Putative L-Triiodothyronine Receptors in the Liver Nuclei of Mature Tropical Toad, *Bufo melanostictus*

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Thyroid hormones exert a major role in growth and differentiation of almost all types of tissues in animals, particularly in amphibian metamorphosis, through its specific nuclear receptor activation followed by gene expression. However, its function in mature tropical amphibians is less studied. The present study revealed the existence of a single class of specific nuclear receptor(s) in the liver nuclei of mature tropical toad, *Bufo melanostictus*, with a dissociation constant of $(3.7 \pm 0.9) \times 10^{-10}$ molar and maximum binding capacity of 0.074 ± 0.013 pmol/mg DNA. The percentage of relative binding affinities for the specific nuclear L-T3 binding site in the liver nuclei of toad were L-triiodothyronine (L-T3) > triiodothyroacetic acid (TRIAC) > L-thyroxine (L-T4) = tetraiodothyroacetic acid (TETRAC) > 3,3′,5′-triiodothyronine (r-T3) > Diiodothyrtonine (L-T2) (100 > 75 > 19.4 = 19.4 > 3.7 > 0.39) and the relative ED_{50} values (in nanomolar) were $0.33 < 0.44 < 1.7 = 1.7 < 9 < 83$.

Key words: Liver Nuclei, L-Triiodothyronine Receptor, Thyroid Hormone Analogs

Introduction

Thyroid hormones (TH) play a pivotal role in growth and differentiation in several tissues and maintain homeostasis in vertebrates. The major active form of TH, L-triiodothyronine (L-T3) is produced from the precursor L-thyroxine (L-T4). TH action is mediated by binding of L-T3 to its specific nuclear receptors followed by gene expression (Samuels and Tsai,1974; Samuels *et al.*, 1988; Yaoita *et al.*, 1990; Dasmahapatra *et al.*, 1990; Zhang and Lazar, 2000). High affinity nuclear TH receptors have been reported in TH-responsive tissue of both mammalian and non-mammalian vertebrates (Samuels and Tsai, 1974; Kistler *et al.*, 1975; Bellabarba and Lehoux, 1981; Oppenheimer, 1983; Dasmahapatra *et al.*, 1990). An increase in malic enzyme (Dey *et al.* 1989), α-glycerophosphate dehydrogenase (Dey and Medda, 1990a) and Na^+-K^+-ATPase activities (Dey and Medda, 1990a) in liver and muscle of mature toad (*Bufo melanostictus*) have been reported after TH administration. Presences of putative nuclear L-T3 receptors in the liver and red blood cells of mature *Rana catesbiana* (Galton, 1985) and Mexican axolotl, *Ambystoma mexicanum* (Galton, 1992), have also been demonstrated. This study reports the presence of saturable nuclear L-T3 receptors in the liver of mature tropical toad (*Bufo melanostictus*).

Materials and Methods

Animals

Mature female toads of 30–40 g body weight were purchased from local supplier in Calcutta, India, and maintained in laboratory conditions at 25 ± 1 °C for one week before experiments. The animals were fed *ad libitum* with ant eggs. Six animals were taken per group for each experiment.

Preparation of liver nuclei

The toads were sacrificed and livers were surgically removed very quickly in cold, weighed and used for homogenization. Liver was washed in isolation buffer containing 0.25 M sucrose, 0.005 M $MgCl_2$, 0.05 M Tris(hydroxymethyl)aminomethane-glycine buffer at pH 8 at a ratio of 1 g equivalent liver nuclei per ml of buffer, homogenized with a teflon homogenizer (10% w/v) and centrifuged at 700 × *g* for 10 min. Melanocytes over the nuclei were removed and the pellet was washed with

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buffer. The liver nuclei were separated from melanin granules by repeated washings, resuspended in 0.25 M sucrose and layered over 2.1 M sucrose solution and centrifuged at 125000 × *g* for 60 min. The pellet obtained was further washed and used as purified nuclei for the binding assay.

In vitro hormone binding assay

Purified nuclear fractions (12–24 mg DNA per assay) were incubated with 10 pM[^{125}I] L-T3 (71 MBq, Amersham International) in a buffer containing 0.05 M Tris, 0.25 M sucrose, 0.001 M $MgCl_2$, 0.001 M EDTA and 0.005 M 2-mercaptoethanol, pH 8, for 2 h with or without increasing concentrations of unlabeled L-T3 and analogs (1×10^{-6} M to 1×10^{-12} M) in a final volume of 1 ml. The binding affinities of the following thyroid hormone analogs relative to L-T3 were determined by their ability to compete with [^{125}I] L-T3 for the nuclear binding sites: L-T4, triiodothyroacetic acid (TRIAC), tetraiodothyroacetic acid (TETRAC), 3,3′,5′-T3 (r-T3) and L-diiodothyronine (T2). All these analogs were dissolved in a minimum volume of 0.1 N NaOH and diluted with 0.05 M Tris-HCl buffer, pH 8. Non-specific binding was determined by adding 15.4 μM L-T3. Bound and free [^{125}I] L-T3 was separated by addition of 1 ml of 24% polyethylene glycol to a final volume of 1 ml of assay mixture (total volume 2 ml). The maximum binding capacity (B_{max}) and the dissociation constant (K_d) were determined by Scatchard plot analysis (Scatchard, 1949). The DNA content was estimated by the method of Croft and Lubran (1965).

Statistical analyses

Statistical Analyses were performed by Student's *t*-test. $P < 0.05$ was considered as significant. Sigmastat software was used for all statistical analyses. All data were presented as mean ± SEM (standard error of mean).

Results

T3 binding site in mature toad liver nuclei

In vitro binding studies revealed the presence of one type of saturable high affinity and limited capacity binding sites for L-T3 in adult toad liver nuclei. The mean values for dissociation constant (K_d) and maximum binding capacity (B_{max}) found in four experiments performed in triplicate were (3.7 ± 0.9) × 10^{-10} M and 0.074 ± 0.013 pmol/mg DNA, respectively (Fig. 1).

Binding affinities of thyroid hormone analogs

The relative order of potencies of binding affinities for the toad liver nuclei binding site in the presence of different TH analogs was as follows: L-T3 > TRIAC > L-T4 = TETRAC > r-T3 > T2 (Fig. 2). The concentrations of analogs required to displace 50% specific binding (ED_{50} value) of [^{125}I] L-T3 to its nuclear binding site were also in the same order (Table I).

Discussion

Adequate reports are not available regarding the presence of L-T3 binding sites in the liver nuclei of mature toad (*Bufo melanostictus*). The find-

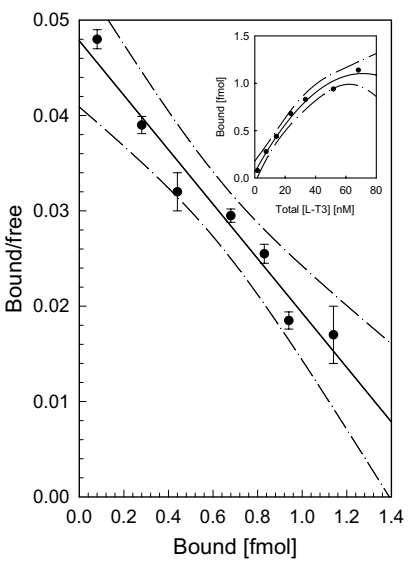

Fig. 1. Scatchard plot of [^{125}I] L-triiodothyronine (L-T3) binding in the mature toad liver purified nuclear fraction. The solid line indicates a single class of high affinity binding sites (•) having a dissociation constant (K_d) of (3.7 ± 0.9) × 10^{-10} M and maximum binding capacity (B_{max}) of 0.074 ± 0.013 pmol/mg DNA. K_d and B_{max} were determined by first-order linear regression analysis (solid line) using Sigma Plot software. Data represent mean ± SEM of four sets of experiments performed in triplicate. The vertical bars denote SEM. Dash-dot line represents 99% confidence interval. Correlation coefficient (r^2) value for the Scatchard plot was 0.96. The inset shows the saturation of the binding sites (solid line) and 99% confidence intervals (dash-dot line) with increasing concentrations of L-T3. Correlation coefficient (r^2) value for the saturation curve was 0.99.

Table I. Relative binding of L-T3 and different TH analogs for the [^{125}I] L-T3 binding sites of the liver nuclei of *Bufo melanostictus*. The results are means of four experiments performed in triplicate. ED_{50} is the concentration of analog that displaces 50% of the bound [^{125}I] L-T3.

Analog	ED_{50} [nM]	Relative nuclear binding (% L-T3) Mean ± SEM
L-Triiodothyronine (L-T3)	0.33	100
Triiodothyroacetic acid (TRIAC)	0.44	75 ± 10
L-Thyroxine (L-T4)	1.7	19.4 ± 3.3
Tetraiodothyroacetic acid (TETRAC)	1.7	19.4 ± 6.1
3,3′,5′-Triiodothyronine (r-T3)	9	3.7 ± 1
Diiodothyronine (T2)	83	0.39 ± 0.05

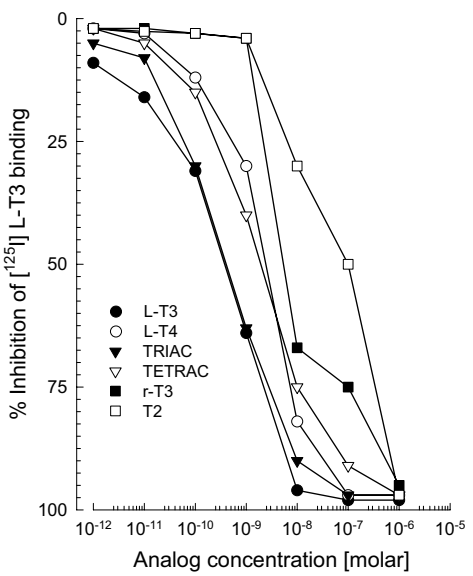

Fig. 2. Representative curves of [^{125}I] L-T3 binding to the purified liver nuclear fraction and its displacement by different thyroid hormone analogs: L-T4, triiodothyroacetic acid (TRIAC), tetraiodothyroacetic acid (TETRAC), 3,3′,5′-triiodothyronine (r-T3) and diiodothyronine (T2). Purified toad liver nuclei were incubated with 10 pM [^{125}I] L-T3 under standard assay conditions alone (total binding) or in the presence of increasing concentrations of unlabeled thyroid hormone analogs. Data were plotted as the percentage of relative inhibition of saturable [^{125}I] L-T3 binding to nuclei in the presence of increasing concentrations of unlabeled analogs. Nonspecific binding was determined in the presence of 15.4 μM of unlabeled L-T3.

ings from our observations suggest availability of high affinity saturable L-T3 binding sites in the liver nuclei of adult tropical female toad, *Bufo melanostictus* (Fig. 1). The Scatchard plot analyses of the present data are in favor with the hypothesis of single class of reversible high affinity [^{125}I] L-T3 binding sites. Presence of L-T3 binding sites in the liver nuclei of adult frog, *Rana catesbiana* (Galton, 1985) has been shown. The TH binding characteristics, particularly the equilibrium dissociation constant, K_d (3.7×10^{-10} M), of isolated liver cell nuclei of toad are closely related to the same order to those in fish livers, for example, in *Heteropneustes fossilis* (Bloch): 0.2×10^{-10} M (Dasmahapatra *et al.*, 1990), in rainbow trout: 1.4×10^{-10} M (Darling *et al.*, 1982), in lake trout: 6.84×10^{-10} M (Weirich *et al.*, 1987), and in a number of other vertebrates indicating an evolutionary conservation of the specific L-T3-binding sites (Bres and Eales, 1988). The maximum binding capacity (B_{max}) of L-T3 for specific nuclear receptors found in this experiment was 0.074 ± 0.013 pmol/mg DNA and was closely related to that found in *Heteropneustes fossilis* (Bloch): 0.17 pmol/mg DNA (Dasmahapatra *et al.*, 1990), in Coho Salmon: 0.13 pmol/mg DNA (Darling *et al.*, 1982), in lake trout: 0.25 pmol/mg DNA (Weirich *et al.*, 1987), in rainbow trout: 0.1 pmol/mg DNA (Bres and Eales, 1988). The B_{max} found in rat was 1.13 pmol/mg DNA, whereas the K_d was 1.08×10^{-9} M (Weirich *et al.*, 1987). Overall, the K_d and B_{max} were in reasonable agreements with those of amphibians and birds. Use of thyroid hormone analogs further suggests the specificity of the nuclear L-T3 binding sites in toad liver. Surprisingly, displacement studies also indicated that TRIAC, a TH analog, was only 25% less potent than L-T3 for the specific [^{125}I] L-T3 binding site in toad liver nucleus. Unlikely in different vertebrate animals, TRIAC showed 50-times more effectiveness than L-T3 in fish brain (Dasmahapatra *et al.*, 1991). However, the TH analog binding studies are in good agreement with other comparable studies in other vertebrates. The K_d value and the B_{max} calculated from our experiment in adult *Bufo*

melanostictus was 200-times more and 22-times less, respectively, than found in hepatic nuclei of *Rana catesbeiana* tadpoles (K_d, 1.65 ± 0.31 × 10^{-12} M and B_{max}, 0.161 ± 0.015 pmol/mg DNA) (Galton, 1986). This is specifically reasonable because during normal amphibian metamorphosis thyroid hormones play crucial role compared to mature frogs. There are ample examples of regulation of metamorphosis in amphibian larvae by TH, but very little is known about the role of TH in adult amphibians. Low doses of L-T3 has been shown to potentiate the activation of vitellogenin gene expression by estradiol-17 beta in primary cultures of adult male and female *Xenopus* hepatocytes and also up-regulated TH beta receptor gene transcript (Rabelo and Tata, 1993). In conclusion, this study described the existence of a saturable and single class of a specific high affinity and limited capacity binding site for L-T3 in the liver nuclei of the adult tropical toad, *Bufo melanostictus*.

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