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Up-regulation of Oxytocin Receptor Gene and Protein in the Sheep Anterior Pituitary by a Dopamine Derivative (Salsolinol)

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ABSTRACT

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Specific oxytocin receptors (OTR) have been identified in the anterior pituitary (AP), and their expression has been shown to change in relation to the animal physiological stage, whereas salsolinol (a derivative of dopamine) has been shown to stimulate the synthesis and release of oxytocin (OT) in lactating sheep. In the present study, the expression of both *OTR* mRNA and OTR protein in the AP were examined by real-time quantitative PCR and enzyme-linked immunosorbent assay, either in anestrous or lactating sheep 48 h after weaning lambs. Moreover, the effect of salsolinol administered via an intracerebroventricular (i.c.v.) infusion was tested in additional sheep at the same physiological stages. The i.c.v. infusions of Ringer-Locke (control) and salsolinol solutions were carried out from 10:00 to 15:00 h in a serial manner, i.e. five 30-min infusions at 30-min intervals. We observed both *OTR* gene and OTR protein expression in the AP, in both anestrous and lactating sheep, but it was significantly ($P < 0.01$ and $P < 0.05$, respectively) higher in the AP of lactating animals compared to anestrous animals. Salsolinol i.c.v. treatment in anestrous sheep evoked a significant ($P < 0.05$) increase in both *OTR* gene and OTR protein expression compared to control animals. In contrast, salsolinol did not affect either *OTR* gene or OTR protein expression in lactating sheep. In conclusion, the expression of OTR in the sheep AP is upregulated by salsolinol. The effect of salsolinol was more pronounced in non-lactating sheep, with a reduced response due to ongoing OTR expression in lactating animals. Increased expression of OTR in the AP of lactating sheep may be related to the stimulation of pituitary lactotrophs by OT following the release of prolactin during suckling.

Keywords: oxytocin; sheep; lactation

The peptide oxytocin (OT) is synthesized in the magnocellular neurosecretory cells of the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, and is stored in the axon terminals located in the posterior pituitary (Gimpl and Fahrenholz 2001; Bealer et al. 2010; Kochman 2013). Activa-

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tion of these hypothalamic neurons leads to OT release into the circulation where it is transported to different peripheral tissues (Gimpl and Fahrenholz 2001). OT released from centrally projecting oxytocinergic neurons forms the active pool of the hormone in the brain (Sabatier et al. 2007). Its actions are mediated by a specific oxytocin receptor (OTR), which is a G protein-coupled receptor. OT has been extensively studied for its numerous peripheral and central functions; the most well-known are inducing uterine contractions during parturition and milk ejection during lactation, as well as its effects on sexual, feeding, maternal, and social behaviours. Indeed, released under the influence of suckling stimulus, OT is essential for the milk ejection reflex, as it acts on myoepithelial cells of the alveoli to induce the movement of milk from the place of storage to the nipple (Marnet et al. 1998).

Evidence exists that OTR are present in the anterior pituitary (AP), and their expression increases during gestation (Breton et al. 1995; Zingg and Laporte 2003). One of the best documented OT actions in this tissue is the stimulation of prolactin (PRL) release (Samson and Schell 1995; Kennett and McKee 2012). During lactation, the OT surge induced by suckling is accompanied by a PRL surge (Kennett and McKee 2012). Kennett et al. (2009) have shown that intravenous administration of an OT antagonist attenuates the PRL surge induced by the suckling stimulus.

In recent years, salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) has emerged as an important neural factor regulating the hormones involved in lactation. As a dopamine derivate, salsolinol has been generally associated with dysfunction of dopaminergic neurons, though presently it is also considered to be a physiological stimulator of PRL release in rodents (Toth et al. 2001) and ruminants (Hashizume et al. 2008; Misztal et al. 2008). In lactating sheep, the suckling stimulus increases the extracellular content of salsolinol in the infundibular nucleus-median eminence, simultaneously with PRL release into the peripheral circulation (Misztal et al. 2008). Intracerebroventricular (i.c.v.) infusion of salsolinol increases PRL release (Gorski et al. 2010), and stimulates *PRL* gene expression in the AP of lactating sheep (Hasiec et al. 2012). Moreover, an i.c.v. infusion of a salsolinol analogue, 1-methyl-3,4-dihydroisoqinoline (1-MeDIQ) in sheep reduces basal PRL release and attenuates the suckling-induced PRL surge (Gorski et al. 2010; Misztal et al. 2010). Our recent studies have shown that salsolinol may also be an OT stimulating factor during lactation. Salsolinol, when i.c.v. administered in lactating sheep, upregulated OT gene expression in the PVN and SON, increased OT peptide content in the posterior pituitary, and stimulated OT release into the peripheral circulation (Gorski et al. 2016).

To further extend our research on the relationship between salsolinol and OT, in the present study, we have determined the changes in *OTR* gene and protein expression in the AP by comparing anestrous and lactating sheep. Additionally, we have investigated the effect of salsolinol administered via i.c.v. infusion on OTR expression.

MATERIAL AND METHODS

Animal management. All experimental procedures were conducted in accordance with the Polish Guide for the Care and Use of Animals and approved by the Local Ethics Committee. The experiment was performed on two groups of Polish Longwool sheep: Group 1, anestrous sheep (n = 12), and Group 2, lactating sheep, 48 h after the weaning of 8-week-old lambs (n = 12). Sheep in Group 2 were mated naturally in September and lambed during the following February. All animals were maintained indoors in individual pens under natural lighting conditions (52°N, 21°E). They were fed twice a day a diet formulated for non-pregnant or pregnant and lactating sheep, according to the recommendations of the National Research Institute of Animal Production, Krakow, Poland (1993 norms), hay and water were available *ad libitum*. Sheep were well-adapted to the experimental conditions, and always had visual contact with neighbouring sheep, even during the experimental period, to prevent stress as a result of social isolation.

Brain surgery. Anestrous and pregnant (third month of pregnancy) sheep were implanted with a stainless steel guide cannula (outer diameter 1.4 mm) into the third ventricle of the brain. The implantation was performed under general anesthesia (a mixture of xylazine 40 mg/kg body weight, and xylapan and ketamine 10–20 mg/kg body weight, administered intravenously; all Biowet, Poland), through a drill hole in the skull, in

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accordance with the stereotactic coordinate system for the sheep hypothalamus (Welento et al. 1969), using the procedure described by Traczyk and Przekop (1963). The guide cannulae were fixed to the skull with stainless steel screws and dental cement Villacryl S (Zhermapol, Poland). The external opening of the cannula was closed with a stainless steel cap. The placement of the cannula was confirmed during surgery by the outflow of the cerebrospinal fluid, and postmortem by the injection of blue ink.

Drug treatment and experimental design. Salsolinol was synthesized and kindly provided by Prof. Ferenc Fülöp from the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged, Hungary. It was dissolved in Ringer-Locke's solution (RL), then aliquoted and stored at –20°C. A new aliquot of the drug solution was used for each infusion to maintain the biological activity of the molecules during the experiment.

Both anestrous and lactating sheep were randomly divided into two groups: control ($2 \times n = 6$) and experimental ($2 \times n = 6$), and were given an i.c.v. infusion of RL or salsolinol solution, respectively. The infusions were carried out from 10:00 to 15:00 h in a serial manner (five 30-min infusions at 30-min intervals) using a BAS Bee™ microinjection pump and calibrated 1.0-ml gastight syringes (Bioanalytical Systems Inc., USA). The salsolinol dosage (5 × 15 µg/60 µl/30 min) was selected based on our previous studies (Gorski et al. 2010; Hasiec et al. 2012). Immediately after the end of the experiment (15:00 h) sheep were deeply anaesthetized with sodium pentobarbital (30 mg/kg body weight; Biowet) and euthanized by decapitation. The AP was rapidly dissected, immediately flash frozen in liquid nitrogen, and stored at –80°C until assayed.

Gene expression assay. Tissue samples were homogenized using a TissueLyser LT bead mill (Qiagen, Germany). Total RNA was extracted and purified using a NucleoSpin RNA II kit (Macherey-Nagel GmbH & Co., Germany) with on-column DNase treatment, in accordance with the manufacturer's instructions. The total RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). Sample integrity was assessed electrophoretically using ethidium bromide-stained 1.5% agarose gels. Total RNA (1 g in a volume of 20 l) was reverse-transcribed into first-strand cDNA using a Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions.

Relative amounts of specific *OTR* transcripts were determined by real-time quantitative PCR (qPCR) analysis, using a Rotor-Gene Q cycler (Qiagen). *OTR* mRNA analysis was performed using the Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific) and HPLC-grade oligonucleotide primers (Genomed, Poland). Specific primers for determining the expression of the gene of interest and endogenous control genes were designed with Primer3 software (Untergasser et al. 2012). The qPCR reactions were run in triplicate using the following protocol: initial denaturation and enzyme activation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, and extension at 72°C for 20 s. After the cycles, a final melting curve analysis with continuous fluorescence measurements was performed to confirm the specificity of the amplification. To confirm that single amplification products were produced, samples were stained with ethidium bromide and electrophoresed on an agarose gel before visualization under a UV light camera. The *OTR* mRNA levels were normalized to the endogenous control gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Initially, three housekeeping genes – *GAPDH*, *β-actin* (*ACTB*), and *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ*) – were tested. The BestKeeper software (Pfaffl et al. 2004) was used to determine the most stable housekeeping gene for normalizing the expression of genes of interest. All primers are listed in Table 1.

ELISA protein quantification. Frozen AP was homogenized in ice cold phosphate-buffered saline (pH 7.4) (Sigma-Aldrich, USA) using the TissueLyser LT bead mill. Afterwards, samples were frozen at –20°C and stored overnight. After two freeze-thaw cycles, the homogenates were centrifuged at 5000 *g* for 5 min at 4°C, then supernatant fluids were removed and assayed immediately. The concentration of OTR protein in the AP extracts was determined with a commercial ELISA kit (Sheep OTR ELISA Kit; Mybiosource, USA). The assay sensitivity was 0.094 ng/ml, and both intra- and inter-assay variability was 10%. The absorbance measurement at 450 nm was performed using a Spectramax M2 microplate reader (Molecular Devices LLC, USA). The obtained results were

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Table 1. Sequences of the primers used in the qPCR analysis

Gene	GenBank Acc. No.	Primer sequence
OTR	NM_001009752	F: TCAGCAACGTCAAGCTCATC R: TCCACATCTGCACGAAAAA
GAPDH	NM_001034034	F: AGAAGGCTGGGGCTCACT R: GGCATTGCTGACAATCTTGA
ACTB	NM_001009784	F: CTTCCTTCCTGGGCATGG R: GGGCAGTGATCTCTTTCTGC
YWHAZ	NM_001267887	F: AGACGGAAGGTGCTGAGAAA R: GAAGCGTTTGGGATCAAGAA

F = forward primer, R = reverse primer

normalized to the total protein concentration in samples, assayed with the Bradford reagent (Sigma-Aldrich).

Data analysis. RotorGene Q software (Qiagen) was used to generate and collate the qPCR data. The relative gene expression was determined using the Relative Expression Software Tool 2008 (Qiagen). The results are presented as the relative gene expression of the target gene compared with the endogenous control gene. The average relative quantity of gene expression in the control group was set to 1.0.

The non-parametric Mann-Whitney U test was used to assess the significance of the differences in *OTR* mRNA and OTR protein expression between the experimental and control groups. Calculations were performed using STATISTICA software (Version 10). All data are expressed as mean ± SEM.

RESULTS

The analyses revealed the presence of specific OTR transcripts and OTR protein in the AP of both anestrous and lactating sheep. *OTR* mRNA expression was up-regulated ($P < 0.001$) in lactating sheep when compared to the AP of anestrous sheep (Figure 1A). Similarly, the OTR protein content in the AP of lactating sheep was increased ($P < 0.05$) compared to the levels in anestrous animals (Figure 1B).

Salsolinol infused into the third ventricle of the brain up-regulated ($P < 0.01$) *OTR* mRNA expression in the AP of anestrous sheep. However, in lactating sheep, treatment with salsolinol was ineffective (Figure 1A). Salsolinol administered via i.c.v. infusion also evoked a significant ($P < 0.05$) increase in the OTR protein content in the AP of

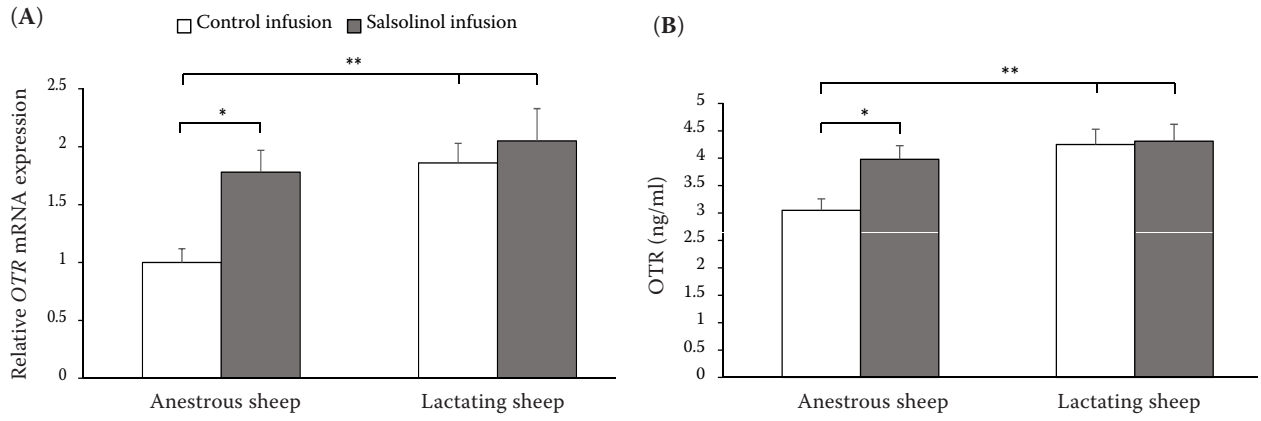

Figure 1. Relative oxytocin receptor (*OTR*) mRNA expression (**A**) and oxytocin receptor (OTR) polypeptide expression (**B**) in the anterior pituitary of anestrous sheep ($n = 12$) and lactating sheep ($n = 12$) infused with vehicle (control; white bars) or salsolinol (grey bars). Data are presented as mean ± SEM

$^{**}P < 0.01$, $^{*}P < 0.05$ according to the non-parametric Mann-Whitney U test

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anestrous sheep, while in lactating animals, OTR protein expression was not significantly changed in response to salsolinol (Figure 1B).

DISCUSSION

The present study demonstrates the variations in *OTR* gene expression and OTR protein concentrations in the sheep AP, which were higher during lactation than in seasonal anestrus. Moreover, the dopamine derivative salsolinol, which is a stimulatory agent for the release of both PRL (Gorski et al. 2010) and OT (Gorski et al. 2016) in lactating sheep, also up-regulated the OTR contents in the AP, which were higher during lactation than during seasonal anestrus.

The central sites of OT synthesis are localized in the hypothalamic PVN and SON. Although most of OT neurons project into the posterior pituitary, where the neurohormone is stored and released into the circulation, OT also reaches the anterior pituitary through the short portal vessels connecting these two parts (Gibbs 1984; Samson and Schell 1995). Additionally, magnocellular oxytocinergic neurons innervate the median eminence, where they can act on the anterior pituitary (Yang et al. 2013). OTR immunoreactivity was found in the rat AP (Adan et al. 1995), and early studies showed that most OTR are present in lactotrophic cells (Breton et al. 1995) where OT plays an important role in stimulating PRL secretion (Samson et al. 1986). In addition, OT is involved in stimulating ACTH release from rat pituitary cells (Schlosser et al. 1994), and the *OTR* gene has also been found in mouse AP corticotrophs (Nakamura et al. 2008). Recently, Gonzales-Iglesias et al. (2015) provided evidence that gonadotrophs and somatotrophs, in addition to lactotrophs, are sensitive to OT stimulation, and responded with transient elevations in the intracellular Ca^{2+} concentration and hormone release in a dose-dependent manner. These responses were also evoked by an OTR agonist, and abolished by an OTR antagonist, suggesting that the OT-induced effects are mediated by OTRs in all three cell types (Gonzalez-Iglesias et al. 2015). It has been suggested that OT may act directly on different AP cell types to coordinate hormone release.

The increased OTR expression in the AP during lactation seen in our study may mediate the biochemical adaptations of the pituitary cells, especially lactotrophs, to facilitate an increase in the PRL synthesis and secretion. Several groups have identified the ability of OT to act directly on AP lactotrophs to release PRL (Samson and Schell 1995; McKee et al. 2007; Tabak et al. 2010). McKee et al. (2007) demonstrated that OT action at the lactotroph is required for PRL surges in cervically stimulated ovariectomized rats. According to Samson et al. (1986), the OT surge precedes that of PRL in response to suckling. Moreover, attenuation of OT action by the administration of a selective OT antagonist (desGly-NH_2-d(CH_2)5[D-Tyr2,Thr4] OVT) is able to block the suckling-induced PRL surge (Kennett et al. 2009). These data further support the thesis that OT stimulates PRL release from AP lactotrophs, and underline the importance of this phenomenon for lactation.

The salsolinol stimulatory effect on both *OTR* mRNA and OTR protein expression in the sheep AP was shown for the first time in this study, and extends our research. Interestingly, *OTR* mRNA levels as well as protein contents reached similar values in anestrous sheep as those seen in lactating animals. Although salsolinol was not detected in seasonally anestrous sheep (Misztal et al. 2008), the increase in OTR in the pituitary cells as a result of salsolinol treatment may indicate their preparedness for the signals associated with lactation. In turn, salsolinol administered via i.c.v. infusion in lactating sheep only slightly enhanced *OTR* mRNA expression. We assume that the level of OTR expression during lactation is so high that additional stimulating factors cannot evoke a further increase in expression. In contrast, a significant upregulation of *PRL* mRNA expression in the AP after i.c.v. treatment with salsolinol was evident 48 h after lamb separation (Hasiec et al. 2012).

Based on available data, the proper mechanism of salsolinol action on OTR expression is difficult to determine. Although a specific salsolinol receptor has not been identified, high-affinity binding sites for salsolinol have been detected in the median eminence, hypothalamus, and AP (Toth et al. 2001; Homicsko et al. 2003). It has also been shown that OTRs are highly upregulated during parturition and lactation. Gonadal steroids are thought to be the key factors mediating this regulation. In the AP, a marked increase in OTR expression was observed after estrogen treatment in ovariectomized female rats (Breton and Zingg 1997; Quinones-Jenab et

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al. 1997). The *OTR* promoter sequence contains elements of the estrogen response element (ERE); however, it is not a completely functional ERE (Gimpl and Fahrenholz 2001). Estrogen modulation probably involves additional factors, and might be mediated via other functional promoter elements (Zingg and Laporte 2003). The sequence analysis of the rat *OTR* gene revealed the presence of cAMP response elements (CRE). Some data suggest that cAMP may induce the up-regulation of *OTR* gene expression. Treatment with forskolin, a compound that elevates the intracellular cAMP concentration, increases OT binding and *OTR* mRNA expression in rabbit amnion cells *in vitro* (Jeng and Soloff 2009). Additionally, dibutyryl-cAMP has been shown to increase *OTR* gene expression *in vitro* (Ivell et al. 2001). In turn, salsolinol has been shown to be a stimulator of cAMP accumulation in the AP (Radnai et al. 2005). Regarding these data, it is assumed that salsolinol may induce *OTR* gene expression in pituitary cells via the activation of intracellular cAMP. Further studies are required, especially concerning the identification of specific salsolinol receptors and the intracellular mechanism of its action.

In conclusion, the expression of OTR in the sheep AP is upregulated by salsolinol. The effect of salsolinol may be more pronounced in non-lactating sheep, with a less robust response observed with ongoing OTR expression in lactating animals. The increased OTR expression in the AP of lactating sheep may be related to the stimulation of pituitary lactotrophs by OT and the release of PRL during suckling.

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