigenic effects of leptin. Physiological and transient reductions in the actions of leptin are phenomena that allow organisms to maintain energy homeostasis in conditions that require predictive/anticipatory adjustment to subsequent metabolic demand, as is necessary in cases of pregnancy or adaptation to seasonal fluctuations in food in the external environment.

In rats, it has been demonstrated that leptin resistance occurs at the hypothalamic level via region-specific losses of hypothalamic LRb mRNA expression (Garcia et al., 2000; Ladyman and Grattan, 2004) and suppressed leptin signal transduction (Garcia et al., 2000; Ladyman and Grattan, 2004, 2005), but information about leptin insensitivity during pregnancy in non-rodent mammals is scarce and incomplete. Considering that rodents are characterized by specific metabolic profiles, patterns of reproduction and gestational physiology, using other animal models to study leptin resistance seems to be essential for obtaining a more detailed picture of this phenomenon. Our present results from a small ruminant model confirm the observations from rodents that a decrease in LRb levels (as in the VMH/DMH after 60 days of gestation) and an increase in the levels of leptin suppressors (as in the ARC at day 120) may lead to insensitivity of the hypothalamus to the effects of leptin. On the other hand, upregulation of LRb expression in the hypothalamus was previously observed in feed-restricted sheep (Dyer et al., 1997) and lactating ewes (Sorensen et al., 2002), representing states with a negative energy balance and suggesting that metabolic status may be linked to the expression of leptin receptors, thus bidirectionally affecting tissue sensitivity to leptin.

It has been repeatedly demonstrated in different species, including sheep (Dyer et al., 1997), that LRb, a signal-transducing isoform of LR, is strongly expressed in specific nuclei of the hypothalamus, and it is

involved in the control of food intake, regulation of energy homeostasis and neuroendocrine function. However, it should be mentioned that gestational changes in LRb levels vary in different hypothalamic areas. Ladyman and Grattan (2005) showed that LRb expression in rats was reduced in the VMH, while in the ARC, DMH, supraoptic nucleus, and paraventricular nucleus (PVN), LRb expression was relatively unchanged during pregnancy. Our results from sheep are compatible with those from rodent studies. In the VMH/DMH, LRb expression in early-pregnant ewes was similar to that observed in non-pregnant ewes, whereas it decreased drastically to undetectable levels at later stages of pregnancy. Simultaneously, the LRb transcript levels in the ARC were relatively constant during pregnancy and before conception. Moreover, significant changes in hypothalamic levels of mRNA were observed in pregnant rats only in relation to LRb levels and not to the levels of other leptin receptor isoforms (LRa, LRc and LRf) (Ladyman and Grattan, 2005). Despite these results, the possibility of a link between the expression of other LR subtypes and leptin insensitivity/resistance should not be disregarded. It has been suggested that, at least in rodents, LRa and other short isoforms could function as a specific transport system for leptin through the blood-brain barrier because they are present at high concentrations in the choroid plexus and brain microvessels (Tartaglia et al., 1995). Short and soluble isoforms, such as LRe, may also act as functional antagonists by sequestering leptin and preventing its binding to the LRb isoform (White et al., 1997).

Among the factors that could be responsible for leptin insensitivity, one of the most frequently mentioned is impaired or inhibited transduction of leptin signalling by negative regulators, such as SOCS suppressors. Among eight known SOCS proteins in the hypothalamus, leptin specifically induces the expression of SOCS-3 (Bjorbaek et al., 1999; Baskin et al., 1999), which makes this suppressor one of the most important regulatory elements in leptin signalling. In hamsters, it was demonstrated that SOCS-3 and many factors that induce its expression are localized in the ARC, VMH, DMH, PVN, suprachiasmatic nucleus and pituitary gland (Tups et al., 2004), suggesting that SOCS-3 plays a pivotal role in the regulation of energy homeostasis and the modulation of neuroendocrine interactions. It has been reported that high SOCS-3 levels are involved in leptin resistance (Bjorbaek et al., 1998, 1999). Zampieri et al. (2015) analysed several genes that could modulate leptin sensitivity, including SOCS subtypes, adapter proteins such as SH2B1, and protein-tyrosine phosphatases, and showed that only SOCS-3 expression within the hypothalamus was significantly affected by pregnancy. However, Ladyman and Grattan (2004, 2005) have shown in rats that the ability of centrally administered leptin to inhibit food intake is lost during pregnancy while the level of activated STAT3 in the VMH is simultaneously decreased, suggesting the occurrence of leptin resistance due to blocking of the JAK/STAT pathway. Trujillo et al. (2011) observed a reduction in the level of STAT3 phosphorylation in pregnant rats after intravenous leptin injection. They also found that the hypothalamic level of SOCS-3 was increased in pregnant rats treated with intracerebroventricular infusions of leptin relative to that in nonpregnant rats, and the expression of LRb was reduced at the end of pregnancy. Moreover, they were unable to determine any significant differences in SOCS-3 and LRb levels in the hypothalamus between pseudopregnant and nonpregnant animals (Trujillo et al., 2011). These data suggest that leptin resistance in rats during pregnancy is induced by factors with placental origins that are not present in pseudopregnant females. As indicated in this paper, overexpression of SOCS-3 occurred preferentially in the VMH/DMH during early- and mid-pregnancy in sheep. In late-pregnant ewes, SOCS-3 mRNA in the VMH/DMH was undetectable in contrast to the ARC, where it was upregulated in this stage of pregnancy.

In adult, non-pregnant ewes, the plasma leptin concentration is positively related to body weight, body condition score and body fatness as well as nutritional status to a lesser extent (Delavaud et al., 2000), but in pregnant individuals, plasma leptin concentrations increase regardless of these factors (Ehrhardt et al., 2001). As shown by Ehrhardt et al. (2001), feed intake was nearly identical during the pre-breeding and mid-pregnancy periods but increased in late pregnancy, similar to maternal body weight, which increased in late-pregnant ewes. Elevated levels of SOCS-3 in the ARC at P120, which were observed in the present study, may suppress leptin signalling via JAK/STAT to regulate neuropeptide expression and stimulate appetite in late-pregnant ewes.

In pregnant ewes, elevated concentrations of leptin, which are a consequence of both increased adiposity and increased leptin mRNA expression in adipose tissue (Ehrhardt et al., 2001) but not secretory activity of the placenta (Thomas et al., 2001), influence many targets besides the hypothalamus. The presence of cells that are immunoreactive to a leptin receptor antiserum in the ovine AP was demonstrated by Iqbal et al. (2000). Considering these facts, we also investigated the gestational profiles of LRb and SOCS-3 in the AP and in the ME, which are integral parts of the pituitary portal system that connects the hypothalamus to the pituitary gland.

Our previous experiments demonstrated that leptin affects the release of PRL, GH and melatonin in pregnant ewes (Szczesna et al., 2018), suggesting that pregnancy-induced hyperleptinemia may be an essential factor in determining the hormonal status of pregnant females, and our present results confirm that the leptin sensitivity of the ovine AP is greater during pregnancy than in the non-gravid state, which contrasts with the findings for the hypothalamus. The expression of LRb during mid-pregnancy (days 60 and 90) in the AP was doubled relative to the expression in non-pregnant ewes, while SOCS-3 levels remained unchanged at this stage of gestation. In late pregnancy, however, the sensitivity of the AP is probably decreased due to the upregulation of SOCS-3 factors, suggesting that the responsiveness of tissues to leptin may be regulated in multiple ways. Moreover, our present results confirm the existence and variable expression of both LRb and SOCS-3 during pregnancy in the ovine ME. In this region, LRb and SOCS-3 mRNA levels were simultaneously downregulated during the first half of pregnancy (P30 and P60), which may indicate blocking of the JAK/STAT pathway that consequently leads to leptin resistance. This observation provides an interesting insight into the interactions between the hypothalamus and pituitary in relation to the effects of leptin.

The results of the current study confirmed that pregnancy affected the expression of LRb in the VMH/DMH, AP and ME or of SOCS-3 in all the analysed tissues, but the question arises of whether it is possible that the direction of these actions depends not only on the stage of pregnancy but also on seasonal changes. Our previous studies (Zieba et al., 2008; Szczesna et al., 2011) implicated the role of SOCS-3 factors in the modulation of the sensitivity of the hypothalamus and pituitary to leptin according to the season. The results of these experiments demonstrated that centrally infusing leptin into the third ventricle of the brain affected the expression of SOCS-3 factors in a season- and tissue-dependent manner. In the hypothalamus, leptin increased SOCS-3 mRNA expression during long days (LD) but not during short days (ShD) (Zieba et al., 2008). In turn, in relation to the pituitary gland, we observed a leptin-induced increase in SOCS-3 transcript levels only during ShD (Szczesna et al., 2011). In terms of seasonal leptin resistance, which occurs in sheep (Clarke et al., 2001; Miller et al., 2002) and hamsters (Tups et al., 2004), these results also suggest that variable levels of SOCS-3 expression may lead to alterations in the sensitivity of the hypothalamus and pituitary to the actions of leptin. However, although seasonal changes in the endogenous level of SOCS-3 expression have been previously shown in Siberian hamsters (Tups et al., 2004), they were not confirmed in our research on sheep. We have not found any significant differences in the endogenous levels of SOCS-3 expression in the ovine hypothalamus and AP when comparing LD and ShD seasons (Zieba et al., 2008; Szczesna et al., 2011).

Although the majority of information about leptin resistance and the role of the different factors involved in this process have been derived from research conducted on genetically mutated, obese rodents and concern the pathological basis of this phenomenon, the fact that

leptin resistance is also a physiological and positive mechanism should not be neglected; it occurs as an adaptive response by an organism that allows it to adequately adjust the energy homeostasis set point in response to specific challenges, e.g., pregnancy or environmental changes. The leptin responsiveness of various tissues may be modulated by different mechanisms, including the regulation of LRb and SOCS-3 expression. According to the above observations, variable expression of LRb and SOCS-3 in different brain regions and the AP, which we detected during pregnancy in sheep, may be important factors responsible for the variable sensitivity of tissues to the actions of leptin. This phenomenon is essential for the coordination of physiological processes that maintain the requirements and capabilities of the mother and foetus in a well-balanced state for successful reproduction and, consequently, the survival of the species.

Declaration of competing interests

None.

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