3,5-di-iodothyronine stimulates tilapia growth through an alternate isoform of thyroid hormone receptor β1

Pamela Navarrete-Ramírez, Maricela Luna, Carlos Valverde-R and Aurea Orozco
Instituto de Neurobiología, Universidad Nacional Autónoma de México (UNAM), Boulevard Juriquilla 3001, Queretaro 76230, Mexico

Abstract
Recent studies in our laboratory have shown that in some teleosts, 3,5-di-iodothyronine (T2 or 3,5-T2) is as bioactive as 3,5,3'-tri-iodothyronine (T3) and that its effects are in part mediated by a TRβ1 (THRB) isoform that contains a 9-amino acid insert in its ligand-binding domain (long TRβ1 (L-TRβ1)), whereas T3 binds preferentially to a short TRβ1 (S-TRβ1) isoform that lacks this insert. To further understand the functional relevance of T2 bioactivity and its mechanism of action, we used in vivo and ex vivo (organotypic liver cultures) approaches and analyzed whether T3 and T2 differentially regulate the S-TRβ1 and L-TRβ1s during a physiological demand such as growth. In vivo, T3 and T2 treatment induced body weight gain in tilapia. The expression of L-TRβ1 and S-TRβ1 was specifically regulated by T2 and T3 respectively both in vivo and ex vivo. The TR antagonist 1–850 effectively blocked thyroid hormone-dependent gene expression; however, T3 or T2 reversed 1–850 effects only on S-TRβ1 or L-TRβ1 expression, respectively. Together, our results support the notion that both T3 and T2 participate in the growth process; however, their effects are mediated by different, specific TRβ1 isoforms.

Key Words
- 3,5-T2
- growth
- thyroid hormone receptor
- 1–850

Introduction
The pleiotropic effects exerted by thyroid hormones (THs) have led to growing interest to identify, besides the amply studied 3,5,3’-tri-iodothyronine (T3), other possible bioactive iodothyronines that could explain this diversity of functions. Accordingly, studies from several laboratories have suggested that 3,5-di-iodothyronine (3,5-T2 or T2), a putative product of the outer ring deiodination pathway involved in T3 metabolism, also possesses bioactivity (Goglia 2005). In fact, studies from our laboratory have shown that both T3 and T2 act directly on TH-dependent genes in teleost liver (García-G et al. 2007) and that these genomic actions are mediated by different isoforms of TRβ1 (Mendoza et al. 2013). Indeed, results from binding and transactivating assays revealed that the effects of T2 on genomic regulation appear to be mediated by a different isoform of TRβ1 that is found only in fish and contains a 9-amino acid insert in its ligand-binding domain, and which we have denominated long TRβ1 (L-TRβ1). By contrast, T3 preferentially binds to and activates the short TRβ1 (S-TRβ1; Mendoza et al. 2013). Furthermore, hepatic in vivo expression of L-TRβ1 is 106-fold higher than that of S-TRβ1, suggesting that, at least in teleosts, the effects of THs are mainly mediated through this isoform. These observations, together with our findings that in vivo,
Effects of 3,5-T2 in tilapia growth

T2 and T3 modulate the expression of L-TRβ1 and S-TRβ1 respectively, suggest a distinct signaling pathway for each TH in teleosts and prompted us to propose an extra level in the cascade of TH signaling for which T2 is specifically made and regulated (Mendoza et al. 2013).

In this study and with the aim of further understanding the functional relevance of T2 bioactivity and its mechanism of action, two approaches were employed. In vivo, we analyzed whether T3 and T2 differentially regulate the S-TRβ1 and L-TRβ1 isoforms during a physiological demand such as body growth. Ex vivo, we closely analyzed the action mechanisms of the observed T3 and T2 effects and explored whether these mechanisms were indeed mediated by a TRβ1 isoform. Our results support the idea that at least in teleosts, T2 is a relevant, physiologically bioactive TH whose genomic effects are mediated by a distinct TRβ1 isoform.

Materials and methods

Animals

Tilapia juveniles (~0.8±0.1 g) were kindly provided by SAGARPA (Querétaro, Mexico). Fish were kept in 101 tanks containing constantly aerated fresh water at a temperature of 25 °C and maintained on a 12 h light:12 h darkness cycle. The fish were fed twice a day (~40 mg/fish per day) with a commercial diet (Sera Marin, Sera, Germany). All animal experimentation (handling and killing) was conducted in accordance with accepted procedures regarding handling and killing of the animals, as reviewed and approved by the Animal Welfare Committee of our Institute.

In vivo experimental design

In all cases, initial fish body weight was recorded before treatments. Experimental groups consisted of ten individuals per group, and all experiments were performed in duplicate tanks (total of 20 fish per experimental group). Fish were treated by immersion in a 1 nM solution of one of the following TH (Sigma): T3, T2, or the inactive isomer rT3. A negative control group was treated with TH previously solubilized in 0.05 M NaOH to the corresponding tank in the morning (0900 h). The treatment was terminated after 8 h by changing the culture water, and this drug administration protocol was repeated three times per week. Treatments were administered for 1 month, after which the fish were weighed and decapitated, and their livers were rapidly removed. In all cases, livers from each experimental group were divided into two and used for RNA and TH extraction (see below).

Ex vivo experimental design

Organotypic cultures of tilapia liver For each experiment, individual juvenile tilapia livers were dissected and placed in ice-cold 50% Hank’s Balanced Salt Solution (Gibco), which contained 50% Minimum Eagle’s Medium (MEM, Gibco), 10 mM HEPES, and 8 mM glucose (pH 7.2). Livers were sectioned at 400 μm thickness on a McIlwain tissue chopper (about 18 slices per liver). For each treatment, at least five tilapia livers were sliced and randomly placed onto inserts of semipermeable membranes made of Biopore CM hydrophilized PTFE, pore size 0.4 μm (six slices per membrane (Millipore, Herts, UK)), and maintained in an incubator at 18 °C in 5% CO2. The culture medium consisted of 50% MEM, 10 mM HEPES, and 35% Hank’s Balanced Salt Solution supplemented with 10% horse serum (Gibco), 8 mM glucose, and 5% penicillin–streptomycin (Gibco).

Treatments After 48 h of stabilization, the medium of each well (1.1 ml/well) was removed and replaced by the appropriate amount of fresh medium, which contained the different drug treatments: T3 or T2 at concentrations of 0.1, 1, 10, and 100 nM previously solubilized in NaOH or the TR antagonist 1–850 (Schapira et al. 2003; Calbiochem, Darmstadt, Germany) previously solubilized in DMSO at concentrations of 0.1, 1, 10, and 20 μM alone or combined with 100 nM T3 or T2. In all cases, after a 24-h incubation, slices from each plate were removed and pooled for mRNA extraction and quantification. All experiments were performed independently at least twice in duplicate.

Quantification of intrahepatic T3

TH extraction Liver segments from five individual fish per experimental groups were pooled (~50 mg tissue/pool) and homogenized in ten volumes of methanol: ammonium hydroxide (99:1) solution, as described previously (Garcia-G et al. 2004). The homogenates were centrifuged for 10 min at 700 g. The supernatants were collected, evaporated in a speed vacuum, and suspended 1:5 (initial w/v) in assay buffer Tris–HCl (0.05 M; pH 8.6).
**T₃ RIA**  Intrahepatic T₃ content was measured by RIA as described previously (Orozco et al. 1992). The inter- and intra-assay coefficients of variation were 9.5 and 6.6% respectively. The incubation mixture contained assay buffer and a working dilution (1:8000) of anti-T₃ serum (Sigma), standard (standard curve, 7.8-1000 pg/dl), the radioactive solution (10 pg/100 μl of the labeled T₃ plus 10 μg/10 ml 8-anilino-1-naphthalene sulfonic acid (Sigma)), and 50 μl of the experimental sample. Free and antibody-bound radioactive T₃ were separated using 0.5% activated charcoal/dextran solution (Sigma). 

**Measurement of mRNA expression**

In all cases, mRNA was quantified by real-time PCR as described previously (Mendoza et al. 2013). Briefly, total RNA was extracted from juvenile tilapia liver (TRIZol, Invitrogen), and RT (M-MLV, Promega) from 2 μg of total hepatic RNA was performed using oligo(dT) primer (final volume of 25 μl). β-actin was used as an internal standard in reactions that contained 1 μl of the RT reaction, 6 μl Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Waltham, MA, USA), and 500 nM forward and reverse primers in a final volume of 12 μl. Oligonucleotides and PCR protocols used for all gene amplification are specified in Table 1. In all cases, the standard curve ranged from 10¹ to 10⁹ molecules/μl and obtained by comparison with the standard curve, normalized to the concentration of β-actin in each experimental sample. Identical PCRs from the RNA samples before the RT reaction yielded no detectable products.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Amplicon (bp)</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Real-time PCR protocol</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>170</td>
<td>ACT TCG AGC AGG AGA TGG</td>
<td>GGT GGT TTC GTG GAT TCC</td>
<td>3 s at 95 °C; 7 s at 52 °C; and 8 s at 72 °C for 45 cycles</td>
<td>1.96</td>
</tr>
<tr>
<td>IGF1</td>
<td>217</td>
<td>AAC CTT GGG TGC TCT TGG CAT G</td>
<td>GTC TGT GGA GAG CGA GGC TTT</td>
<td>3 s at 95 °C; 8 s at 56 °C; and 9 s at 72 °C for 45 cycles</td>
<td>1.80</td>
</tr>
<tr>
<td>L-TRβ1</td>
<td>232</td>
<td>GTG AAG GAA GCT AAG CCT GA</td>
<td>CAC AAG GCA GCT CAC AGA AC</td>
<td>3 s at 95 °C; 10 s at 52 °C; and 10 s at 72 °C for 55 cycles</td>
<td>1.96</td>
</tr>
<tr>
<td>S-TRβ1</td>
<td>135</td>
<td>GCG GAA ATT CCT GCC TGA G</td>
<td>GCA GCT CAC AGA ACA</td>
<td>2 s at 95 °C; 8 s at 52 °C; and 7 s at 72 °C for 45 cycles</td>
<td>2.01</td>
</tr>
<tr>
<td>D2</td>
<td>249</td>
<td>GAA ACT TGG CTG TGA GGC</td>
<td>TGG GC CTC GTC GAT GTA GAC CAG</td>
<td>2 s at 95 °C; 7 s at 58 °C; and 10 s at 72 °C for 35 cycles</td>
<td>2.08</td>
</tr>
</tbody>
</table>

R, amplification efficiency for the standard curve.

**Statistical analysis**

Results obtained in all experiments were analyzed using a one-way ANOVA coupled with the Tukey’s multiple-comparison test (control vs treatments). Differences were considered statistically significant at P values of ≤0.05.

**Results**

**In vivo experiments**

**Validation of iodothyronine treatment**  An initial aim of this study was to test T₂ bioactivity. To this end, effects of T₂ on body growth were measured as weight gain. As this is a long-term treatment, we designed an administration protocol that consisted in exposing the fish to very low doses of TH (1 nM in water culture) and limited the exposure to 8 h/day, 3 days/week for 1 month. This hormone concentration is at least ten times lower than that employed in other studies (Lam 1980, Nacario 1983, Lam & Sharma 1985, Reddy & Lam 1992, De Jesus et al. 1998, Lam et al. 2005). Furthermore, the administration protocol had a positive effect on fish growth and no negative effects on fish survival, while it induced a burst in growth of ~30% in both T₃- and T₂-treated fish (Table 2). Most importantly, the low TH concentrations used did not alter the thyroidal homeostasis of the fish, as judged by the intrahepatic T₃ levels, which were similar to those recently reported for intramuscular zebrafish T₃ and T₂ (1.8±0.2 and 2.8±0.4 ng/g respectively; Little et al. 2013). Indeed, despite the fact that intrahepatic T₃ concentrations remained within the euthyroid range in all tilapia treated with the different THs, only fish treated with 1 nM T₃ or T₂ presented a four fold body weight gain compared with the control group (P<0.001; Fig. 1).
By contrast, weight gain of rT3-treated fish was similar to that observed in euthyroid tilapia, while those exposed to MMI exhibited the lowest intrahepatic concentration of T3 (P!0.001) and the lowest rate of weight gain (Fig. 1).

Regulation of TRβ1 isoforms by T3 and T2

Given that T3 and T2 treatment induced growth to a similar extent, we then analyzed whether their effects were differentially mediated by the S-TRβ1 and L-TRβ1 isoforms. To this end, hepatic expression of the mRNA for these two TRβ1s was quantified. As shown in Fig. 2, hepatic expression of the short isoform of TRβ1 was higher only in fish exposed to T3. By contrast, expression of the L-TRβ1 isoform was upregulated only in the liver of tilapia treated with 1 nM T2; no upregulation was observed in the group of rT3-treated fish (Fig. 2). mRNA levels of both TRβ1 isoforms in MMI-treated fish were significantly decreased compared with the control group. The fact that T2 and T3 exclusively upregulate the expression of the long and short isoforms of TRβ1 respectively suggests that the effects of these two THs are mediated by two distinct isoforms of TRβ1.

Effect of T3 and T2 on hepatic IGF1 and D2 expression

In order to analyze whether the differential observed effects of T3 and T2 on the expression of L-TRβ1 and S-TRβ1s were specific, hepatic expression of two other TH-regulated genes was quantified. IGF1 (igf1) and D2 (d2) genes were chosen as they are positively and negatively regulated by THs respectively (Schmid et al. 2003). As depicted in Fig. 2, treatment with T3 and T2 equivalently enhanced hepatic expression of IGF1 and blocked that of D2; thus, T3 and T2 do not differ in how they regulate the expression of these two genes. rT3-treated fish showed euthyroid levels of hepatic expression of IGF1 and D2, while those in MMI-treated fish were significantly decreased (IGF1) and increased (D2).

Ex vivo experiments

Regulation of gene expression by T3 and T2

Organotypic liver cultures responded to TH treatment. Expression of mRNA for IGF1 and D2 was up- and downregulated by T3 and T2 treatments respectively (Fig. 3). This pattern of regulation was similar to that observed in the in vivo experiments, validating the use of organotypic liver cultures to study the regulation of TH-regulated genes. We then analyzed whether cultures treated with T3 or T2 showed differential regulation of the two TRβ1 isoforms. Interestingly, the pattern of TRβ1 regulation by T3 and T2 was inverted when compared with the in vivo studies. Indeed, the expression of the short isoform of TRβ1 was lower only in fish exposed to T3, whereas the expression of the L-TRβ1 isoform was downregulated only in the liver cultures treated with T2 (Fig. 4). Furthermore, in agreement with the reported affinity of the tilapia TRβ1 isoforms for their corresponding ligands (Mendoza et al. 2013), T2 treatment did not influence

Table 2  Juvenile tilapia weights before and after a 30-day treatment with the different thyroid hormones (1 nM) or methimazole (4.5 mM). Values are means ± S.E.M. (n=20)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial weight (g)</th>
<th>Final weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.885±0.015</td>
<td>1.019±0.625</td>
</tr>
<tr>
<td>rT3</td>
<td>0.916±0.006</td>
<td>1.041±0.710</td>
</tr>
<tr>
<td>T3</td>
<td>0.853±0.0375</td>
<td>1.609±0.880</td>
</tr>
<tr>
<td>T2</td>
<td>0.810±0.010</td>
<td>1.628±0.865</td>
</tr>
<tr>
<td>MMI</td>
<td>0.788±0.0025</td>
<td>0.865±0.350</td>
</tr>
</tbody>
</table>

Figure 1
(Top panel) Intrahepatic T3 concentrations. (Bottom panel) Body weight gain. Tilapia were treated for 30 days with 1 nM T3, T2, or rT3. A negative control group was treated with 4.5 mM MMI. Values are means ± S.E.M. (n=20). Significance is indicated (*P!0.05 and **P!0.001).
S-TRβ1 expression even at the highest concentrations tested (100 nM), although this concentration of T3 did downregulate L-TRβ1. The fact that T2 and T3 specifically downregulated the expression of the long and short isoforms of TRβ1 respectively further supports the notion that the effects of these two THs are mediated by two distinct isoforms of TRβ1 (Fig. 4).

**Effects of the TR antagonist 1–850 upon gene expression** As shown in Fig. 3, and consistent with their regulation by THs, 1–850 effectively blocked IGF1 expression while it increased that of D2, further supporting the idea that both hormones act through a TR pathway. Furthermore, the co-treatment with 100 nM T3 or T2 reversed the effects of the TR antagonist 1–850, an effect that was blunted at 10 and 20 µM antagonist, probably because 1–850 has a 1000-fold lower affinity than T3 for the receptor (Schapira et al. 2003). The expression of the long and short isoforms of TRβ1 was not affected by 1–850 treatment; however, T3 and T2 co-administration downregulated S-TRβ1 and L-TRβ1 respectively only at low (0.1 and 1 µM) concentrations of 1–850 (Fig. 4).

**Discussion**

Our previous studies support the notion that T2 bioactivity in fish is mediated by the L-TRβ1 isoform, whereas T3 acts by binding to the S-TRβ1 isoform (Mendoza et al. 2013). As we used either mammalian GH3 cells, or fish acutely treated with T3 and T2 in those experiments, the observed TRβ1 isoform specificity for a given ligand could not be functionally corroborated. Thus, one of the goals of this study was to analyze whether T2, like T3, could regulate a physiological demand such as growth and whether this regulation was mediated by the L-TRβ1 isoform. We here show for the first time that treatment with T2 effectively stimulates growth in tilapia fingerlings in the same manner as observed for T3. Therefore, and although the effects of THs on growth have been solely attributed to T3 (Power et al. 2001), our present results clearly show that T2 is also a physiologically active player in this process, at least in tilapia, validating fish growth as a useful marker to analyze the possible differential regulation of the TRβ1 isoforms that mediate T3 and T2 effects.

Results from both *in vivo* and *ex vivo* T3 or T2 treatments revealed an equivalent enhancement of IGF1 and blockade of D2 liver expression, showing that T3 and T2 do not differ in how they regulate the transcription...
of these two genes. By contrast, results regarding the regulation of the TRβ1 gene by T3 and T2 under the two experimental approaches were different. In previous studies, in which tilapia were acutely treated with either T3 or T2, the classical downregulatory response of the liver S-TRβ1 and L-TRβ1 mRNAs respectively was observed (Mendoza et al. 2013); this effect was also seen here in the organotypic liver cultures (Fig. 4) and was previously reported for the TRβ1 gene in other vertebrates treated with T3 (Sadow et al. 2003). Interestingly, however, in the present long-term (1 month) in vivo studies, the expression of mRNAs for both the L-TRβ1 and S-TRβ1 was upregulated by T2 and T3 treatment respectively (Fig. 2). This regulatory pattern resembles that of tadpoles (Sachs et al. 2000, Tata 2000) and flatfish (Yamano & Miwa 1998, Marchand et al. 2004, Galay-Burgos et al. 2008), in which a pre-metamorphic rise in circulating THs is followed by an increase in the expression of TRβ1. The auto-induction of TR and other nuclear receptors is linked to developmental actions and seems to be a prerequisite for a sequential activation of target genes involved in a specific biological action (Tata 2002). As there were no age or environmental distinctions between tilapia in previous study (Mendoza et al. 2013) and this study, it is reasonable to suggest that the observed differences in TRβ1 regulation are mainly due to the administration protocols. Thus, we hypothesize that the intermittent administration protocol used in the present set of experiments partially mimics the pulsatile physiological stimulus of THs and increases the number of receptors, which in turn activate growth-related target genes to stimulate body growth, an event that, like metamorphosis, involves proliferative effects.

We had previously shown that MMI treatment induced the classic upregulation of both hepatic TRβ1 isoforms in juvenile tilapia (Mendoza et al. 2013). Surprisingly, the partial blockade of TH synthesis which results from the intermittent MMI administration protocol elicited the opposite regulatory effect, despite the fact that intrahepatic T3 concentrations were below euthyroid levels in both experiments. Interestingly, others have reported a TRb, but not TRa, downregulation after up to 15 days of exposure to the goitrogen thiourea (400 μM) in metamorphosing Solea senegalensis larvae (Manchado et al. 2009). The fact that both THs and MMI intermittent treatments regulated the TRβ gene in a manner similar to that observed in metamorphosing fish or amphibian is intriguing, especially as none of the other studied genes elicited such a regulatory response. The underlying mechanisms for this opposite regulation

![Figure 3](image-url)

**Figure 3**

mRNA expression from organotypic liver cultures of IGF1 and D2. Tilapia organotypic liver cultures were treated for 24 h with 0.1–100 nM T3 (top panel) or T2 (bottom panel), or with 0.1–20 μM of 1–850 without or with 100 nM T3 (top panel) or T2 (bottom panel, n = 6 slices per experimental group). Values are means ± S.E.M. of three independent experiments. Significance is indicated (*P < 0.01 and **P < 0.001).
are not known, and further studies would be needed to clarify this issue.

To further support the notion that the actions of T₂ upon body weight gain were indeed mediated by the L-TRβ₁ isoform, we treated organotypic liver cultures with the TR antagonist 1–850. This antagonist has been reported to bind with high affinity to both TRα and TRβ in mammalian systems (Schapira et al. 2003). In these studies, 1–850 treatment effectively blocked transcription of a positively regulated gene (IGF1); however, it either enhanced (D2) or had no effect (TRβ1s) on transcription of negatively regulated genes. A similar lack of effect on TRβ1 expression by the TH antagonist NH3 had previously been observed in Xenopus TRβA (Opitz et al. 2006). However, the different response patterns of the two negatively regulated genes are intriguing. Several factors may contribute to this response, i.e. unliganded TRs have ligand-independent actions; thus, unliganded- or 1–850-liganded TRs may adopt altered conformations, which could change coregulator recruitment and specific gene expression independently of the effects on TH binding (Shah et al. 2008). Nonetheless, in all cases, the effects of 1–850 were blunted after treatment with T₃ or T₂. Importantly, T₃ reversed the effects of 1–850 only upon the expression of the S-TRβ₁ isoform and T₂ had similar effects only upon that of the L-TRβ₁ isoform, further demonstrating that this L-TRβ₁ isoform selectively mediates T₂ actions.

Aside from its well-described extranuclear effects upon metabolism in mammals (Goglia 2005), the physiological relevance of T₂ has been unnoticed until recently, and T₃ has been accepted as the foremost TH capable of genomic action. A major finding in this study consists in the demonstration that, through interacting with a specific and distinct TRβ₁ isoform, T₂ effectively triggers an outburst of body growth in tilapia fingerlings, with an efficiency comparable to that of T₃. These results confirm and extend previous reports from our laboratory and others suggesting that T₂ is also an important transcriptional regulator, at least in teleosts (García-G et al. 2004, 2007, Little et al. 2013, Mendoza et al. 2013).

The lack of available data on circulating or intratissular T₂ concentrations has masked the importance of this TH. However, very recent studies have shown that zebrafish intramuscular T₂ concentrations closely resemble those of T₃ (Little et al. 2013). These data together with our previous findings that T₂ preferentially binds to and activates the L-TRβ₁, an isoform that is expressed in vivo at 10⁴-fold higher levels than S-TRβ₁, support the notion that, at least in teleosts, T₂ is physiologically relevant.

**Figure 4**

mRNA expression from organotypic liver cultures of short TRβ1 and long TRβ1. Organotypic tilapia liver cultures were treated for 24 h with 0.1–100 nM T₃ (top panel) or T₂ (bottom panel) or with 0.1–20 µM, 1–850 without or with 100 nM T₃ (top panel) or T₂ (bottom panels; n = 6 slices per experimental group). Values are means ± S.E.M. of two independent experiments. Significance is indicated (*P<0.05 and **P<0.001).
THRA

Lam TJ 1980 Thyroxine enhances larval development and survival in that the two TRβ isoforms are differentially regulated by these two ligands raises the possibility that additional genes may also be differentially regulated, a suggestion that warrants further attention.

Declaration of interest

P N-R is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México (UNAM), and received fellowship 216637 from CONACYT.

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