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Short communication

The interactions of season, leptin and melatonin levels with thyroid hormone secretion, using an in vitro approach

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ABSTRACT

Leptin's actions on the hypothalamic–pituitary–thyroid axis occur both centrally and peripherally. In addition, the thyroid activity may also be stimulated by melatonin. The mechanisms through which leptin, melatonin (MT) and the thyroid hormones co-operate in the regulation of the seasonal physiology in sheep, however, remains unclear. The aim of the study was to evaluate the interactions of season, leptin and melatonin levels with thyroid hormone secretion in sheep, using an in vitro approach. Thyroid glands from 18 ewes were collected during long-day (LD, $n = 9$) and short-day (SD, $n = 9$) photoperiods. The glands were dissected into approximately 30 mg samples. Each sample was equilibrated in RPMI 1640 (Roswell Park Memorial Institute 1640) + F12 (Nutrient Mixture F-12) medium (1/1) for 90 min, followed by an additional 3 h incubation. At the end of the equilibration period, all tissue samples were treated with a medium containing either 0 or 50 ng/ml leptin, with or without MT (40 ng/ml) and a medium containing MT (40 ng/ml) only. Secretions of thyroxine (T4) and triiodothyronine (T3) were higher ($P < 0.05$) in the control group during the short-day (SD) period, compared to the long-day (LD) period. Melatonin tented to reduce the T4 secretion during the LD and SD periods. However, leptin stimulated ($P < 0.001$) T4 secretion in both seasons. Leptin, MT, and both leptin and MT increased the secretion of T3 during LD period ($P < 0.001$). Results demonstrated leptin to have a stimulatory effect on T3 and T4 secretion in sheep, however the effect of melatonin is dependent on the season.

1. Introduction

Biological rhythms of seasonal species regulate the annual changes in feed intake, body weight and reproductive ability. The main secretory product of the pineal gland is the hormone melatonin (N-acetyl-5-methoxytryptamine, MT) and it is known to be the principal inner biological timer for the circadian and circannual activities to co-ordinate the reproductive cycles of seasonal breeders (Morgan et al., 2003). There are however a growing number of studies indicating that in seasonal breeders, several hormones, in addition to melatonin (MT) play a role in regulating the synchronization of the seasonal cues. Seasonal sensitivity to leptin has been demonstrated in sheep and it appears to serve as a mechanism of adaptation to feed availability. Recently, it was reported that leptin is able to act directly on pineal gland tissue (Zieba et al., 2008), and it was found that leptin modulates MT secretion in vivo, following central infusions (Zieba et al., 2007). The relationship between these two hormones however seems to be species-specific. The effects of leptin on MT-release are also different, depending on the route of administration and the dose utilized (Zieba et al., 2007, 2008).

Several laboratories have considered the roles of the thyroid hormones in the control of seasonal reproduction. Triiodothyronine (T3) and thyroxine (T4) appear to play

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critical roles in the transition between the natural breeding and anestrous seasons in ewes (Karsch et al., 1995). Thyroidectomized sheep failed to undergo the transition from the breeding to non-breeding season, and showed almost the same pulse release pattern of GnRH during anestrus, as during the breeding season (Webster et al., 1991). Yasuo et al. (2006) demonstrated male Saanen goats, a short-day breeder, that long-day suppressed the expression of the type 2 deiodinase (Dio2) gene in the mediobasal hypothalamus – the target of both MT and T4 activity. Leptin was further shown to stimulate the pro-TRH neurons through direct or indirect pathways in the region of the hypothalamic paraventricular nucleus – PVN (Perello et al., 2006). The current study was designed to test the hypothesis that leptin and MT act independently, but also interact to regulate the thyroid activity in seasonally-breeding sheep, using an in vitro approach.

2. Materials and methods

2.1. Animals and treatment

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

Eighteen multiparous Polish Longwool ewes, a breed that exhibits strong seasonal reproduction activities (Zieba et al., 2000), were used in this study. Animals were 2–3 years of age and had a mean body weight of 60 ± 5 kg. All animals were housed in individual pens under natural photoperiodic and thermoperiodic conditions (longitude: 19°57′E, latitude: 50°04′N) and had a BCS of 3 (on a scale from 0 to 5; where O = emaciated and 5 = obese; Russel et al., 1969). Ewes were fed twice daily at 07:00 and 16:00 with a diet formulated to supply 100% of the nutritional requirements according to the National Research Institute of Animal Production recommendations, for maintenance (Norms, 1993). Water was available *ad libitum*.

Sheep were sacrificed by exsanguination following captive bolt stunning. The thyroid glands were aseptically dissected from the ewes 10–15 min post mortem. The glands were collected from 9 ewes chosen randomly during the long days (LD; March, April and May) and from an additional 9 ewes during the short days (SD; September, October and November). The thyroid lobes were placed on ice and transported to the laboratory and all subsequent procedures were performed under sterile conditions.

2.2. Thyroid tissue cultures

Before incubation, the thyroid lobes were washed 3 times in Hank's Balanced Salt Solution (HBSS; Laboratory of Vaccines, Lublin, Poland). The thyroid glands collected from each ewe (3 ewes per month) were then dissected and sliced sagittally into samples of approximately 2.0 × 4.0-mm (30 mg). Incubations were carried out in a 6-well Corning tissue culture dish (Corning Glass Works, New York, USA), in a gas-liquid interface. Randomly allotted thyroid samples was placed on a microporous filter, supported on a stainless steel grid (Trowell, 1959). The stainless steel grid was then immersed in a 2.5 ml incubation buffer, composed of Roswell Park Memorial Institute-1640 medium (RPMI 1640) + L-glutamine, $NaHCO_3$ (RPMI 1640, Laboratory of Vaccines, Lublin, Poland) and a F12 Nutrient Mixture (Ham) + L-glutamine, 1:1 (F12, Invitrogen Ltd, Paisley, UK), with 0.5% fetal calf serum (FCS; Laboratory of Vaccines, Lublin, Poland). Incubation was carried out in 95% humidified air and 5% CO_2 atmosphere, at 37 °C, for a total period of 4.5 h. After a 90-min period of equilibration, all thyroid tissue samples were treated 3 times. Samples were either treated with 0 (control), or 40 ng/ml melatonin (MT; Sigma Chemical Co., St. Louis, MO, USA) or 50 ng/ml recombinant ovine leptin (Lep; Ray Biotech, Inc., Norcross, GA, USA) or both hormones. Samples were then incubated for another 3 h, according to Zieba et al. (2007). Each treatment group consisted of 6 replicates. The dose of leptin was selected, based on previous studies (Zieba et al., 2003). Thus, the treatments following the 90-min equilibration period were: (1) control; (2) Lep, leptin; (3) MT, melatonin; (4) MT + Lep; a combination of both treatments (groups 2 and 3). One ml of culture medium was harvested every 30 min from each experimental group, and replaced with a fresh medium. This harvesting and replacing procedure continued for a total of 3.0 h. Samples were stored at -20 °C for RIA of T3 and T4.

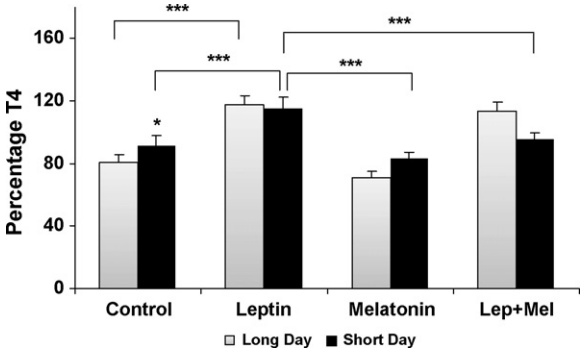

Fig. 1. Mean (± SEM) thyroxine (T4) concentrations in culture media after Mel (melatonin), leptin, and combined leptin and Mel (Lep + Mel) treatment during LD (long-day) and SD (short-day) periods. $^{*}P < 0.05$, $^{***}P < 0.001$.

2.3. Radioimmunoassay (RIA)

Tissue concentrations of T4 and T3 were determined by RIA, using a commercial T4 or T3 RIA kit (B.R.A.H.M.S. T4 RIA, Berlin, Germany). After completion of the hormonal assay, concentrations were recorded in a 1470 Wizard gamma counter (PerkinElmer, Germany). The sensitivity of the assay for thyroxine was less than 9.8 ng/ml. The intra and interassay coefficients of variation were 6 and 7%, respectively. Sensitivity of the assay for T3 was 97.6 pg/ml and the intra and interassay coefficient of variation was 4.5 and 8%, respectively.

2.4. Data analysis and statistical tests

Thyroid hormone concentrations in the culture media were analyzed for repeated measurements using the PROC MIXED procedures (SAS 9.1; SAS Institute Inc., Cary, NC, USA). The statistical model included: treatment as a random effect, treatment and its interaction with time, and season as fixed effects. As the effect of season on T3 and T4 was significant ($P = 0.001$ for T4 and $P < 0.001$ for T3, respectively), separate analyses for T3 and T4 were performed. Following determinations, a significant *F*-value for the treatment × time interaction, means at each time point were separated using PROC PDIFF, corrected by the Tukey method. Mean concentrations of each hormone for the whole experimental period were subjected to a one-way analysis of variance using PROC GLM. Due to random differences in the T3 and T4 concentrations between groups at the onset of the experiments, hormonal values were converted to a percentage of time 0 values. The Tukey's test was used whenever it was appropriate to separate means.

3. Results

3.1. Tissue secretion of thyroxine (T4)

In the control group, the mean concentrations of T4 were approximately 10% higher ($P < 0.05$) during the SD (short-day), than during the LD (long-day) period. The addition of leptin to the culture medium, with (not significant) or without ($P < 0.001$) melatonin, increased the T4 secretions, when compared to the controls – during both the LD and SD (Fig. 1) periods. Melatonin as such had no effect on the T4 secretion during both the LD and SD, periods when compared to the control group (Fig. 1). In the MT (melatonin) group a decrease in T4 secretion was recorded ($P < 0.001$), compared to cultures treated with leptin during