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Effect of Alloxan and Insulin Immunoneutralization on Circulating Thyroid Hormone Levels in Larval Landlocked Sea Lampreys, *Petromyzon Marinus*

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ABSTRACT—The effects of alloxan, an insulin (INS)-secreting cell toxin, and INS immunoneutralization on circulating levels of thyroid hormones (thyroxine, T_4; triiodothyronine, T_3) were examined in larval landlocked sea lampreys, *Petromyzon marinus*. Animals were injected intraperitoneally with either (Experiment 1) saline (0.6%) or alloxan (20 or 200 μg/g body weight), or with (Experiment 2) normal rabbit serum or anti-lamprey INS. Alloxan (200 μg/g) decreased plasma T_3, but not T_4, in larvae by about 45–80%. Three, six, or nine hr after acute immunoneutralization of lamprey INS with anti-lamprey INS, plasma T_3 levels decreased by 13–30%, relative to controls. These data indicate that INS deficiency can regulate the thyroid system of larval lampreys. There is some suggestion that INS may mediate the metamorphic processes by modulating thyroid hormone concentrations.

Key words: insulin, thyroxine, triiodothyronine, alloxan, lamprey

INTRODUCTION

The endocrine system of lampreys, like other poikilothermic vertebrates that possess a metamorphic episode as part of their life history (Dickhoff, 1993), is inextricably linked to the metamorphic process (Youson, 1997). It has been suggested that thyroid hormones (TH; thyroxine, T_4, and triiodothyronine, T_3) are important factors in the metamorphic development of lampreys (Youson, 1997). This notion is supported by the observation that TH deficiency, caused by treatment with goitrogens, induces precocious metamorphosis of four Northern Hemisphere lamprey species, *Lampetra planeri* (Hoheisel and Sterba, 1963), *L. reissneri* (Suzuki, 1986), *P. marinus* (Youson *et al*., 1995), and *L. appendix* (Holmes *et al*., 1999). In addition, TH treatment completely blocked $KClO_4$-induced metamorphosis (Manzon *et al*., 1998) and inhibited $KClO_4$-induced changes in lipid metabolism that are similar to those observed during spontaneous metamorphosis (Kao *et al*., 1999a). A recent report indicated that, with the exception of propylthiouracil, several different goitrogens (i.e., potassium thiocyanate, sodium perchlorate and methimazole) induced metamorphosis in *P. marinus* larvae (Manzon *et al*., 2001).

Numerous reports, consistent with the aforementioned goitrogen experiments, have shown that premetamorphic larval lampreys maintain relatively high plasma TH titers which decline rapidly at the onset of spontaneous metamorphosis (Lintlop and Youson, 1983; Suzuki, 1986; Leather-

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land *et al.*, 1990; Youson *et al.*, 1994). The falling plasma TH levels observed during the metamorphosis of lamprey were coincident with the rising concentrations of insulin (INS) in the serum (Youson *et al.*, 1994). In the hagfish (another agnathan), *Eptatretus stouti*, INS insufficiency, achieved 24 hr following the administration of guinea pig anti-human INS serum, lowered plasma T_3, but had a tendency to increase plasma T_4 levels (Plisetskaya *et al.*, 1983). To date little is known about the regulatory influences which INS has on TH titers in lampreys nor the ultimate role this plays in lamprey metamorphosis.

The objective of this study was to examine the effects of alloxan and INS immunoneutralization TH levels in larval sea lamprey in order to gain further insight into the evolution of TH regulatory systems and the endocrine control of metamorphosis.

MATERIALS AND METHODS

Sea lamprey, *P. marinus*, larvae (body weight, 2.7–2.9 g; body length, 116–130 mm) were housed and injected intraperitoneally (10 μl/g body weight) as previously described by Kao *et al.* (2001). Briefly, larvae were administered 0.6% of saline (control) or alloxan (5, 6-dioxyuracil monohydrate; dissolved in 0.6% saline; Sigma) at a low dose of 20 μg or high dose of 200 μg per gram body weight once per day for 2 days. Alloxan is a β-cell cytotoxin (Lukens, 1948; Morris and Islam, 1969). Twelve to 14 hr after the last injection, individual animals were anesthetized with 0.05% tricaine methanesulfonate. Acute neutralization of pancreatic hormones was induced essentially as previously described by Plisetskaya *et al.* (1989). Groups of 10 larvae received an intraperitoneal injection of 10 μl/g body weight of 1:1 mixture of 0.6% saline and either anti-lamprey INS or normal rabbit serum (control). The antisera were preabsorbed with excess thyroglobulin before injection. The validation of the antisera was described by Plisetskaya *et al.* (1988), and Youson *et al.* (1992). Blood was collected into heparinized-capillary tubes from the severed caudal vasculature 3, 6, or 9 hr after the injection. Plasma was collected and stored at –70°C for later determination of TH concentrations. Plasma TH was measured by the radioimmunoassay (RIA) method described by Manzon *et al.* (2001). Inter- and intra-assay variance was about 10% for both assays, and assay sensitivities were 8.0 and 0.24 nM for T_4 and T_3, respectively. The T_4 antiserum used in the RIAs has a cross-reactivity of 12% with T_3, and the T_3 antiserum a cross-reactivity of 0.3% with T_4. Data are expressed as mean±SEM. Unpaired Student *t*-test was used to examine differences between two groups. Analysis of variance (ANOVA) and Duncan's multiple range test were used to examine differences among the various groups in the dose-dependent experiment. A probability level of 0.05 was used to indicate significance. All statistics were performed using SigmaStat (Jandel Scientific, Palo Alto, CA).

RESULTS AND DISCUSSIONS

Plasma concentrations of T_4 and T_3 differed in larva; T_4 levels were 3–5 times greater than T_3 levels (Fig. 1A). The high dose (200 μg/g body weight) of alloxan, a β-cell toxin and oxidant, decreased plasma T_3 concentrations in larvae, but had no effect on plasma T_4 levels in larval animals (Fig. 1A). Acute neutralization of lamprey INS into larvae treated with anti-lamprey INS for 3, 6 or 9 hr tended to reduce the concentrations of plasma T_3 and T_4; however, the elevation of plasma T_4, above control levels, was observed 9 hr after the administration (Fig. 1B).

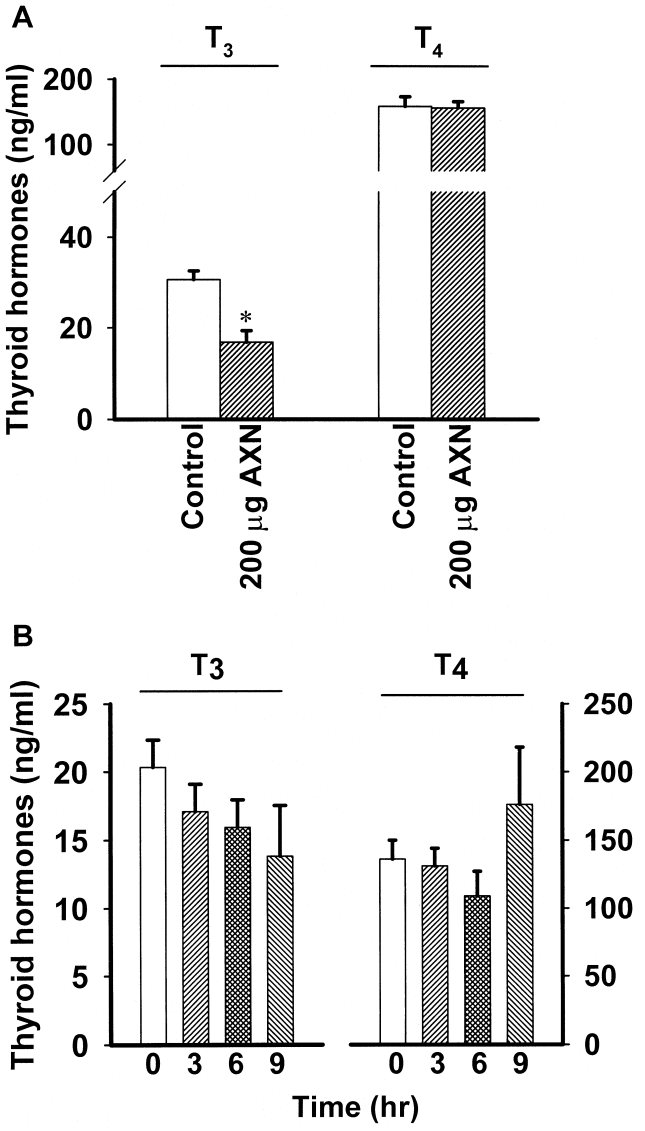

FIG. 1. Effect of alloxan (AXN; **A**) and acute immunoneutralization with lamprey INS antiserum (**B**) on plasma thyroid hormone (T_3, triiodothyronine; T_4, thyroxine) levels of larval lampreys, *Petromyzon marinus*. Data presented as mean±SEM. (n=5–6 animals in **A** and n=10 animals in **B**). In (**B**), the Arabic numbers, 3, 6 and 9 indicate hours after injection of anti-lamprey INS serum. An asterisk symbol indicates a significant (a critical probability value<0.05) difference from the control group.

The present study provides the observations that acute INS deficiency, brought about by anti-lamprey INS serum or by alloxan treatments, reduced plasma T_3, but not T_4, concentrations in larval sea lampreys. These data are consistent with those for hagfish, another cyclostome, in which INS insufficiency 24 hr after guinea pig anti-human INS serum administration resulted in a decline of plasma T_3 levels (Plisetskaya *et al.*, 1983). In contrast, salmon had a higher concentration of plasma T_3 12 hr after the administration of

anti-salmon INS serum (Plisetskaya *et al*., 1989). This suggests that the mechanism of TH regulation by INS in teleosts and cyclostomes is fundamentally different.

The mechanism by which INS deficiency modulates thyroid system in lamprey is not known. However, the lamprey INS antiserum-induced decrease in larval plasma TH titers is clearly a response to the neutralizing endogenous INS because plasma INS levels were reduced by 100% after injection with lamprey INS antiserum (Youson *et al*., 1992). Whether the low concentrations of plasma TH in larval lamprey caused by INS deficiency due to the increase in the deiodinase activity (Eales *et al*., 2000) needs further investigations.

The observed alloxan-induced decrease of plasma TH in lamprey is consistent with that reported in mammals (Kumaresan and Turner, 1966). We used alloxan in this study to provide additional insight into INS action and assumed that alloxan would alter thyroid system of sea lamprey by ablating INS. This assumption was supported by previous observations that alloxan damaged β cells and increased plasma glucose in larval *L. planeri* (Morris and Islam, 1969) and adult *L. fluviatilis* (Bentley and Follett, 1965). Many of our results, indeed, including those on increased plasma FA and plasma glucose (Kao *et al*., 1999b and 2001) were consistent with INS disruption. Whether oxidative stress generated by alloxan oxidant (Dulin *et al*., 1983) and diabetic glucose (Evans *et al*., 2002) affects lamprey β cells is unknown. On the other hand, our immunohistological findings (Kao *et al*., 1999b), as reported in lamprey (Morris and Islam, 1969; Biuw, 1970) and in mammals (Dulin *et al*., 1983), suggest that in the time frame of our experiment (2 days, 0.2 mg/g body weight), alloxan toxicity in larval sea lampreys is primarily on extra-islet tissues and/or enzymes. This effect may also result in alterations in β-cell functions and/or thyroid functions as well as explain why alloxan injection in this study did not oppose all INS-induced alterations in TH concentrations of larvae and stage 6 transformers (unpublished data). Marked species differences to alloxan treatment in terms of the β cell toxicity have been observed (Lukens, 1948).

It appears that INS plays a role in lamprey metamorphosis. This conclusion is supported by the following observations. First, INS deficiency as reported here modulates TH concentrations. Second, TH deficiency induces precocious metamorphosis of *P. marinus* (Youson *et al*., 1995; Manzon *et al*., 2001); whereas, TH treatment blocks $KClO_4$-induced metamorphosis (Manzon *et al*., 1998). Finally, alterations in plasma levels of INS and TH are spontaneously associated with lamprey metamorphosis (Youson *et al*., 1994) and both hormones play an anabolic role in the metamorphosis-associated changes in lamprey lipid metabolism (Kao *et al*., 1999a and 1999b).

Given the ancient lineage of lamprey, these findings suggest that the thyroidoregulatory roles of INS emerged early during the evolution of vertebrates. Furthermore, the findings suggest a role for INS in lamprey metamorphosis and support the view that T_3 is the most important TH at this phase of the life cycle (Youson, 1997).

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