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Molecular mechanisms of corticosteroid synergy with thyroid hormone during tadpole metamorphosis

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ABSTRACT

Corticosteroids (CS) act synergistically with thyroid hormone (TH) to accelerate amphibian metamorphosis. Earlier studies showed that CS increase nuclear 3,5,3′-triiodothyronine (T_3) binding capacity in tadpole tail, and 5′ deiodinase activity in tadpole tissues, increasing the generation of T_3 from thyroxine (T_4). In the present study we investigated CS synergy with TH by analyzing expression of key genes involved in TH and CS signaling using tadpole tail explant cultures, prometamorphic tadpoles, and frog tissue culture cells (XTC-2 and XLT-15). Treatment of tail explants with T_3 at 100 nM, but not at 10 nM caused tail regression. Corticosterone (CORT) at three doses (100, 500 and 3400 nM) had no effect or increased tail size. T_3 at 10 nM plus CORT caused tails to regress similar to 100 nM T_3. Thyroid hormone receptor beta (TRβ) mRNA was synergistically upregulated by T_3 plus CORT in tail explants, tail and brain *in vivo*, and tissue culture cells. The activating 5′ deiodinase type 2 (D2) mRNA was induced by T_3 and CORT in tail explants and tail *in vivo*. Thyroid hormone increased expression of glucocorticoid (GR) and mineralocorticoid receptor (MR) mRNAs. Our findings support that the synergistic actions of TH and CS in metamorphosis occur at the level of expression of genes for TRβ and D2, enhancing tissue sensitivity to TH. Concurrently, TH enhances tissue sensitivity to CS by upregulating GR and MR. Environmental stressors can modulate the timing of tadpole metamorphosis in part by CS enhancing the response of tadpole tissues to the actions of TH.

1. Introduction

Amphibian metamorphosis is dependent on thyroid hormone (TH), which induces the suite of molecular and cellular changes that cause a tadpole to transform into a frog. Thyroid hormone actions are mediated by TH receptors (TRs, TRα and TRβ) that are members of the nuclear receptor (NR) superfamily and function as ligand-activated transcription factors (Mangelsdorf et al., 1995). In *Xenopus laevis*, TRα is expressed shortly after hatching and is maintained at a relatively constant level throughout tadpole life and metamorphosis (reviewed by Shi, 2000). TRα may establish tissue competence to respond to TH once the thyroid gland matures and starts to secrete hormone, and may mediate the actions of TH on cell proliferation (Denver et al., 2009; Furlow and Neff, 2006). By contrast, TRβ is expressed at a low level throughout tadpole life, and increases dramatically at the onset of metamorphosis when the plasma TH concentration rises (Shi, 2000). The expression of TRβ is dependent on the rise in plasma TH; i.e., it is autoinduced, and the level of TRβ expression in target cells is predicted to play a central role in determining the responsiveness of the cell to TH (reviewed by Furlow and Neff, 2006; Tata, 2006).

In addition to the amount of TR made by a cell, an important determinant of TH action during metamorphosis is the activity of intracellular enzymes (deiodinases) that establish the concentration of bioactive TH within the cell (St Germain et al., 2009). The major product of the amphibian thyroid gland is thyroxine (T_4) with minor amounts of 3,5,3′-triiodothyronine (T_3) produced (Buscaglia et al., 1985; Rosenkilde, 1978). The biologically active form of TH is T_3, which has up to ten times greater activity than T_4 (Frieden, 1981; Lindsay et al., 1967; Rosenkilde, 1978; Wahlborg et al., 1964; White and Nicoll, 1981). Of the three deiodinases expressed in amphibian tissues, two appear to play dominant roles during metamorphosis: the 5′ deiodinase type 2 (D2), which converts T_4 to T_3, and the 5 deiodinase type 3 (D3), which converts T_4 to reverse T_3 (3,3′,5′-triiodothyronine; rT_3), and T_3 to 3,5-diiodo-L-thyronine (T_2), both of which are inactive in that they do not bind to TRs (reviewed by Denver, 2009a). The balance of D2 and D3 activity within a cell is hypothesized to establish the intracellular level

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of bioactive hormone, although precisely how this balance is achieved is not well understood (St Germain et al., 2009).

Hormones other than TH play important roles in amphibian metamorphosis, in part by modifying the production and actions of TH. For example, pituitary prolactin may reduce tissue responsiveness to TH by blocking TRβ autoinduction. This action of PRL may be important during metamorphic climax when PRL expression increases, possibly to control the morphogenic actions of TH (reviewed by Denver, 2009a). Corticosteroids (CS), hormones produced by adrenocortical cells (interrenal glands in frogs), synergize with TH at target tissues to promote morphogenesis (Denver, 2009b; Kikuyama et al., 1993). The production of CS changes with development, rising throughout metamorphosis and reaching a peak at metamorphic climax (Kikuyama et al., 1993). Also, because CS are stress hormones, their production is increased by exposure to environmental stressors such as habitat dessication, competition for resources, predation risk, etc. (Denver, 2009b). Various environmental stressors have been shown to accelerate tadpole metamorphosis, and this developmental acceleration may be mediated by stress hormones produced both centrally (central nervous system – CNS and pituitary gland) and peripherally (interrenal glands; Denver, 2009b). At the level of the CNS and pituitary gland, hypothalamic corticotropin-releasing factor (CRF) has a dual hypophysiotropic role in tadpoles, stimulating the secretion of pituitary adrenocorticotropic hormone (ACTH), which increases CS production by adrenocortical cells, and thyroid stimulating hormone (TSH), which increases hormone secretion by the thyroid gland. At the level of hormone target cells in peripheral tissues, CS have been shown to synergize with TH to promote morphogenesis (Denver, 2009b; Kikuyama et al., 1993).

Like TH, CS actions are mediated by NRs encoded by two different genes: the type I (mineralocorticoid receptor; MR) and the type II (glucocorticoid receptor; GR). Two mechanisms have been proposed to explain CS synergy with TH during tadpole metamorphosis. Work from Kikuyama's group showed that CS increase nuclear T_3 binding capacity in tadpole tail (reviewed by Kikuyama et al., 1993). Galton (1990) found that CS influence deiodinase activity in tadpole tissues, increasing 5′ deiodinase and decreasing 5 deiodinase. These findings suggest that CS act to enhance cellular responsiveness by upregulating TRs and at the same time increasing conversion of the precursor T_4 to the biologically active hormone T_3. Thus, stress hormones mediate environmental effects on development, and they interact with the thyroid axis at both central and peripheral levels.

In the current study we examined morphological and gene expression changes in tadpole tissues and cell lines caused by TH, CS, or combined treatment with the two hormones. We investigated whether the synergy between TH and CS occurs at physiological doses of the hormones, and is seen at the level of TR and deiodinase gene expression. We also investigated the expression of CS receptors, to determine if they are autoregulated, or crossregulated by TH. Our findings provide a molecular basis for understanding the enhanced sensitivity of tadpole tissues to TH caused by increased CS, and support a mechanism for accelerated metamorphosis via interactions among the thyroid and stress hormone axes at the hormone target tissues.

2. Materials and methods

2.1. Animal care

We raised *Xenopus laevis* and *Silurana tropicalis* tadpoles obtained from in-house breeding in dechlorinated tap water (water temperature 21–23 °C – *X. laevis*; 24–25 °C – *S. tropicalis*) under a 12L:12D photoperiod and fed frog brittle (*X. laevis*; NASCO, Fort Atkinson, WI) or Seramicron (Sera North America, Inc., Montgomeryville, PA) plus boiled spinach (*S. tropicalis*). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan.

2.2. Tissue explant culture and hormone treatment

We initiated tadpole tail explant cultures to investigate the actions of T_3 and corticosterone (CORT) on tail regression and gene expression. We treated Nieuwkoop–Faber stage 52–54 (Nieuwkoop and Faber, 1956) *X. laevis* tadpoles with oxytetracycline (100 μg/ml in aquarium water) for 24 h prior to dissection. Dissections were carried out under semi-sterile conditions. Tadpoles were anesthetized in 0.01% benzocaine and dipped in 70% ethanol to sterilize the epidermis. The tails were dissected into sterile 6-well tissue culture dishes containing 2 ml ice-cold tissue culture medium ($n = 7$/treatment for morphological measurements, $n = 6$/treatment for RNA analyses). Tail explants were cultured in Dulbeco's Modified Eagle's Medium (DMEM; Gibco BRL) diluted 1:1.5 for amphibian tissues at 25 °C in an atmosphere of 5% CO_2 and 95% O_2 with gentle rotation (50 rpm). The T_3 was dissolved in 0.01 N NaOH and CORT was dissolved in 100% ethanol. All treatments received an equivalent amount of ethanol vehicle (0.001%). Tail explants were treated with T_3 (10 or 100 nM), CORT (100, 500 or 3400 nM) or 10 nM T_3 plus CORT (100, 500 or 3400 nM). The medium was replaced every 12 h. For RNA analysis, three tails were harvested per treatment 2 days after initiation of hormone treatments; the remaining three tails per treatment were harvested at 5 days. Tails were rinsed twice in ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma Chemical Co., St. Louis, MO), snap frozen in a dry ice-ethanol bath and stored at −80 °C until RNA extraction.

We conducted morphometric analysis of tail explants by measuring tail area and final dry weight. For tail area, we captured images every day for seven days with a digital camera and then used Scion Image software (v. 3.0 Scion Corporation, Frederick, MD, USA) to trace the perimeter of each tail and calculate the area. At the end of the experiment we dried the tails in a drying oven and recorded the final dry weight.

2.3. Treatment of tadpoles with hormones in vivo

To investigate gene expression responses to T_3 and CORT *in vivo* we treated NF stage 52 *S. tropicalis* tadpoles with T_3 and CORT for 24 h before sacrifice. We maintained tadpoles in 4 L aquaria (6 tadpoles per aquarium) and hormones were added to the aquarium water. All treatments received an equivalent amount of ethanol vehicle (0.001%). Tadpoles were treated with T_3 (1, 5 or 50 nM), CORT (100 nM) or 100 nM CORT plus T_3 (1 or 5 nM) and the aquarium water was changed and hormones replenished at 12 h. At sacrifice tail and brain (a region encompassing the preoptic area/diencephalon) were harvested, snap frozen in a dry ice-ethanol bath and stored at −80 °C for subsequent RNA isolation.

2.4. X. laevis tissue culture cells

To further investigate gene expression responses to T_3 and CORT, and to establish a cell culture model system for future detailed molecular analyses of hormone synergy we treated the *X. laevis* embryonic fibroblast-derived cell line XTC-2 and the tadpole tail myoblast-derived cell line XLT-15 with hormones and measured gene expression by reverse transcriptase-polymerase chain reaction (RT-PCR). We cultured cells in Leibovitz L-15 medium (diluted 1:1.5 for amphibian tissues) plus 10% Fetal Bovine Serum (Invitrogen, Carlsbad, CA; growth medium) that was stripped of