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Effect of Salsolinol on ACTH and Cortisol Response to Handling Stress in Early Anestrous Sheep

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ABSTRACT

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Inhibition of hypothalamic–pituitary–adrenal (HPA) axis activity by salsolinol was demonstrated in lactating sheep. We assessed whether salsolinol regulates, besides lactation, also adrenocorticotropic hormone (ACTH) and cortisol release, and if its action is prolactin-dependent. We examined two groups of early anestrous sheep, which received for three days salsolinol or vehicle-only intracerebroventricular injections, and a group of lactating sheep injected with the vehicle only. On day 3, blood samples were collected for over six hours and the anterior pituitary was dissected. Plasma ACTH, cortisol, and prolactin concentrations, and *proopiomelanocortin* (*POMC*) and *prolactin* (*PRL*) mRNA expression within the anterior pituitary were assayed. In all groups, ACTH and cortisol concentrations were higher ($P < 0.05$ and $P < 0.001$) during the first half of sampling than in the second half; there were no differences in prolactin concentration. Lactating sheep had lower ($P < 0.05$ and $P < 0.001$) plasma ACTH and cortisol concentrations and higher ($P < 0.001$) plasma prolactin concentration than both groups of anestrous sheep during the first half of sampling. In the second half, there were no differences in ACTH and cortisol concentrations between all groups, but prolactin concentration was still higher ($P < 0.001$) in lactating animals. Salsolinol treatment decreased ACTH and cortisol concentrations during the first half of sampling ($P < 0.05$ and $P < 0.001$) compared to the anestrous controls, but had no effect on prolactin concentration. *POMC* mRNA expression was lower ($P < 0.05$) and *PRL* mRNA expression was higher ($P < 0.05$) in lactating sheep than in anestrous sheep. Salsolinol did not affect *POMC* and *PRL* mRNA expression. In conclusion, increased ACTH and cortisol concentrations during the first half of sampling occurred in response to handling stress. Salsolinol inhibited the HPA axis response to stress in early anestrous sheep, and it was unrelated to prolactin secretion.

Keywords: adrenocorticotropic hormone; cortisol; prolactin; stress response; sheep

Lactation is characterized by adaptive changes in both basal and stress-induced maternal hypothalamic–pituitary–adrenal (HPA) axis activities. The changes are essential due to metabolic demands of lactating females and prevention of developing offspring against corticosteroids that enter maternal milk (Angelucci et al. 1985). Numerous mammalian species, including sheep, have been

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observed to exhibit reduced stress-induced HPA axis activity during lactation (Altemus et al. 1995; Reeder et al. 2004; Tilbrook et al. 2006; Windle et al. 2013). Further, studies on lactating rats revealed elevated basal secretion of adrenocorticotropic hormone (ACTH) and corticosterone, with a flattened diurnal rhythm for corticosterone secretion (Windle et al. 2013). However, no changes in basal cortisol secretion have been observed in lactating sheep (Kabaroff et al. 2006;Tilbrook et al. 2006).

The mechanism of the attenuated HPA axis response to stress is not well understood, but there is no doubt that suckling is involved in this phenomenon. An early study revealed that rats suckled by their offspring exhibited lower ACTH and corticosterone responses to stress compared to dams with removed nipples (Stern and Levine 1972). In women, 15 min after breastfeeding plasma cortisol concentration was lower than prior to beginning of nursing (Amico et al. 1994), and the cortisol response to the Trier Social Stress Test was suppressed shortly after breast-feeding (Heinrichs et al. 2001). The importance of suckling has also been demonstrated in sheep, in which this stimulus was involved in the attenuation of the cortisol response to isolation and restraint stress (Tilbrook et al. 2006).

Observing the impact of suckling on the HPA axis, an intriguing question arises: what biological factors mediate the inhibitory effect of this stimulus on the HPA axis? Prolactin was the first suspect because this hormone is released from the anterior pituitary (AP) under the influence of both suckling and stress (Freeman et al. 2000). The inhibitory role of prolactin has been confirmed by intracerebroventricular (ICV) infusion of antisense nucleotides against the long form of prolactin receptor in lactating rats, which resulted in enhancement of the stress-induced ACTH secretion (Torner et al. 2002). Moreover, prolactin infusion into the paraventricular nucleus (PVN) reduced cortisol secretion in response to acute stress in lactating sheep (Cook 1997).

Our previous study revealed that suckling caused an increase in the concentration of salsolinol (1-metyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) in the infundibular nucleus/median eminence (IN/ME) of lactating sheep (Misztal et al. 2008). This derivative of dopamine has been considered to be a physiological prolactin-releasing factor in rodents (Toth et al. 2001) and ruminants (Hashizume et al. 2008; Misztal et al. 2008). Additionally, in lactating sheep, salsolinol increases both prolactin release into the bloodstream and *prolactin* (*PRL*) mRNA expression in the AP cells (Hasiec et al. 2012). The prolactin-releasing effect is not the only physiological function of salsolinol. We also demonstrated that ICV infused salsolinol suppressed stress-induced increases in plasma ACTH and cortisol concentrations in lactating sheep (Hasiec et al. 2014). In turn, the ICV infusions of an antagonistic analogue of salsolinol, 1-MeDIQ, during the fifth week of lactation, elevated the basal plasma concentrations of both ACTH and cortisol (Hasiec et al. 2015).

Considering the above, it is likely that prolactin mediates the salsolinol inhibitory effect on HPA axis activity. Surprisingly, we demonstrated that the ICV infusions of salsolinol in lactating sheep decreased ACTH and cortisol response to isolation stress without changing the plasma prolactin concentration (Hasiec et al. 2014). This observation contradicts the prolactin mediation of the salsolinol inhibitory effects on stress-induced HPA axis activity. Continuing our research, we investigated the effect of salsolinol on the HPA axis in anestrous sheep during the early period of increasing day length, when prolactin secretion is low.

We hypothesized that salsolinol administered into the third ventricle of the brain (IIIv) of early anestrous sheep inhibits ACTH and cortisol release without changing plasma prolactin concentration. Therefore, sheep were treated for three days with the ICV injections of salsolinol. During the last day, blood samples were collected to measure the concentrations of selected hormones, and the AP was dissected to check whether expected changes in plasma hormone concentrations resulted from changes in their genes expression.

MATERIAL AND METHODS

Animal management. All animal procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals and were approved by the Local Ethics Committee. The experiment was performed using Polish Longwool sheep (3- to 4-year old, n = 15), which were maintained indoors in individual pens under natural lighting conditions (52°N, 21°E). They were fed

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twice a day with a diet formulated according to the recommendations of the National Research Institute of Animal Production, Krakow, Poland (1993 norms); hay and water were available *ad libitum*. Animals were divided into three groups. One group of sheep (n = 5) was mated naturally in September, and they lambed in the following February. The other two groups of sheep remained unmated (n = 10). The experiment was performed in March, when lactating sheep were in the fifth week of lactation. During the experimental procedures, ewes were kept in individual pens, but lactating sheep were housed together with their lambs, which had free access to the udder.

Brain surgery. A stainless steel guide cannula was implanted into the IIIv (1.2 mm outer diameter) during the third month of pregnancy. Implantation was performed under general anaesthesia (xylazine: 40 mg/kg of body weight, intravenously; xylapan and ketamine: 10–20 mg/kg of body weight, intravenously; Bioketan; Vetoquinol Biowet, Poland) through a hole drilled in the skull, in accordance with the procedure described by Traczyk and Przekop (1963). The cannulas were positioned according to the stereotaxic coordinates of the sheep hypothalamus (Welento et al. 1969): frontal 29.5–31 mm and sagittal 0.3–0.5 mm. Guide cannulas were fixed to the skull with stainless steel screws and dental cement. The external opening of the canal was closed with a stainless steel cap. After the surgery, ewes were injected daily with antibiotics for five days (1 g streptomycin and 1 200 000 IU benzylpenicillin; Polfa, Poland) and with analgesics for four days (metamizole sodium 50 mg/animal; Biovetalgin, Biowet Drwalew, Poland or meloxicam 1.5 mg/animal; Metacam, Boehringer Ingelheim, Germany).

Experimental design and drug treatments

Drugs. Salsolinol was synthesized and kindly provided by Prof. Ferenc Fulop from the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary. The compound was dissolved in Ringer-Locke's solution (RLs), aliquoted, and stored at –20°C. A new aliquot of the drug solution was used for each injection to maintain the biological activity of the molecule during the experiment.

Experimental procedures. The experiment was performed over three consecutive days: lactating sheep (n = 5) and one group of anestrous sheep (n = 5) received ICV injections of the vehicle (RLs), and the other group of anestrous sheep (n = 5) received analogical injections of salsolinol. During the first two days, injections were performed every 2 h, from 8:00 to 20:00, and on the third day from 8:00 to 14:00. A single dose of salsolinol was 5 µg/30 µl (daily 35 and 20 µg/animal), and was selected based on previous studies (Hasiec et al. 2014, 2015). The injections were performed using a microsyringe with an approximate flow rate of 10 µl/min. Additionally, on the third day, blood samples were collected every 15 min, from 8:15 to 14:30 h, through a catheter that was inserted into the jugular vein the day before the collection. Immediately after the last blood sampling, sheep were slaughtered. The brains were rapidly removed from the skull, and AP tissue was dissected, frozen in liquid nitrogen, and stored at –80°C until assay. The experimental schedule is presented in Figure 1.

Analytical techniques

Hormone concentration assay. The plasma ACTH concentration was determined using a commercial

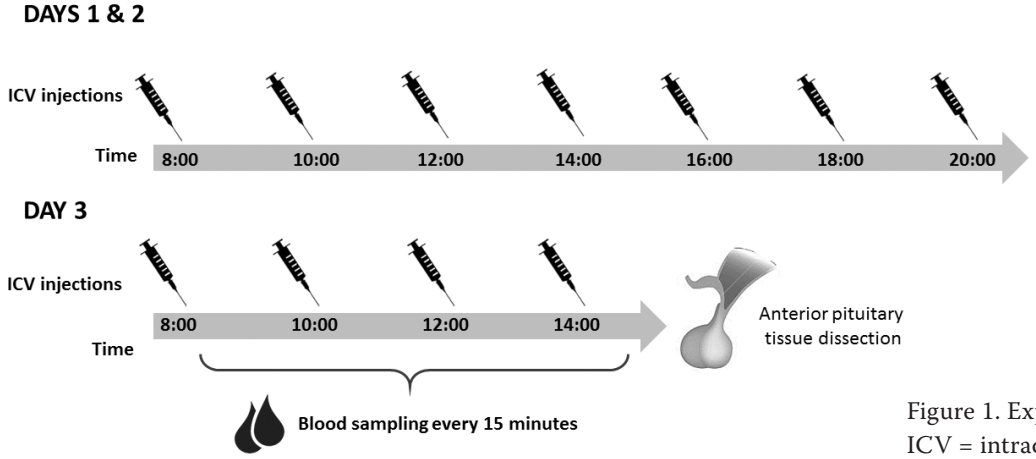

Figure 1. Experiment scheme
ICV = intracerebroventricular

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ELISA kit CSB-EQ027618SH (Cusabio, China) according to the manufacturer's protocol. The minimum detectable dose of ACTH was less than 1 pg/ml, and the intra-assay and inter-assay coefficients of variation (CVs) were < 15% and < 15%, respectively.

The plasma cortisol concentration was measured using a radioimmunoassay (RIA), according to Kokot and Stupnicki (1985) using rabbit anti-cortisol antisera (R/75) and a cortisol standard (Sigma-Aldrich, USA). The assay sensitivity was 0.95 ng/ml, and the intra- and inter-assay CVs were 10% and 12%, respectively.

The plasma prolactin concentration was assayed by the RIA double-antibody method, using anti-ovine prolactin and anti-rabbit γ-globulin antisera, as described by Wolinska et al. (1977). The prolactin standard was synthesized and kindly provided by Prof. Kazimierz Kochman from The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences. The assay sensitivity for prolactin was 2 ng/ml and the intra- and inter-assay CVs were 9% and 12%, respectively.

Relative gene expression assay. Total RNA from the AP tissue was isolated using TRIzol® Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The concentration and purity of the isolated RNA were determined with a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific). RNA integrity was verified with electrophoresis using 1.7% agarose gels containing ethidium bromide. Complementary DNA (cDNA) was synthesized using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Fisher Scientific) according to the manufacturer's instructions. For cDNA synthesis, 3 mg of total RNA was used in a reaction volume of 20 ml. Quantitative polymerase chain reaction (qPCR) was performed with 5 × HOT FIREPol® EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia). Each PCR reaction contained 2 µl cDNA template, 0.5 µl primers (0.25 µl each; 10 µM working concentration), 3 µl PCR Master Mix, and 9.5 µl H_2O. Reaction conditions were as follows: initial denaturation at 95°C for 15 min, denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s (35 cycles). Primers designed using the Primer3 software (http://primer3.ut.ee; Whitehead Institute, USA) are presented in Table 1.

Data were analyzed with the Rotor Gene 6000 v. 1.7 software (Qiagen, Germany) using the comparative quantification option. To compensate for variation in cDNA concentration and PCR efficiency among tubes, three endogenous control genes were assayed in each sample. In the experiment, *beta-2 microglobulin* (*B2M*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *peptidylprolyl isomerase C* (*PPIC*) genes were tested as housekeeping genes, and using the BestKeeper software (http://www.gene-quantification.de/bestkeeper.html), *B2M* was chosen as the best endogenous control gene to normalize gene expression in the study. The results are presented as relative gene expression of the target gene vs housekeeping gene (*B2M*), with the relative gene expression for the group of anestrous sheep that received vehicle-only infusion set to 1.0.

Table 1. Sequences of specific primers used in the qPCR and size of obtained amplicons

Gene	GenBank Acc. No.	Sequence 5'→3'	Amplicon size (bp)
POMC	NM_001009266.1	F: GTAACCTGCTGGCGTGCAT R: GAAGCTGCTGCTACCATTCC	167
PRL	NM_001009306.1	F: TGGCCAAGTTATTCCTGGAGC R: CTTGCTTGAATCCCTGCGC	142
B2M	NM_001009284.2	F: ATCCAGCGTATTCCAGAGGTC R: CTTCTCCCCGTTCTTCAGCA	135
GAPDH	NM_001190390.1	F: GGGTCATCATCTCTGCACCT R: GGTCATAAGTCCCTCCACGA	176
PPIC	XM_004008676.1	F: TGGAAAAGTCGTGCCCAAGA R: TGCTTATACCACCAGTGCCA	158

POMC = proopiomelanocortin, PRL = prolactin, B2M = beta-2 microglobulin, GAPDH = glyceraldehyde-3-phosphate dehydrogenase, PPIC = peptidylprolyl isomerase C (*cyclophilin C*), F = forward, R = reverse

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Statistical analysis. Mean hormone concentrations and relative gene expression data were analyzed using one-way analysis of variance (ANOVA) followed by the least significant differences *post hoc* test. Additionally, Student's *t*-test was applied in the case of *PRL* mRNA expression for comparison of only two groups: lactating sheep and anestrous sheep injected with vehicle-only.

All data are expressed as mean ± standard error of the mean.

RESULTS

Hormone concentration

ACTH. The mean concentration of ACTH in salsolinol-treated anestrous sheep was 237.69 ± 29.65 pg/ml, and was not significantly different from the ACTH concentration in anestrous sheep that received the injections of vehicle-only (289.65 ± 36.26 pg/ml). The mean concentration of ACTH detected in lactating sheep was 157.55 ± 15.24 pg/ml, and it was significantly lower ($P < 0.01$ and $P < 0.05$, respectively) than in anestrous sheep that received either the vehicle only or salsolinol injections.

Analysis of the hormone concentrations in two time intervals of sampling revealed that in all groups, ACTH concentrations were significantly ($P < 0.001$ in anestrous sheep, $P < 0.05$ in lactating sheep) higher during the first half of sampling compared with the second half (Figure 2A). During the first interval of sampling, anestrous sheep that received salsolinol injections had significantly lower ($P < 0.05$) ACTH concentration (355.96 ± 53.68 pg/ml) than anestrous sheep that received the vehicle only (464.93 ± 64.60 pg/ml). In lactating sheep, the mean ACTH concentration detected during the first half of sampling (223.8 ± 27.76 pg/ml) was significantly lower ($P < 0.001$ and $P < 0.05$, respectively) than in anestrous sheep that received

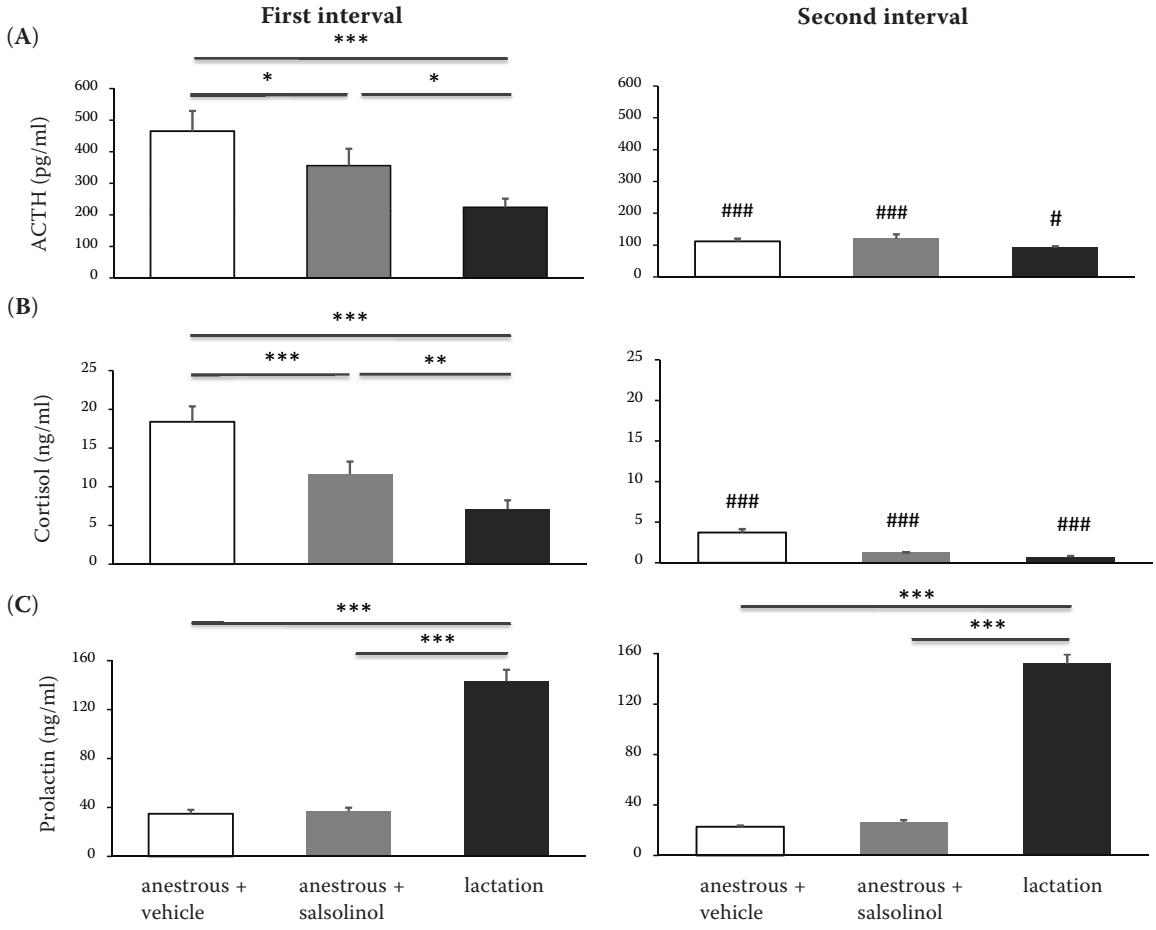

Figure 2. Mean ACTH (**A**), cortisol (**B**), and prolactin (**C**) concentrations in all experimental groups: anestrous + vehicle, anestrous + salsolinol, and lactation during two consecutive periods of the experiment

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ within time interval; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ between time intervals in the same group

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either the vehicle only or salsolinol. There were no significant differences in mean ACTH concentrations during the second half of sampling between all groups: sheep injected with the vehicle only (111.63 ± 8.96 pg/ml) or salsolinol (119.4 ± 14.8 pg/ml), and lactating sheep (91.29 ± 5.3 pg/ml).

The mean plasma ACTH concentration time profile is illustrated in Figure 3A.

Cortisol. The mean plasma concentration of cortisol in anestrous sheep that received the salsolinol injections was 6.45 ± 0.94 ng/ml, which was significantly lower ($P < 0.001$) than in anestrous sheep that received the vehicle-only, 11.11 ± 1.22 ng/ml. The mean plasma cortisol concentration detected in lactating sheep was 3.89 ± 0.66 ng/ml, i.e. significantly ($P < 0.001$) lower than in anestrous sheep that received the vehicle only, but it did not significantly differ from cortisol concentration in anestrous sheep that received salsolinol.

Analysis of the hormone concentrations in the two time intervals of sampling revealed that in all groups of sheep mean cortisol concentrations were significantly ($P < 0.001$) higher during the first half of sampling (Figure 2B). During the first half of sampling anestrous sheep that received salsolinol injections had significantly lower ($P < 0.001$) mean plasma cortisol concentration than anestrous sheep that received the vehicle only (11.62 ± 1.64 ng/ml vs 18.38 ± 2.02 ng/ml). The mean concentration of cortisol in lactating sheep during the first half of sampling (7.08 ± 1.19 ng/ml) was significantly lower ($P < 0.001$ and $P < 0.01$) than in anestrous sheep that received either the vehicle only or salsolinol injections. There were no significant differences in mean cortisol concentrations during the second half of sampling between all groups: sheep injected with the vehicle (3.72 ± 0.43 ng/ml) or salsolinol (1.28 ± 0.2 ng/ml), and lactating sheep (0.7 ± 0.14 ng/ml).

The mean plasma cortisol concentration time profile is illustrated in Figure 3B.

Prolactin. The mean plasma concentration of prolactin in anestrous sheep that received salsolinol injections was 31.9 ± 1.63 ng/ml, which was not significantly different from the prolactin concentration in anestrous sheep that received the vehicle-only injections (28.86 ± 1.79 ng/ml). The mean plasma concentration of prolactin detected in lactating sheep was 147.6 ± 6.03 ng/ml, which was significantly ($P < 0.001$) higher than in both groups of anestrous sheep.

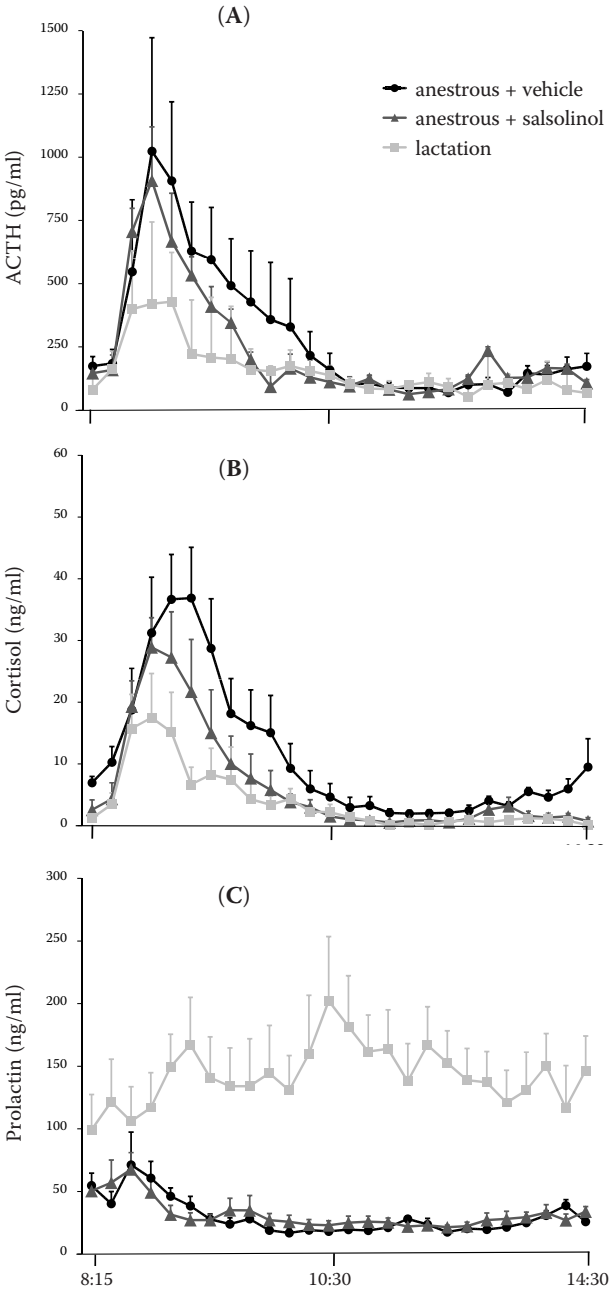

Figure 3. Mean ACTH (**A**), cortisol (**B**), and prolactin (**C**) concentrations in consecutive plasma samples in all experimental groups of sheep: anestrous + vehicle, anestrous + salsolinol, and lactation

Analysis of the hormone concentrations in the two time intervals of sampling revealed that there were no differences in prolactin concentration between the first and second halves of sampling for any group (Figure 2).

The mean plasma prolactin concentration time profile is illustrated in Figure 3C.

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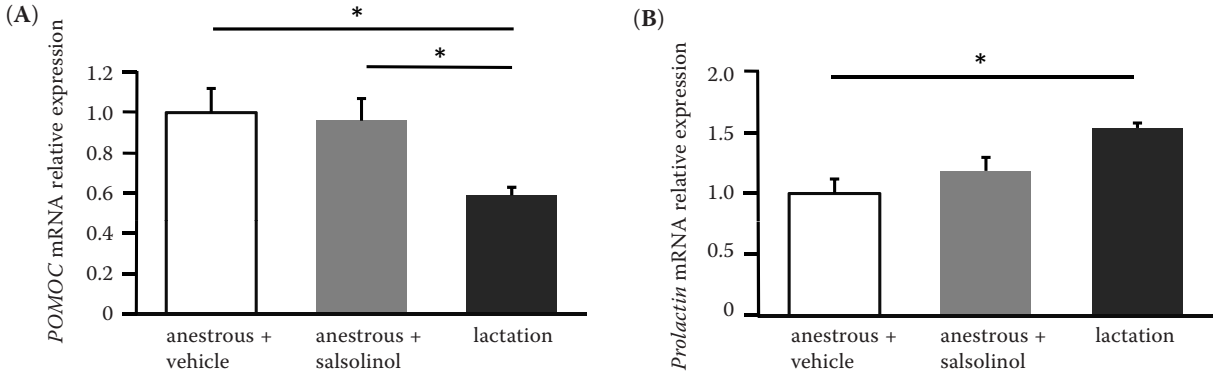

Figure 4. Relative *proopiomelanocortin* (*POMC*) (**A**) and *prolactin* (*PRL*) (**B**) mRNA expression within the anterior pituitary of sheep from all experimental groups: anestrous + vehicle, anestrous + salsolinol, lactation
*$P < 0.05$

Relative POMC and PRL mRNA expression

The relative expression of *proopiomelanocortin* (*POMC*) mRNA within the AP was significantly ($P < 0.05$) lower in lactating sheep than in anestrous sheep injected with the vehicle-only or salsolinol (Figure 4A). ANOVA revealed no significant difference in relative *prolactin* (*PRL*) mRNA expression between lactating sheep and anestrous sheep injected with the vehicle-only or salsolinol. However the Student's *t*-test revealed that *PRL* mRNA expression was significantly ($P < 0.05$) higher in lactating sheep than in anestrous sheep injected with vehicle-only (Figure 4B).

There were no differences in the relative amount of *POMC* and *PRL* mRNA in the AP between anestrous sheep that received the vehicle only or salsolinol (Figure 4A, B).

DISCUSSION

The inhibitory tone of salsolinol in respect to HPA axis activity has been demonstrated in lactating sheep (Hasiec et al. 2014, 2015), and the present study expands these findings to the anestrous period. The hormone concentration time profiles showed a clear response to stress in both anestrous and lactating sheep, which was manifested by increased ACTH and cortisol secretion during the first half of sampling. In addition, an increase in the plasma concentration of prolactin, which is known to be elevated during response to stressors (Freeman et al. 2000), was noted at the beginning of the sampling in anestrous sheep, although statistically insignificant. This stress response occurred likely due to handling, arising from blood collection. The greater stress response in anestrous ewes compared to the lactating ones confirms lower sensitivity of the HPA axis to the stressors in lactating animals (Altemus et al. 1995; Reeder et al. 2004; Tilbrook et al. 2006; Windle et al. 2013). In turn, lack of differences in plasma ACTH and cortisol concentrations among these animals during the second half of sampling suggests that basal HPA axis activity in anestrous and lactating sheep is similar, as it was observed in other studies (Kabaroff et al. 2006; Tilbrook et al. 2006). Despite the fact that stress was not intended, the experiment allowed to study the influence of salsolinol, not only on basal HPA axis activity, but mainly on the reaction to stress. The presented data show that centrally injected salsolinol is able to inhibit the hormonal stress reaction induced by handling in early anestrous sheep. Salsolinol reduces stress response of the HPA axis in lactating ewes induced by isolation from their offspring and flock (Hasiec et al. 2014). Because salsolinol has been found in the hypothalamus of lactating sheep, where its concentration increases in response to suckling (Misztal et al. 2008), it is highly probable that this compound mediates the inhibitory effect of suckling on the HPA axis response to stress. On the other hand, salsolinol did not influence ACTH or cortisol concentrations in the second half of sampling, suggesting no impact of salsolinol on basal HPA axis activity in anestrous sheep, differentially affecting lactating sheep (Hasiec et al. 2015).

Considering the physiological significance of salsolinol inhibition of the ACTH and cortisol

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response to stress in anestrous sheep, it should be noted that the presence of salsolinol within the hypothalamus has not been documented in these animals. A previous study showed a lack of extracellular salsolinol in the IN/ME of sheep 10 weeks after weaning (Misztal et al. 2008). However, the study was performed during May and June, when late anestrous sheep experience a predominant dopamine release within the mediobasal hypothalamus/median eminence (Misztal et al. 2011). Furthermore, salsolinol content has not been studied in the other hypothalamic areas of sheep, especially in the PVN, where corticotropin-releasing hormone and arginine vasopressin are synthetized for regulating hormonal response to stress (Herman et al. 2003). Nevertheless, it cannot be precluded that salsolinol is present in the various areas of the ovine hypothalamus to fulfil physiological functions in other states, besides lactation. Especially, because it was found to be present in the median eminence of ovariectomized rats (Toth et al. 2001).

Prolactin is considered to be a factor regulating stress-induced HPA axis activity (Cook 1997, Torner et al. 2001, 2002). However, high prolactin concentration in lactating sheep, and similarities in ACTH and cortisol concentrations between lactating and anestrous animals during the second half of sampling indicate that prolactin is not a major factor affecting basal HPA axis activity during lactation. Moreover, the injections of salsolinol into the IIIv of our early anestrous sheep failed to increase *PRL* gene expression and hormone secretion, thereby contradicting the possibility of prolactin mediation of the salsolinol effect on HPA axis activity. A lack of prolactin response to salsolinol is in contrast to the observation in late anestrous sheep, performed during the long photoperiod, in which salsolinol infusion into IIIv roughly doubled the mean plasma concentration of prolactin (Misztal et al. 2011). Interestingly, ICV infusions of salsolinol in these sheep diminished the extracellular concentration of dopamine in the IN/ME to an undetectable level, thereby triggering prolactin release. This discrepancy in prolactin response to salsolinol might result from variable seasonal regulation of prolactin secretion, in which the dopaminergic system and melatonin signal have predominant roles (Curlewis 1992). Under a short photoperiod, the long signal of melatonin keeps the prolactin secretion at a minimal level (Wood and Loudon 2014), but dopaminergic suppression of prolactin secretion increases in a long photoperiod because the prolactin-releasing response to an antagonist to the dopamine D2 receptor was smaller in an 8-hour photoperiod compared with a 16-hour photoperiod in goats (Yaegashi et al. 2012). Hence, this kind of stress, as well as salsolinol signalling within the central nervous system (CNS) was insufficient to evoke clear changes in the prolactin secretion in early anestrous sheep. Similarly, in goats, the prolactin-releasing response to salsolinol was considerably lower under a short photoperiod (Yaegashi et al. 2012). Hashisume et al. (2013) showed that daily treatment with melatonin during a long day photoperiod greatly reduced the prolactin-releasing response to salsolinol. Another reason for the lack of prolactin response to salsolinol in our study might have been a "stress effect", which prevented the prolactin-releasing effect of salsolinol, as indicated by a study on lactating sheep, where ICV infusion of salsolinol inhibited ACTH and cortisol response to stress without increasing the plasma prolactin concentration (Hasiec et al. 2014). Although the plasma concentration of this hormone remained at a relatively high level, typical for lactation, the modulation of the HPA axis by salsolinol most likely occurred without the participation of the plasma prolactin (Hasiec et al. 2014). Nevertheless, the anestrous sheep may be used to examine other potential physiological effects of salsolinol within the CNS, which could be independent of the effect on the plasma prolactin concentration.

Considering the mechanism by which salsolinol affects the plasma ACTH and cortisol concentration, we also measured *POMC* mRNA expression. Comparing the relative genes expression between anestrous and lactating sheep that received vehicle-only injections, a decrease in *POMC* mRNA levels was noticeable in lactating ones. Nevertheless, the lower *POMC* mRNA expression could partially explain lower hormonal response to stress in lactating sheep. To our knowledge, there has been no other research on this topic for sheep. Interestingly, in rats there is no influence of lactation on the *POMC* mRNA expression in the pituitary (Kim et al. 1997). Further, our study showed that salsolinol injections into the IIIv did not change *POMC* mRNA levels. This excludes the possibility that the effect of salsolinol on plasma ACTH concentration resulted from reduced *POMC* mRNA level within the corticotrophs.

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Although the central mechanism of HPA axis regulation is beyond the scope of this study, it should be considered as the most probable mechanism of salsolinol action. Our previous research indicated that suppression of hormonal responses to stress in lactating sheep might result from the inhibitory effect of salsolinol on the noradrenergic system (Hasiec et al. 2014). Noradrenaline is considered to be the one of main neurotransmitters regulating HPA axis activity within the PVN, an integrative centre for converting inputs from various brain regions (Herman et al. 2003). It has been observed that stress increases noradrenaline release, reuptake, metabolism, and synthesis within the PVN in rats (Pacak et al. 1992) and noradrenaline concentration in the cerebrospinal fluid in sheep (Torner et al. 2002). Moreover, mother's adaptations in the HPA axis during lactation are caused by changes in noradrenergic system activity within the CNS; lower basal release of noradrenaline within the PVN was detected in lactating rats compared to virgin ones (Toufexis et al. 1998). Likewise, non-reduced HPA axis responses to stress by neurotoxic lesions on the noradrenergic input to the PVN were observed in lactating rats but not in virgin rats (Toufexis and Walker 1996). Finally, salsolinol inhibits the activity of the rate-limiting enzyme of catecholamine biosynthesis, tyrosine hydroxylase (Briggs et al. 2013). Hence, it is possible that salsolinol could be a factor inhibiting the HPA axis stress response in sheep by regulation of the central noradrenergic system activity. However, further research, which will focus on salsolinol action on the noradrenergic system within the PVN, is needed.

CONCLUSION

Salsolinol injected into IIIv inhibits ACTH and cortisol response to handling stress in early anestrous sheep. However, this compound affects the release of ACTH rather than its synthesis. Moreover, peripheral prolactin does not mediate the presented inhibitory action of salsolinol. These observations provide further evidence for the role of salsolinol in the inhibition of HPA axis activity in sheep, but more complex investigations at the central nervous system level are necessary to understand the mechanism controlling ACTH release in sheep.

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