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Localization and Role of Leptin in the Thyroid Gland of the Lizard *Podarcis sicula* (Reptilia, Lacertidae)

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ABSTRACT Leptin, the product of the ob gene, is a hormone secreted by adipocytes that regulates food intake and energy expenditure. The hypothalamus–pituitary–thyroid axis is markedly influenced by the metabolism status, being suppressed during food deprivation. The present study was designed to ascertain whether (1) lizard thyroid gland expresses the long form of leptin receptor (Ob-Rb) and (2) the leptin administration affects the thyroid gland activity in this species (and to verify whether leptin plays a similar role in reptiles as observed in the other vertebrates). The presence of leptin receptor in the thyroid gland of *Podarcis sicula* was demonstrated by immunohistochemical technique (avidin–biotin–peroxidase complex—ABC method). The role of leptin in the control of thyroid gland activity was studied in vivo using light microscopy (LM) technique coupled to a specific radioimmunoassay for thyroid-stimulating hormone (TSH) and thyroid hormones (T4 and T3). Leptin (0.1 mg/100 g body wt)/day increased T4 and T3 release for 3 days but decreased the plasma concentration of TSH; using LM clear signs of stimulation in the thyroid gland were observed. These findings suggest that systemic administration of leptin stimulates the morphophysiology of the thyroid gland in the lizard through a direct mechanism involving Ob-Rb. *J. Exp. Zool. 303A:628–634, 2005.*

Leptin is a 16-kDa protein hormone originally identified as the product of the obese gene (ob) expressed in the adipose tissue of mammals (Zhang et al., '94). Leptin is expressed proportionally to the fat mass, and is important in controlling the amount of fat stored by inhibiting appetite (for reviews see Flier, '98; Houseknecht et al., '98). Leptin travels, bound to a carrier protein, through the bloodstream and acts via a feedback mechanism that operates at the level of the hypothalamus, where it binds to an Ob-R receptor (Tartaglia et al., '95) and inhibits neuropeptide Y (NPY) release. Since NPY is known to stimulate appetite, the final leptin effect is to decrease food intake. It is now accepted that NPY is an important neuroendocrine target for leptin (Stephens et al., '95; Mercer et al., '96; Schwartz et al., '96). Leptin exerts its effects on different endocrine axes and in particular on the hypothalamic–pituitary–thyroid axis (Wauters et al., 2000). Leptin receptors are found outside the central nervous system, supporting the view that leptin may act peripherally as well.

Prolonged leptin administration in Wistar rats is associated with growth of the thyroid gland; morphometry showed that the increase in the weight of the gland is coupled to a net increase in the epithelium/colloid ratio and increase in the levels of circulating T3 and T4 (Nowak et al., 2002). Compelling evidence indicates that the thyroid gland weight increase occurs as a result of a leptin-stimulated release of thyroid-stimulating hormone (TSH), at least in euthyroid rats

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(Seoane et al., 2000; Iossa et al., 2001), probably by enhancing the hypothalamic production of TRH (Powis et al., '98; Nillni et al., 2000; Harris et al., 2001; Sone and Osamura, 2001).

The presence and function of leptin in the thyroid gland in vertebrates, other than mammals are scanty. High levels of leptin have been found in the brain of several teleosts (Johnson et al., 2000) and in the lizard *Sceloporus undulatus* (Niewiarowski et al., 2000), suggesting that the central nervous system is a target organ of leptin also in these species. Recently, leptin-like immunoreactivity has also been reported in the sea lamprey *Petromyzon marinus*, suggesting that leptin is highly conserved among vertebrates (Yaghoubian et al., 2001). Leptin levels have been monitored in the plasma, liver and fat bodies of the lizard *Podarcis sicula* throughout the year (Paolucci et al., 2001). *P. sicula* is a temperate-zone adapted lizard, characterized, at this latitude, by a life cycle alternating periods of semi-hibernation to periods of activity. In temperate-zone lizards there is a pronounced seasonal change in fat deposition (Sexton et al., '72; Sciarrillo et al., 2000). Lipid is stored in discrete fat bodies (corpora adiposa) located in the abdominal cavity close to the pelvis. At this latitude, in early spring *P. sicula* leaves its winter shelters and starts eating. However, during the period of maximum activity the mass of fat bodies decreases, concomitantly with a decrease in leptin in both the plasma and fat bodies. It is likely that fat body depletion during the period of activity is due to the fat body utilization in support of reproduction (Paolucci et al., 2001).

The presence of leptin receptors in the lizard thyroid gland has not yet been studied and evidence is not available that leptin may act directly on this gland. It therefore seemed worthwhile to investigate whether lizard thyroid gland expresses the long form of leptin receptors (Ob-Rb) and to study the effects of leptin administration on the structure and function of the thyroid gland in the lizard *P. sicula*.

MATERIALS AND METHODS

Animals and experimental design

Adult male lizards of *P. sicula* (weighing 13–15 g) were captured in the neighborhood of Naples in May, when the thyroid glands undergo functional stimulation (Sciarrillo et al., 2000). After capture, the animals were housed in large soil-filled terraria containing heather, and exposed to natural temperature and photoperiod corresponding to the season. Water dishes were present in the terraria and the animals were fed on live fly larvae daily. Captivity lasted 20 days before sampling to reverse capture-related stress (Manzo et al., '94).

The specimens were divided into two groups of 20 animals each and underwent the following experimental design.

Group A: Animals were injected with a single intraperitoneal injection of mouse recombinant leptin (LINCO, St. Louis, MO) (0.1 mg/100 g body wt)/day for 1 day. Leptin was dissolved in reptilian physiological solution (NaCl 0.75%) with an injection volume of 0.1 ml. Injections were between 9:00 and 10:00 a.m. and the animals were sacrificed 2 hr after the injection.

Group B: Animals were given a single intraperitoneal injection of mouse recombinant leptin (LINCO, St. Louis, MO) (0.1 mg/100 g body wt)/day for 3 consecutive days. Leptin was dissolved in reptilian physiological solution (NaCl 0.75%) with an injection volume of 0.1 ml. Injections were between 9:00 and 10:00 a.m. and the animals were sacrificed 2 hr after the injection.

A control group for each leptin treatment group was kept under the same conditions as the treated groups, but it was intraperitoneally injected with saline solution. Animals were sacrificed 2 hr after the last injection.

The animals were anaesthetized by hypothermia chilling in chipped ice. Blood samples were collected by intracardiac puncture and put in a heparinized capillary. They were then centrifuged at 1,500*g* for 10 min at 4°C to obtain plasma.

Tissue preparation

Immediately after collection of blood samples, the animals were decapitated, and the thyroid glands were removed and fixed in Bouin's fixative for 24 hr. The tissues were then washed in 75% ethanol overnight, dehydrated through graded ethanols and embedded in paraplast. They were then cut into 7 μm sections with a rotary microtome, separated into parallel series, mounted on slides and stained by Galgano I stain (Beccari and Mazzi, '66).

Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running

the Kontron Elektronik KS 300 image analysis system and Adobe Photoshop.

The height of the follicular cells was measured in 30 cells every three slides, always on the second section of normal and treated specimens using a digital system of image (KS 300).

Radioimmunoassay

Plasma levels of T3 and T4 were determined by the method described by Sciarrillo et al. (2000) and Virgilio et al. (2003). In brief, they have used sensitive and highly specific radioimmunoassay kits (Byk-Sangtec Diagnostica, Dietzenbach, Germany).

In the T3 assay, a measured amount of sample serum and standards was added to a tube coated with anti-T3 rabbit antibody, along with a trace amount of radioactively labelled T3 ([^{125}I] T3), 4.4 μCi (Byk-Sangtec Diagnostica, Dietzenbach, Germany), and a blocking agent, Tris buffered saline 4 mM, ANS (8-anilino-1-naphthalenesulfonic acid) 6 mM sodium salicylate with 0.2% sodium azide as a preservative (Sigma Chemical Co., St. Louis, MO) to release T3 from serum binding proteins. The Sensitivity was 0.1 ng/ml with an accuracy of 97%. The range of intra-assay variance in 20 assays was 1.0–2.6%, while the interassay variance was 3.9–5.7% in 12 assays.

For T4, a measured amount of sample serum and standards was added to a tube coated with anti-T4 rabbit antibody, along with a trace amount of radioactively labelled T4 ([^{125}I] T4), 4.4 μCi (Byk-Sangtec Diagnostica, Dietzenbach, Germany), and a blocking agent, Tris buffered saline 4 mM, ANS 6 mM sodium salicylate with 0.2% sodium azide as a preservative (Sigma Chemical Co., St. Louis, MO) to release T4 from serum binding proteins. The Sensitivity was 0.45 ng/ml with an accuracy close to 100%; the mean intra- and interassay coefficients of variance were 4.6% and 4.3%, respectively. A logit–log curve fit using a % B/B_0 calculation was used. T4 and T3 concentrations were determined by computing the % B/B_0 for each sample and then finding the results on the standard curve. Cross-reactivity for T4 in the T3 RIA (1.3%) was not considered for data calculations, neither was that for T3 in the T4 RIA (0.1%).

Plasma TSH concentrations were measured by a two-site immunoradiometric assay using mouse monoclonal antibodies (Byk-Sangtec Diagnostica, Dietzenbach, Germany). In the TSH procedure, sample plasma and standards were added to anti-ligand coated tubes. The Tracer/Capture Reagent, a blend of ligand-tagged TSH-specific antibody and ^{125}I labelled TSH (10 μCi), was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose was 0.01 μIU/ml, with an accuracy close to 100% and mean intra- and interassay coefficients of variance of 5.0% and 7.5%, respectively.

Statistical analysis

All data are presented as means ± standard error of mean (SEM). Statistical analyses were performed by one-way analysis of variance with repeated measures followed by Duncan's multiple range test for pairwise comparisons. Differences were considered significant if $P < 0.05$.

Immunohistochemistry

The thyroid glands of non-treated *P. sicula* animals were examined by immunohistochemistry with the avidin–biotin–peroxidase complex (ABC) method (Hsu et al., '81) to localize the Ob-Rb. The thyroid glands were fixed in Bouin's solution for 2 hr, then dehydrated and embedded in paraplast. Rehydrated sections were incubated for 30 min in a solution of 3% H_2O_2 in methanol (1:5 vv) to inhibit endogenous peroxidases. The sections were incubated with normal goat serum for 20 min and then incubated with a polyclonal goat anti-Ob-Rb antibody (Biogenesis Ltd., Poole, England). (1:300) overnight at 4°C in a moist chamber.

After washing in PBS, the sections were incubated for 30 min with diluted biotinylated rabbit anti-goat IgG (1:400) (Vecta-lab ''Elite'' (ABC) kit, Vector Laboratories, Burlingame, CA) and, after washing in PBS, incubated for 30 min with Vectastain Elite ABC reagent. Immunoreactivity was visualized by reaction with 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) and 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.2, for 10 min to reveal the brown immunoreactive cells. After DAB reaction, the sections were counterstained with hematoxylin.

To confirm the specificity of the immunostaining, the following control staining procedures were carried out: (1) replacing specific antiserum with normal rabbit serum, (2) omitting primary

antibody, and (3) pre-absorbing primary antiserum with 10 nmol of antigen per ml of optimally diluted serum.

RESULTS

Morphological observations of the thyroid gland

The thyroid gland of the lizard *P. sicula* control specimens is a single discrete ribbon-like structure which transversely crosses the middle of the trachea. It is formed by follicles that are connected by an interfollicular connective tissue which contains blood vessels. A superficial connective tissue capsule envelops the gland and sends branches which form a network that surrounds the follicles. These thyroids showed a medium–high follicular epithelium ($8.15 \pm 0.03\,\mu m$) and there were reabsorbing vacuoles in the colloid (control group) (Fig. 1A).

All samples treated with leptin revealed morphological signs of stimulation of thyroid activity. In Group A, the follicles were irregular in shape with a very high epithelium ($16.8 \pm 0.03\,\mu m$, $P < 0.05$) and roundish nuclei at the base of the follicular cells. The colloid filled up the follicular lumen, showing numerous reabsorption vacuoles (Fig. 1B).

In Group B (Fig. 1C), there was a major stimulation of the thyroid gland, which revealed an increase in follicular epithelium ($24.9 \pm 0.03\,\mu m$, $P < 0.05$). Data about the height of follicular epithelium are shown in Fig. 2.

Hormonal variations of the T3, T4 and TSH

Leptin treatment increased the plasma levels of thyroid hormones. In fact, plasma T3 increased significantly ($P < 0.05$) from 3.63 ± 0.05 ng/ml in the control specimens to 8.10 ± 0.03 ng/ml in the specimens treated with three leptin injections (Group B). Moreover, plasma T4 increased significantly ($P < 0.05$) from 4.32 ± 0.04 ng/ml in the control specimens to a maximal value of 9.42 ± 0.05 ng/ml in the specimens of Group B, following a trend similar to that of T3 (Fig. 3A).

After leptin treatment, TSH showed a significant decrease ($P < 0.05$). It decreased from $4.13 \pm 0.05\,\mu IU/ml$ in the control group to $1.26 \pm 0.02\,\mu IU/ml$ in Group B (Fig. 3B).

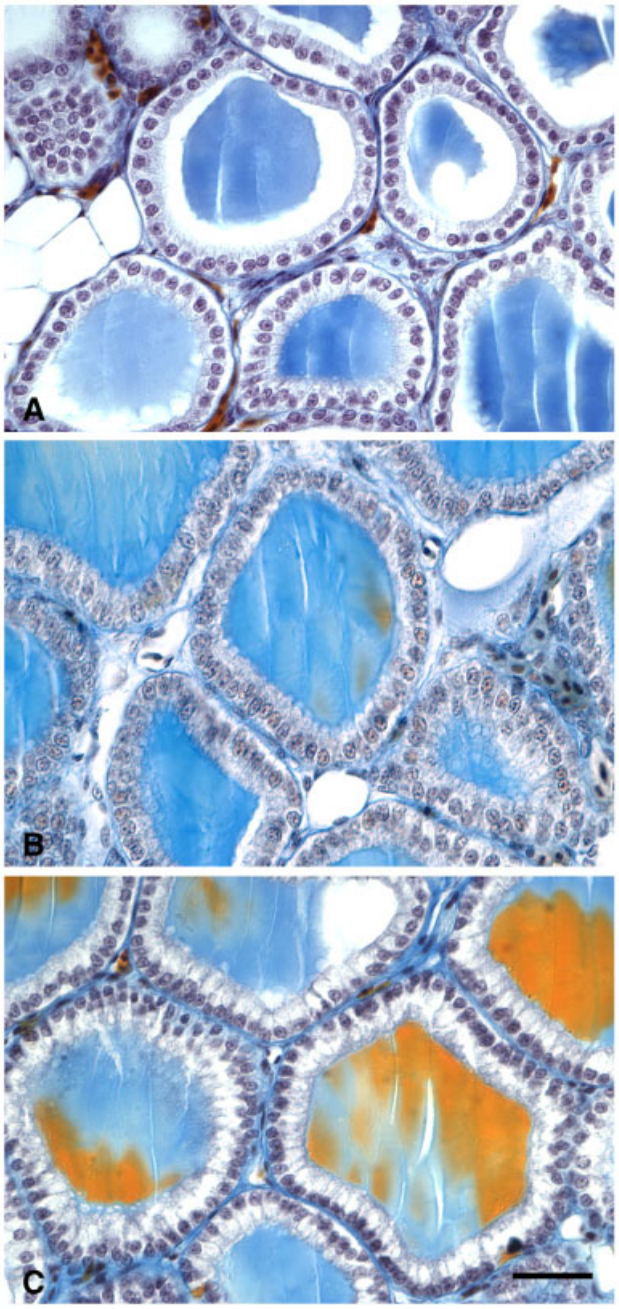

Fig. 1. Section of thyroid of normal and leptin-treated *Podarcis sicula* (stain Galgano I; scale bar: 20 μm). (**A**) Normal specimen; note the medium–high follicular epithelium; (**B**) specimen treated with one injection of leptin (0.1 mg/100 g body wt); the follicular epithelium is higher than in normal specimens; (**C**) specimen treated with three injections of leptin (0.1 mg/100 g body wt); note the follicular epithelium which is very high and the colloid that appears very vacuolized.

Immunohistochemistry

Control sections exhibited no staining (Fig. 4A). Ob-Rb-like immunoreactivity in the thyroid gland

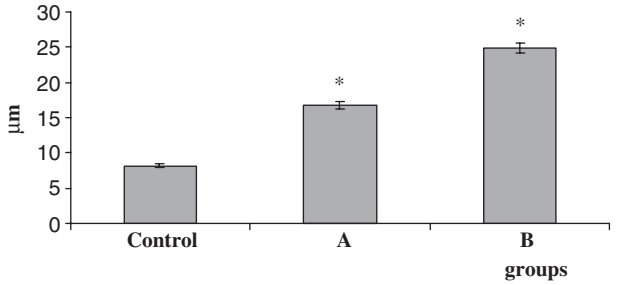

Fig. 2. Variations of epithelium height of the follicular cells of the thyroid gland in *Podarcis sicula* subjected to different experimental treatments (see Materials and Methods). Values are shown as means ± SEM. *Significant at $P < 0.05$.

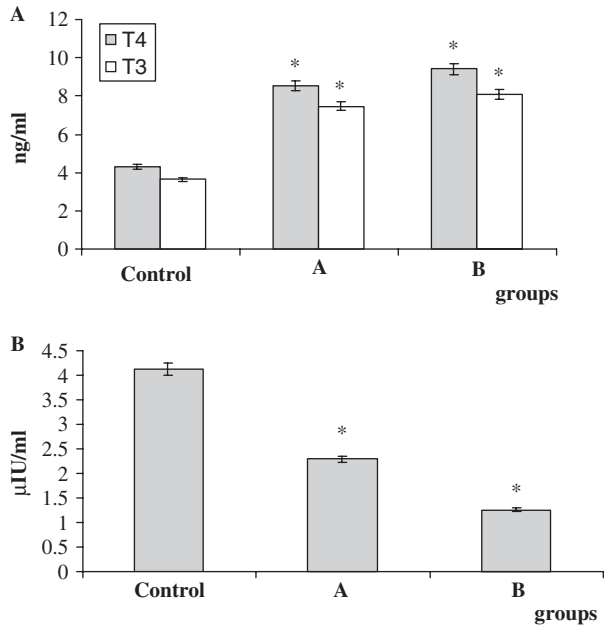

Fig. 3. Variations of T3, T4 (**A**) and TSH (**B**) levels in the plasma of *Podarcis sicula* subjected to different experimental treatments (see Materials and Methods). Values are shown as means ± SEM. *Significant at $P < 0.05$.

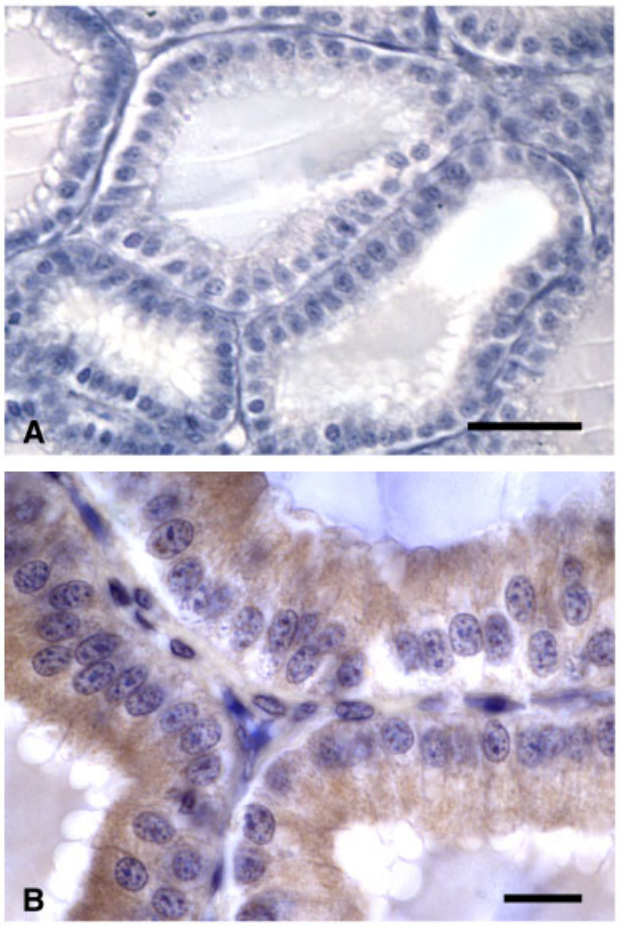

Fig. 4. Localization of leptin receptor in *Podarcis sicula* thyroid gland. (**A**) Negative control of ABC reaction showing no signal (scale bar: 30 µm); (**B**) immunostaining for leptin receptor was present in the apical cytoplasm of thyroidal cells (scale bar: 40 µm).

of *P. sicula* appears in the apical cytoplasm of the follicular cells; therein no immunopositivity to leptin in the nerve fibers surrounding blood vessels and running between and along thyroid follicles (Fig. 4B).

DISCUSSION

The results of the present study indicate that in *P. sicula* thyroid gland the long form of leptin receptor (Ob-Rb) is located in the apical cytoplasm of follicular cells. They also demonstrate that leptin can influence the activity of the thyroid gland.

Leptin receptors (Ob-R) belong to the gp 130 family of cytokine receptor superfamily and are present in both the central nervous system and peripheral organs. All Ob-R isoforms have transmembrane domains, of which Ob-Rb is predominant (the long receptor isoform) and is predominantly expressed in the hypothalamus (Tartaglia et al., '95; Cioffi et al., '96; McCowen et al., '98). Several studies have revealed the presence of Ob-Rb in the thyroid gland. In fact, the first evidence (Lin et al., 2000) has demonstrated that Ob-Rb are expressed in the thyroid gland in pigs. Besides, coupled RT-PCR, Western blotting and immunohistochemical findings (Nowak et al., 2002) have shown the expression of Ob-Rb as mRNA and

protein in the thyroid gland of normal rats. In agreement with these studies in mammals, our light microscopic immunohistochemistry results have shown that there is Ob-Rb immunoreactivity in the follicular cells of the thyroid gland in the lizard.

Taken together, these results make it likely that systemically injected leptin may exert a direct effect on the thyroid function in the lizard. Our results indicate that the leptin administration stimulates the thyroid gland; morphology showed that the increase in the height of follicular epithelium is coupled to a net increase of reabsorption vacuoles. Accordingly, our RIA assay indicated an enhanced secretory activity, as indicated by the increased levels of circulating T4 and T3.

Besides, we studied the effect of leptin administration on plasma TSH levels and observed that plasma levels of this hormone are markedly reduced in leptin-treated lizards. The decrease in TSH is probably dependent on the negative feedback exerted by the leptin-induced raised levels of thyroid hormones.

In conclusion, in reptiles leptin stimulates the morphology and the secretion of the thyroid gland, through a direct mechanism involving Ob-Rb with which follicular epithelium cells are provided.

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