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Differential effects of TR ligands on hormone dissociation rates: Evidence for multiple ligand entry/exit pathways

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ABSTRACT

Some nuclear receptor (NR) ligands promote dissociation of radiolabeled bound hormone from the buried ligand binding cavity (LBC) more rapidly than excess unlabeled hormone itself. This result was interpreted to mean that challenger ligands bind allosteric sites on the LBD to induce hormone dissociation, and recent findings indicate that ligands bind weakly to multiple sites on the LBD surface. Here, we show that a large fraction of thyroid hormone receptor (TR) ligands promote rapid dissociation ($T_{1/2}$ < 2 h) of radiolabeled T_3 vs. T_3 ($T_{1/2} \approx 5$–7 h). We cannot discern relationships between this effect and ligand size, activity or affinity for TRβ. One ligand, GC-24, binds the TR LBC and (weakly) to the TRβ-LBD surface that mediates dimer/heterodimer interaction, but we cannot link this interaction to rapid T_3 dissociation. Instead, several lines of evidence suggest that the challenger ligand must interact with the buried LBC to promote rapid T_3 release. Since previous molecular dynamics simulations suggest that TR ligands leave the LBC by several routes, we propose that a subset of challenger ligands binds and stabilizes a partially unfolded intermediate state of TR that arises during T_3 release and that this effect enhances hormone dissociation.

1. Introduction

Nuclear receptors (NRs) regulate gene expression in response to small signaling molecules [1]. The NR family includes receptors for thyroid hormone (TH) [2], steroid hormones, vitamins A and D, cholesterol and fatty acid derivatives, heme, glucose and other molecules. Since NRs play widespread roles in development and disease, they are important targets for pharmaceutical discovery. TH receptors (TRs) are the subject of efforts to develop selective agonists to ameliorate aspects of metabolic syndrome without harmful effects on heart and antagonists to treat hyperthyroidism and other conditions [3,4]. Improved understanding of mechanisms of NR ligand association and dissociation will provide insights into receptor function and could suggest ways to stabilize or destabilize bound hormone, improve antagonism and facilitate development of drugs that interact tightly and selectively with cognate NRs.

NRs harbor a single ligand binding cavity (LBC) whose location, relationship to gene activation and organization have been extensively studied [1,5,6]. X-ray structures of NR LBDs with agonists reveal that the LBC is buried in the C-terminal ligand binding domain (LBD). Agonists promote packing of C-terminal helix (H) 12 against the LBD to complete a coactivator binding surface, activation function 2 (AF-2) [5,7]. Close investigation of the LBCs of the two TRs (TRα and TRβ) revealed one subtype specific amino acid in the TR LBC involved in ligand contact (TRβN331/TRαS277) and it has been possible to exploit this difference to obtain TRβ selective ligands [3,8]. X-ray structures also reveal that the buried pocket is flexible; the TR LBC can expand to accommodate a bulky 5′ iodine substituent in the parental form of TH, thyroxine (T_4), and a bulky 3′ phenyl group in the TRβ selective agonist, GC-24 [6,9,10].

In contrast, mechanisms of ligand binding and dissociation from the LBC are only partly understood [11,12]. X-ray structures of NR LBDs reveal that H12 can move to expose the LBC, and this probably constitutes one ligand escape route [5,7,11,12]. However, our analyses of regions of instability in X-ray structures [13–15] and molecular dynamics simulations [11,12] suggest that active TH (triiodothyronine, T_3) can escape from the LBD in three ways:

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under H12 (Path I, described above); between H8 and H11 near the dimer/heterodimer surface at the H10/H11 junction (Path II); and through the H1–H3 loop (Path III). Our simulations also suggest that escape routes vary with ligand and receptor; T_3 prefers Path III whereas the TRβ-selective GC-24 prefers Path I [12].

While the notion that there are multiple ligand escape paths await definitive verification, a number of data are consistent with this conclusion. Structural elements that permit ligand escape through each pathway are implicated in stable agonist binding [11,12]. For Path I, suboptimal packing of TRβ H12 against the T_4–LBD complex is associated with rapid ligand dissociation [9]. Conversely, point mutations and coactivators that stabilize estrogen receptor (ER) or TR H12 in the active position reduce hormone dissociation rates [16–18]. For Path II (involving residues near the dimer surface), resistance to thyroid hormone syndrome (RTH) mutations that affect this region enhance T_3 dissociation rates [19]. For Path III, X-ray structural analysis of other RTH mutants reveals that increased T_3 dissociation rate is associated with disorder in the H1–H3 loop [14,15,19].

In spite of strong evidence for a single high affinity hormone binding site, early studies raised the possibility that NRs harbor auxiliary ligand binding sites that exert allosteric effects on bound hormone. Some NR interacting compounds displace bound high affinity ligands more rapidly than the high affinity ligand itself. Progesterone, for example, binds glucocorticoid receptors (GRs) with lower affinity than dexamethasone, and acts as an antagonist, yet displaces this higher affinity agonist more rapidly than dexamethasone [20]. The mechanism of this effect is not clear, but it was proposed that progesterone binds to an undefined allosteric site to promote dexamethasone dissociation.

Recent evidence confirmed that there are multiple ligand binding sites on NR LBD surfaces. Several compounds bind to NR (including TR) AF-2 sites [21–23]. Other compounds were found at another location on the androgen receptor surface (BF-3) in X-ray screens and ligand binding to BF-3 may exert allosteric effects on AR AF-2 [24]. Finally, the TR agonist GC-24 binds to at a location near the TR dimer/heterodimer surface at the junction of TRβ H10 and H11 [10].

In this paper, we show that TR ligands (including GC-24) displace bound hormone at different rates and investigate this phenomenon. The effect is not related to ligand affinity, activity or size and does not appear to involve surface ligand interactions. Instead, several lines of evidence suggest that the challenger interacts with the LBC to displace bound hormone. We propose that challengers promote ligand release by binding partially unfolded conformational intermediates that occur in ligand release and blocking refolding of the hormone/receptor complex around labeled ligand. Implicit in this hypothesis is the concept that different ligands associate with TRs via different pathways.

2. Materials and methods

2.1. Plasmids

Expression vectors for TRs (CMX-TRβ, CMX-TRα), TRβ mutants (CMX-TRβP419R, L422R, M423R, N331S) and the TRα mutant (TRαS277N) are described [8,19]. TRs were expressed in TNT T7 Quick *in vitro* coupled transcription/translation kits, according to manufacturer's protocols (Promega, Madison, WI).

2.2. T_3 binding

T_3 binding affinities were determined by saturation binding assays [19]. Approximate amounts of TRs were determined by measurement of T_3 binding activity in single point binding assays; TR preparations were incubated overnight at 4 °C with 1 nM L-3,5,3′-^{125}I-T_3 (NEN Life Science Products) in 100 μl binding buffer (400 mM NaCl, 20 mM KPO_4, pH 8, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 10% glycerol) containing 1 mM monothioglycerol and 50 μg calf thymus histones (Calbiochem). Bound ^{125}I-T_3 was separated from free ligand by gravity flow through a 2 ml course Sephadex G-25 column (Pharmacia Biotech) and quantified on a γ-counter (COBRA, Packard Instruments, Meriden, CT). The number of binding sites per unit volume were calculated from specific activity of radiolabeled T_3 (3824 cpm = 1 fmol). For saturation binding, 10–20 fmol of TR protein were incubated overnight at 4 °C with varying concentrations of ^{125}I-T_3. Amount of ^{125}I-T_3 was verified by precount in each aliquot, prior to addition of protein. Next morning, bound vs. free ^{125}I-T_3 was determined by passage over the Sephadex G-25 column, as above. In these conditions, non-specific binding of ^{125}I-T_3 to unprogrammed reticulate lysates was negligible; >1% observed in the presence of 20 fmol TRs, as was residual binding of 1 nM ^{125}I-T_3 obtained with a 1000-fold excess of unlabeled T_3 (not shown). T_3 applied to the column in the absence of TRs only dissociates after several hours of washing, and does not contribute to measurements of bound T_3 (not shown). Thus, most (>99%) of labeled ligand that passes through the Sephadex G-25 column corresponds to TR bound to T_3. K_d values were calculated by fitting saturation curves to the equations of Swillens using the GraphPad Prism program (GraphPad Software V3.03, San Diego, CA).

T_3 association (k_{on}) and dissociation (k_{off}) rates were determined using methods similar to saturation binding assays, with the following modifications. For k_{off}, TRs were incubated overnight with saturating (1 nM) ^{125}I-T_3 at 4 °C [9,19]. Unlabeled T_3 or challenger was added to a final concentration of 1 μM (1000-fold excess) the following morning and aliquots were taken at various times and applied to Sephadex G-25 columns to determine how rapidly ^{125}I-T_3 dissociates from TR. Binding curves and k_{off} values were calculated using the GraphPad Prism one phase exponential decay model. For k_{on}, unliganded TR preparations were added to binding buffer containing 1.5 nM ^{125}I-T_3 to a final concentration of 20 fmol TRs per 100 μl of buffer. 100 μl aliquots were then applied at various times to Sephadex G-25 columns to separate bound from unbound T_3. In these conditions, T_3 is in excess of receptor, only about 10% of T_3 present in the initial mix associates with the TR at equilibrium and the remainder remains unbound. Binding curves and k_{on} values were calculated, where possible, by non-linear regression analysis using one and two phase association growth models with Graph Pad Prism Software. The program identifies the best fit (one/two phase) for each curve.

2.3. Gel shifts

Binding of TR to TREs was assayed by mixing 20 fmol of ^{35}S-labeled TRs produced in a reticulocyte lysate (TNT T7; Promega), with 10 ng oligonucleotide and 1 μg poly(dI-dC) (Amersham Pharmacia Biotech) in final volume of 20 μl 1× binding buffer (25 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, 10 μM $ZnSO_4$, 0.1% NP-40, 5% glycerol). After 30′ incubation, the mixture was loaded onto a 5% non-denaturing polyacrylamide gel that was pre-run for 30 min at 200 V and run at 4 °C for 120 min at 240 V, in a running buffer of 6.7 mM Tris (pH 7.5), 1 mM EDTA, and 3.3 mM sodium acetate. The gel was then fixed, treated with Amplify (Amersham Pharmacia Biotech), dried and exposed for autoradiography. TRs used in assay were quantified with ^{125}I-T_3 binding assay and SDS-PAGE analysis of ^{35}S-TRs.

2.4. GST-pulldowns

Full-length hRXRα was prepared in *Escherchia coli* BL21 as a fusion with glutathione S-transferase (GST) as per the manufac-

turer's protocol (Amersham Pharmacia Biotech). The bindings were performed by mixing glutathione-linked Sepharose beads containing 4 μg of GST fusion proteins (Coomassie Plus protein assay reagent, Pierce) with 1–2 μl of the ^{35}S-labeled wild type hTRβ in 150 μl of binding buffer (20 mM HEPES pH 7.5, 150 mM KCl, 25 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors) containing 20 μg/ml bovine serum albumin for 1.5 h. Beads were washed three times with 200 μl of binding buffer, and the bound proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

3. Results

3.1. TR ligands vary in effects on T_3 dissociation

We examined abilities of different TR ligands to displace bound hormone from the TRβ LBC in kinetic studies [14,15,19]. For these assays (in schematic in Fig. 1A), TR preparations were incubated overnight with saturating labeled T_3 (1 nM) to allow stable hormone–TR complex formation and challenged with excess unlabeled ligand to prevent reassociation with radiolabeled T_3. Displacement of radiolabeled hormone was monitored by size exclusion chromatography, which separates T_3–TR complexes from free hormone.

TRβ selective agonists vary in effects on T_3 displacement (Fig. 1B). The half-life ($T_{1/2}$) of the TRβ–T_3 complex at 4 °C was 5–6 h; consistent with previous measurements [9]. Similar values were obtained when the TRβ–T_3 complex was challenged with excess GC-1, a synthetic TRβ selective ligand that binds TRβ with similar affinity to T_3 [8]. However, T_3 dissociated more rapidly in the presence of excess GC-24 ($T_{1/2}$ varied between 30 and 120 min over these studies). Differential effects of GC-1 and GC-24 on T_3 release were not related to affinities for TR; all three agonists displaced T_3 with similar K_d values (0.1–0.15 nM inferred from K_i determinations, see Section 2) in equilibrium hormone binding assays, in accordance with previous results (not shown).

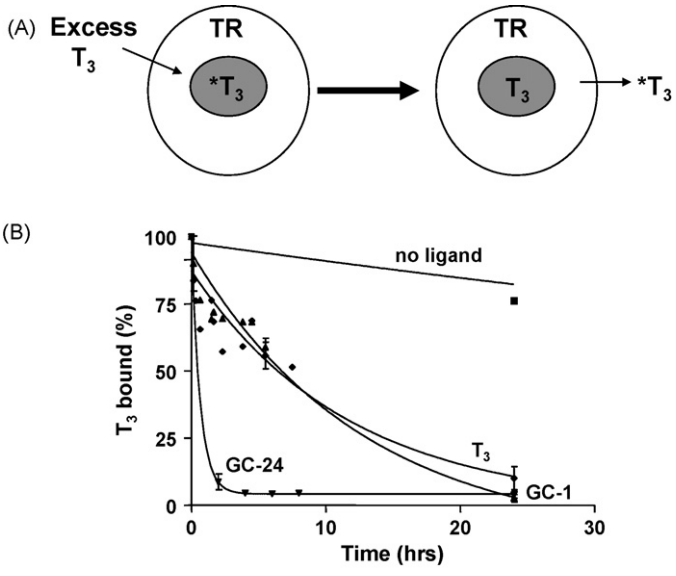

Fig. 1. GC-24 promotes rapid T_3 dissociation. (A) Schematic of T_3 dissociation assay. TR is incubated with saturating (1 nM) amounts of radiolabeled (*) T_3 and the system is allowed to reach equilibrium. The complexes are then challenged with excess (1 μM) unlabeled T_3 or alternate ligand, so that bound ligand is displaced. (B) Results of typical experiment in which rates of T_3 dissociation from TRβ are measured in response to challenge with T_3, GC-1 and GC-24.

Other TR ligands promoted rapid T_3 release. We previously described GC-1 derivatives with bulky 5′ extensions that exhibit diverse activities, from full agonist to full antagonist, and bind TRs with a range of affinities [25,26]. Of this series, eight of ten compounds displaced T_3 more rapidly than native hormone (Fig. 2, GC-24 with a 3′ extension is also shown as a reference). This effect was not obviously related to ligand activity; rapid T_3 release was observed with an agonist (NH-1), partial agonists (GC-14, NH-2, NH-6) and full antagonists (NH-3, NH-5, NH-7 and HY-4). Rapid T_3 release was unrelated to affinity; the same phenomenon was observed with compounds that bind TRs with low and high affinity. For example, HY-4 (K_d = 146 nM) displaced T_3 as rapidly as NH-2 (K_d = 0.52 nM).

TR interacting compounds that lack large extensions also varied in their ability to displace bound hormone (Fig. 3A). The synthetic TR agonist DIMIT displaced T_3 at the same rate as T_3 itself, but three other TH derivatives displaced T_3 more rapidly than T_3. These were (a) Triac, a low abundance active TH that binds TR with high affinity and is produced by deamination of thyroid hormone in the liver [27]; (b) thyroxine, T_4, the parental form of thyroid hormone which binds TR with moderate affinity [9]; and (c) reverse T_3, a product of thyroid hormone metabolism that binds weakly to TRs and acts as a partial agonist [18]. Ligands that bind other NRs with high affinity, but not TRs, did not enhance T_3 dissociation, including progesterone, testosterone, the synthetic androgen R1881, the mineralocorticoid receptor antagonist spironolactone and estradiol (Fig. 3B).

3.2. TR dimer mutations enhance T_3 dissociation but do not abolish GC-24 effects

TRs exist as a mix of dimers and monomers in solution with T_3 promoting monomer formation. Since the GC-24 surface binding site lies near the dimer surface [10], we examined the possibility that GC-24 interactions with this surface were involved in rapid T_3 dissociation. In accordance with previous results [19,28], mutant TRs that only form monomers (P419R, L422R, M423R) bound T_3 with similar affinity to wild type TRs (data not shown). However, dimer surface mutations did alter ligand binding kinetics; T_3 dissociated more rapidly from TRβL422R than wild type TRβ (Fig. 4A) and TRβL422R also exhibited increased rates of T_3 association (Fig. 4B), with more than half the mutant TRs occupied by T_3 within 5 min. Similar results were obtained with TRβP419R and TRβM423R that impair dimer and heterodimer formation (Fig. 4C and not shown). This resembles studies with ERs, which showed that estradiol dissociated more rapidly from monomers than homodimers [29]. Thus, mutations in the TR dimerization surface affect ligand binding kinetics. However, GC-24 continued to promote rapid release of labeled T_3 from each TR mutant that exists as an obligate monomer (Fig. 4C). This implies that GC-24 does not increase TR ligand dissociation by blocking residual TR–TR dimer interactions that occur in the presence of this ligand.

RXR–TR heterodimer formation involves the same TR surface that mediates homodimer formation, including residues implicated in GC-24 surface contact [28]. However, by contrast to TR–TR homodimer formation, RXR–TR heterodimer formation is not affected by hormone [2,19]. RXR did not affect the rate of radiolabeled T_3 dissociation, in the presence of T_3 or GC-24 (Fig. 5A). Control assays confirm that RXR–TRs to form in these conditions and that GC-24 did not disrupt heterodimer formation in these conditions, either in gel shift assays on DNA or pulldown assays in solution (Fig. 5B and C). Thus, assuming that RXR heterodimer formation does occlude the surface GC-24 binding site, our data suggest that GC-24 interactions at the TR surface are not necessary for increased rates of T_3 release.

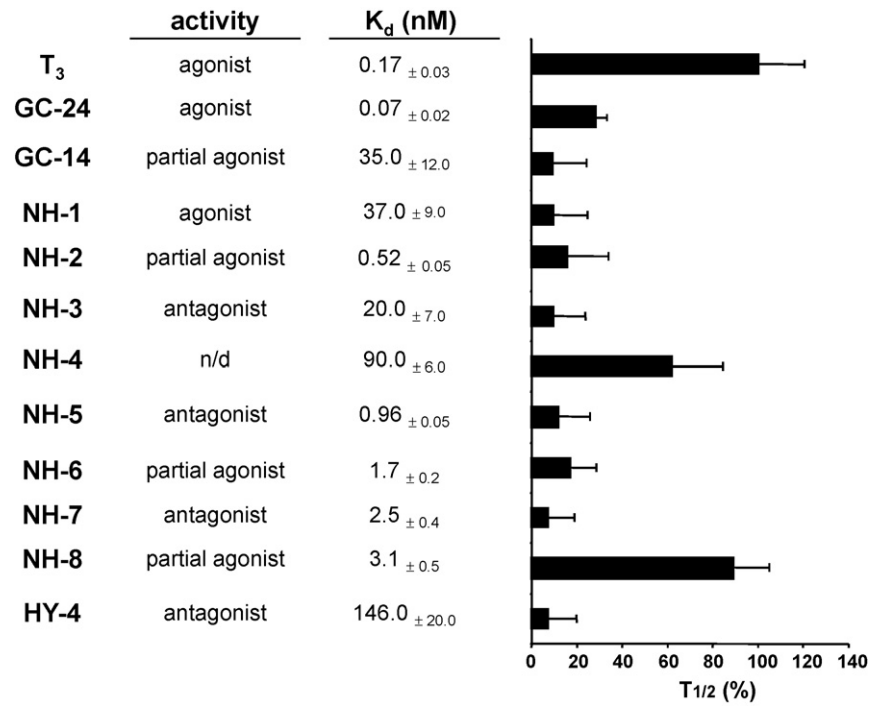

Fig. 2. Multiple GC-1 derivatives rapidly displace bound T_3. Comparative $T_{1/2}$ values of TRβ–T_3 complex determined in response to challenge with GC-1 derivatives. The graph represents averages of multiple experiments, compared to half-life of the TRβ–T_3 complex determined in parallel in the presence of excess T_3 (100%). Activity of ligand and K_i values are listed at the side of the graph.

3.3. TR isoform-specific ligand effects on T_3 dissociation

To test whether effects of ligands on T_3 release were TR isoform-specific, we compared effects of different challengers on TRβ and TRα. Radiolabeled T_3 dissociated from both TRs at similar rates ($T_{1/2} \approx 4$–5 h; Fig. 6). Similar results were obtained with GC-1, even though this ligand binds more tightly to TRβ. By contrast, GC-24 (which binds TRβ about 40–100 times more tightly than TRα [10]) promoted more rapid release of radiolabeled T_3 from TRβ than T_3, but not from TRα. Similar results were obtained with the weakly TRβ selective antagonist NH-3 (not shown).

Interestingly, TR isoform-specific effects of GC-24 on T_3 dissociation were partly sensitive to mutation of the buried LBC [8]. T_3 dissociation was not altered by mutations that reverse the LBC subtype specific residue (TRβN331S and TRαS277N) in response to excess T_3 or GC-1 (Fig. 6). However, the TRα S277N mutant (which converts the LBC to that of TRβ) exhibited more rapid T_3 dissociation than native TRα in the presence of GC-24. The converse TRβN331S mutant (which converts the TRβ LBC cavity to that of TRα) did not reduce T_3 dissociation rates. Thus, a strongly TRβ-selective challenger ligand (GC-24) exerts TRβ selective effects on release of a non-TR isoform selective hormone, T_3, and this effect is partly sensitive to mutation of the buried pocket.

3.4. T_4 associates rapidly with TRs

Finally, we assessed association rates of T_4 with TRs. This ligand binds TR with relatively low affinity vs. T_3 and dissociates rapidly from both TRs yet also displaces radiolabeled T_3 more rapidly from TRβ (Fig. 3) and TRα (not shown) than T_3 [9]. Fig. 7 reveals that T_4 associates very rapidly with TRs; whereas half the TRs were occupied with T_4 within 2 min, more than 50 min were needed to obtain similar levels of TR occupancy with T_3. Thus, a compound that displaces T_3 rapidly from TR also associates rapidly with the TR.

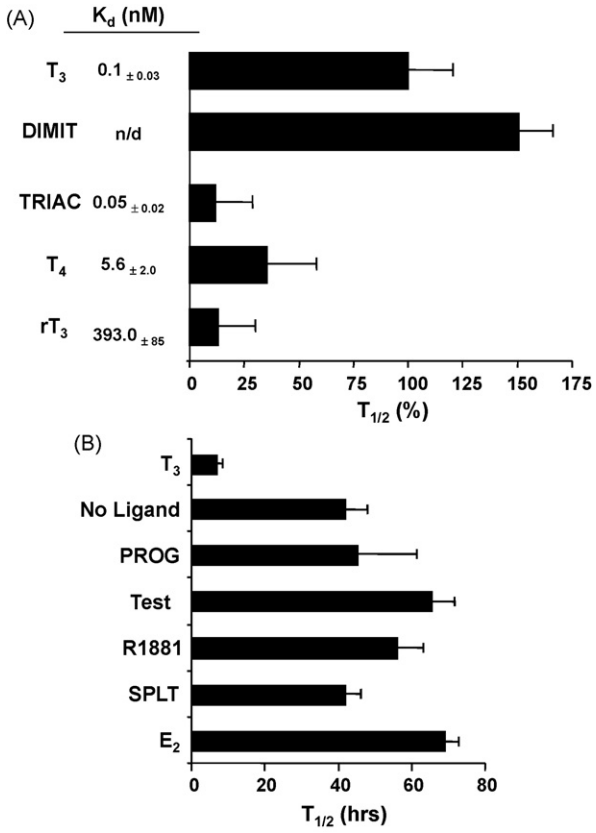

Fig. 3. Rapid T_3 displacement with thyroid hormones, but not ligands that bind other NRs. (A) Comparative $T_{1/2}$ values of TRβ–T_3 complex determined in response to challenge with thyroid hormones, as in Fig. 2. K_i values for each ligand listed at the side of the graph. (B) As above, with ligands that bind to other NRs, PROG: progesterone, Test: testosterone, R1881 (androgen receptor agonist), SPLT: spironolactone, E_2: estradiol.

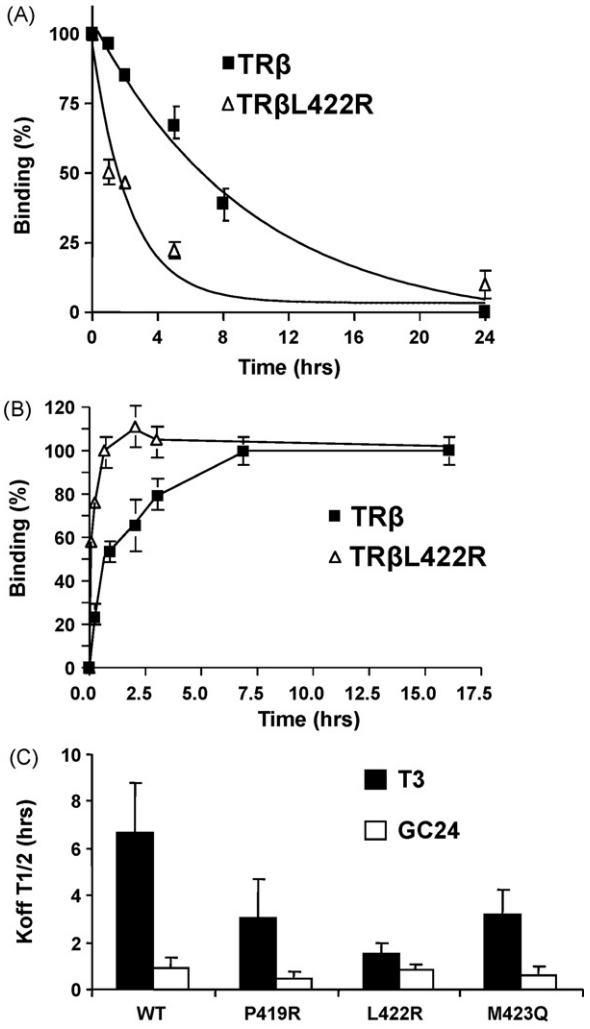

Fig. 4. TRβ mutations that block homodimer formation enhance T_3 association and dissociation. (A) T_3 dissociation rates determined in response to challenge with unlabeled T_3 for wild type TRβ and TRβL422R. (B) T_3 association rates for TRβ and TRβL422R. (C) T_3 dissociation rates determined for TRβ and TRβ dimer surface mutants (expressed as $T_{1/2}$ values in hours) in response to challenge with T_3 or GC-24.

4. Discussion

In this study, we examined the basis of an observation that was made in the 1970s [20], ligands (challengers) that bind NRs with low affinity displace radiolabeled bound ligands more rapidly than non-labeled versions of bound hormone itself. Since early hypotheses suggested that the low affinity challenger interacts with an undefined allosteric site to promote hormone release, and emerging evidence confirms that NR ligands weakly interact with the LBD surface at functionally important sites, we tested whether this phenomenon could be observed with TRs and whether we could understand the effect in terms of recent evidence about TR structure, function, ligand interaction and dynamics.

A large subset of ligands that bind to TR displace bound T_3 more rapidly than T_3 itself. Generally, $T_{1/2}$ for the TR–T_3 complex varied between 5 and 7 h in response to T_3 challenge. Of seventeen TR interacting compounds investigated, twelve displaced T_3 with $T_{1/2}$ from 20 min to 2 h. There is no obvious correlation between effects of TR ligands and their affinity for TR, activity or molecular weight. More rapid T_3 dissociation was observed with compounds that bind TRβ tightly (GC-24, K_d = 0.07 nM) or weakly (rT_3, K_d = 393 nM), with agonists (GC-24, NH-1, Triac, T_4 and rT_3), partial agonists (GC-14, NH-2, NH-6 and NH-8) and antagonists (NH-1, NH-3, NH-5, NH-7 and HY-4) and with compounds that are of similar size to T_3 (Triac and rT_3) or contain bulky extension groups (GC-24, GC-14, the NH series, HY-4 and T_4). However, compounds that bind to other NRs did not enhance T_3 dissociation relative to T_3 challenger, including one compound (progesterone) which displaces dexamethasone rapidly from GR even though it interacts weakly with the GR LBC [20]. Thus, our data suggest that only compounds that bind to the TR LBC enhance T_3 dissociation rates.

Since one of the ligands that rapidly displaces T_3 from the LBC, GC-24, was found at a site in the vicinity of the TR–TR dimerization and TR–RXR heterodimerization surface [10], we examined the possibility that surface interactions could influence T_3 dissociation rates. However, RXR, which should occlude the site through heterodimer formation, fails to alter effects of GC-24 on T_3 dissociation rates. Moreover, mutations in the TR dimer/heterodimer surface enhance T_3 dissociation rates, but do not abolish GC-24 effects. In addition, we have not observed other compounds that promote rapid T_3 dissociation (including Triac) at surface sites in our structures [8]. Thus, we do not think that surface ligand binding to the dimer surface explains rapid T_3 dissociation.

Why does T_3 dissociation rate vary with different challenger ligands? Three lines of evidence suggest that challenger ligands interact with the buried LBC to promote T_3 release. As mentioned above, there is no correlation between affinity of the challenger ligand for TR and its ability to displace T_3 in kinetic studies with TRβ, but only challenger ligands that are T_3 analogues are effective. Ligands that interact with other NRs fail to enhance T_3 dissociation. Moreover, two TRβ selective challengers (GC-24 and NH-3) promote rapid T_3 dissociation (relative to T_3) from TRβ but not TRα and this effect is partly sensitive to mutation of the TR LBC. Finally, T_4, which binds TRs with low affinity, induces rapid T_3 dissociation

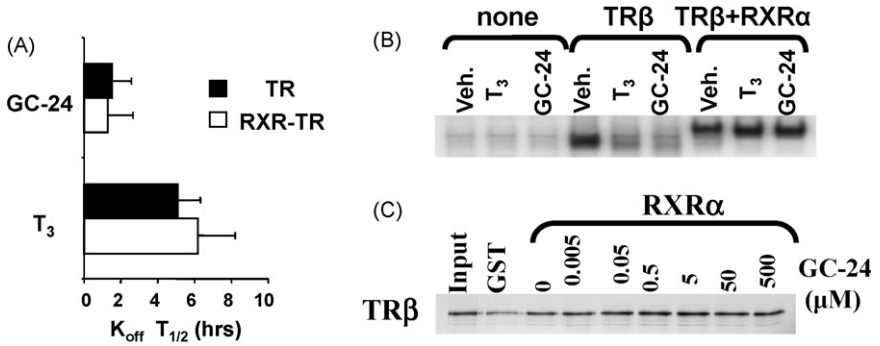

Fig. 5. RXR–TR heterodimer formation does not alter GC-24 effects on T_3 dissociation rates. (A) $T_{1/2}$ values for TRβ–T_3 and RXR–TRβ–T_3 complexes in the presence of excess T_3 or GC-24. (B) Image of autoradiogram of non-denaturing gel used to visualize TR–TRE and RXR–TRE complexes +/− T_3 and GC-24. (C) Image of denaturing SDS-polyacrylamide gel used to separate products of pulldown assay in which radiolabeled TRβ was incubated with GST-RXRα beads or control.

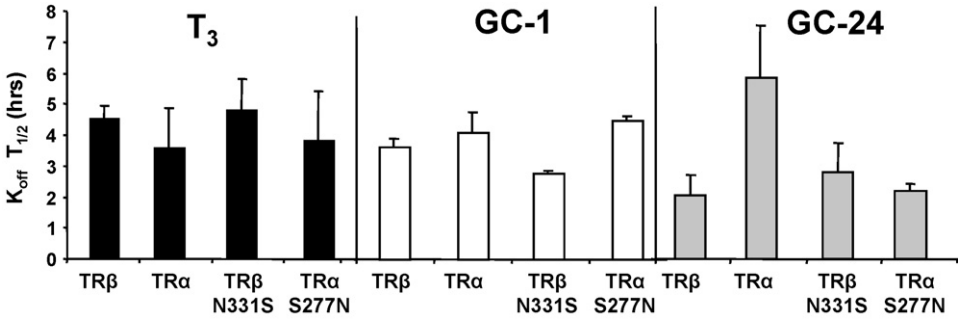

Fig. 6. TR isoform-selective effects on T_3 dissociation. $T_{1/2}$ values of TRβ–T_3, TRα–T_3, TRβN331S–T_3 and TRαS277N–T_3 complexes determined in response to challenge with different ligands. T_3, black bars; GC-1, white bars; GC-24, grey bars.

and associates with the TR much more rapidly than T_3. This finding suggests that a ligand that displaces T_3 rapidly from TRs also binds rapidly to TRs. Presently, we have not been able to examine association rates of other ligands due to the lack of availability of radiolabeled compounds but we predict that variations in ligand association rates will be common. Together; our data implies that rapid T_3 dissociation observed with selected challenger ligands is associated with processes involved in binding of these ligands to the buried LBC.

As described in Section 1, we have suggested that there are multiple ligand entry and exit pathways for TRs and other NRs and we propose that our findings can be explained in terms of differential utilization of entry and exit pathways (Fig. 8). Our MD simulations suggest that the TR LBD is a highly mobile protein and that bound T_3 is constantly probing potential escape routes on the receptor surface [11,12]. Usually, escape routes close before T_3 release, but T_3 can also escape from partially unfolded intermediates before the LBD refolds into the active state. We have also found that preference of escape route varies with receptor oligomeric state and ligand [11,12,29]. Thus, for standard T_3 dissociation assays (Fig. 8A), we predict that the TR–T_3 complex constantly rearranges and unfolds to open ligand exit routes (a). At this point, labeled T_3 can dissociate (b) and be sequentially replaced by unlabeled T_3 (c), or the hormone–receptor complex refolds (a′). We propose that alternate ligands with preferences for different entry/exit routes bind the partially unfolded TR intermediate complex before T_3 leaves, blocking T_3 re-entry into the LBC and promoting rapid hormone dissociation (Fig. 8B). Accordingly, our previous simulations with T_3 and GC-24 reveal strong energetic differences in pathway utilization; whereas T_3 prefers Path III (through the H1–H3 loop) GC-24 prefers Path I (under H12). In this event, the second ligand will inhibit refolding of the T_3–TR complex and enhance T_3 dissociation through step b.

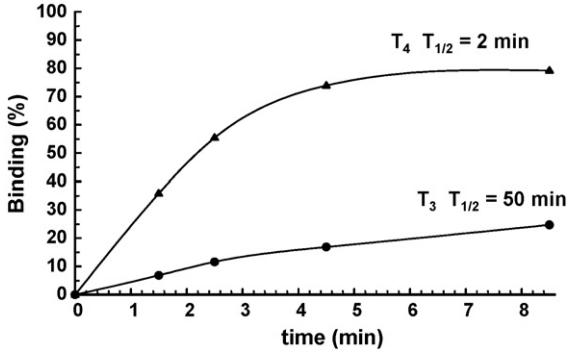

Fig. 7. Rapid T_4 association with TRβ. T_3 and T_4 association curves with TRβ.

Our model suggests explanations for several puzzling observations. First, it explains how the challenger interacts with an inaccessible LBC to promote T_3 release; TR will partly unfold to expose entry routes to the pocket. Second, it explains why effects of the challenger ligands correlate poorly with their affinities for TR; the key interaction involves a partially unfolded TR and not the native receptor observed in X-ray structures. Finally, our model explains why T_4 associates with TRs more rapidly than T_3; different ligands bind TRs in different ways. Our model does not predict detailed molecular events involved in ligand escape or conformations of partially unfolded intermediate states. However, we think that this model accounts for many aspects of previous observations about ligand release.

Our data also support to the notion that there may be different modes of ligand escape from TRs and that patterns of TR ligand association and dissociation resemble other NRs. As discussed earlier, ER dimerization reduces hormone dissociation rates and our MD simulations suggest that this may be because pathways of ligand release are occluded in the dimer [29]. We find here that ligand association and release rates are elevated in TR mutants that are obligate monomers. Perhaps dimerization selectively occludes TR ligand association and release pathways either directly (Path II involves H8 and H11 near the dimer surface) or indirectly through

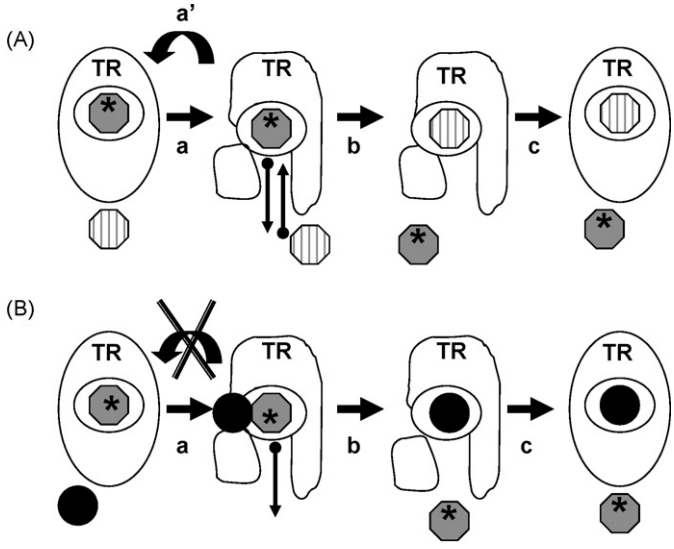

Fig. 8. Model for ligand-selective effects on T_3 dissociation. See text for detailed description. (A) Upper panel represents T_3 dissociation. Folded TR LBD is represented with a circle, the LBP a smaller unfilled circle, T_3 as an octagon and radiolabel designated with (*). (B) The lower panel represents events involved in rapid T_3 release. As in (A) with challenger ligand represented as dark ovals. Note that the challenger ligand can occupy TR at the same time as radiolabeled T_3, thereby preventing refolding of the native TR–T_3 complex.

stabilization of LBD conformation. Improved understanding of relationships between NR LBD surfaces and hormone binding to the buried LBC could help us explain variations in ligand release pathways and exploit these findings in drug design.

We do not think that effects observed in this paper will prove to be physiologically relevant, as circulating T_3 and T_4 concentrations are much lower than 1 μM used to obtain radiolabeled T_3 displacement in these assays (see Ref. [9]). However, given that high affinity for the TR LBC does not always correlate with the ability of challenger ligands to rapidly displace bound ligand, it is interesting to consider the possibility that some compounds which bind TRs with low affinity, but are present in cells at high concentrations, could regulate T_3 dissociation rate. Since T_3 is an unusual amino acid derived from tyrosine, it may be interesting to measure effects of physiological amino acids on TR ligand binding kinetics.

5. Conclusion

A large fraction of available TR ligands trigger release of bound T_3 from the buried LBC more rapidly than an excess of T_3 itself. While previous explanations of this phenomenon suggested that such ligands interact with a poorly defined allosteric interaction site, our data suggest that the challenger interacts with the LBC to promote ligand release, implying that it binds to a partially unfolded TR intermediate. This hypothesis suggests that different ligands associate with, and dissociate from, the TR LBD in different ways.

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References

[1] V. Laudet, H. Gronemeyer, The Nuclear Receptor Facts Book. Factsbook Series, 1st ed., Academic Press, London, 2002.
[2] P.M. Yen, Physiological and molecular basis of thyroid hormone action, Physiol. Rev. 81 (3) (2001) 1097–1142.
[3] J.D. Baxter, P. Webb, Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes, Nat. Rev. Drug Discov. 8 (4) (2009) 308–320.
[4] P. Webb, et al., Design of thyroid hormone receptor antagonists from first principles, J. Steroid Biochem. Mol. Biol. 83 (1–5) (2002) 59–73.
[5] K.W. Nettles, G.L. Greene, Ligand control of coregulator recruitment to nuclear receptors, Annu. Rev. Physiol. 67 (2005) 309–333.
[6] M. Togashi, et al., Conformational adaptation of nuclear receptor ligand binding domains to agonists: potential for novel approaches to ligand design, J. Steroid Biochem. Mol. Biol. 93 (2–5) (2005) 127–137.
[7] C.K. Glass, M.G. Rosenfeld, The coregulator exchange in transcriptional functions of nuclear receptors, Genes Dev. 14 (2) (2000) 121–141.
[8] R.L. Wagner, et al., Hormone selectivity in thyroid hormone receptors, Mol. Endocrinol. 15 (3) (2001) 398–410.
[9] B. Sandler, et al., Thyroxine-thyroid hormone receptor interactions, J. Biol. Chem. 279 (53) (2004) 55801–55808.
[10] S. Borngraeber, et al., Ligand selectivity by seeking hydrophobicity in thyroid hormone receptor, Proc. Natl. Acad. Sci. U.S.A. 100 (26) (2003) 15358–15363.
[11] L. Martinez, et al., Molecular dynamics simulations reveal multiple pathways of ligand dissociation from thyroid hormone receptors, Biophys. J. 89 (3) (2005) 2011–2023.
[12] L. Martinez, et al., Molecular dynamics simulations of ligand dissociation from thyroid hormone receptors: evidence of the likeliest escape pathway and its implications for the design of novel ligands, J. Med. Chem. 49 (1) (2006) 23–26.
[13] R.L. Wagner, et al., A structural role for hormone in the thyroid hormone receptor, Nature 378 (6558) (1995) 690–697.
[14] B.R. Huber, et al., Thyroid hormone receptor-beta mutations conferring hormone resistance and reduced corepressor release exhibit decreased stability in the N-terminal ligand-binding domain, Mol. Endocrinol. 17 (1) (2003) 107–116.
[15] B.R. Huber, et al., Two resistance to thyroid hormone mutants with impaired hormone binding, Mol. Endocrinol. 17 (4) (2003) 643–652.
[16] K.E. Carlson, et al., Altered ligand binding properties and enhanced stability of a constitutively active estrogen receptor: evidence that an open pocket conformation is required for ligand interaction, Biochemistry 36 (48) (1997) 14897–14905.
[17] A.C. Gee, et al., Coactivator peptides have a differential stabilizing effect on the binding of estrogens and antiestrogens with the estrogen receptor, Mol. Endocrinol. 13 (11) (1999) 1912–1923.
[18] M. Jeyakumar, et al., Quantification of ligand-regulated nuclear receptor corepressor and coactivator binding, key interactions determining ligand potency and efficacy for the thyroid hormone receptor, Biochemistry 47 (28) (2008) 7465–7476.
[19] M. Togashi, et al., Rearrangements in thyroid hormone receptor charge clusters that stabilize bound 3,5′,5-triiodo-L-thyronine and inhibit homodimer formation, J. Biol. Chem. 280 (27) (2005) 25665–25673.
[20] M.B. Suthers, L.A. Pressley, J.W. Funder, Glucocorticoid receptors: evidence for a second, non-glucocorticoid binding site, Endocrinology 99 (1) (1976) 260–269.
[21] Y. Wang, et al., A second binding site for hydroxytamoxifen within the coactivator-binding groove of estrogen receptor beta, Proc. Natl. Acad. Sci. U.S.A. 103 (26) (2006) 9908–9911.
[22] A.L. Ambrosio, et al., Ajulemic acid, a synthetic nonpsychoactive cannabinoid acid, bound to the ligand binding domain of the human peroxisome proliferator-activated receptor gamma, J. Biol. Chem. 282 (25) (2007) 18625–18633.
[23] E. Estebanez-Perpina, et al., Structural insight into the mode of action of a direct inhibitor of coregulator binding to the thyroid hormone receptor, Mol. Endocrinol. 21 (12) (2007) 2919–2928.
[24] E. Estebanez-Perpina, et al., A surface on the androgen receptor that allosterically regulates coactivator binding, Proc. Natl. Acad. Sci. U.S.A. 104 (41) (2007) 16074–16079.
[25] N.H. Nguyen, et al., Hammett analysis of selective thyroid hormone receptor modulators reveals structural and electronic requirements for hormone antagonists, J. Am. Chem. Soc. 127 (13) (2005) 4599–4608.
[26] N.H. Nguyen, et al., Rational design and synthesis of a novel thyroid hormone antagonist that blocks coactivator recruitment, J. Med. Chem. 45 (15) (2002) 3310–3320.
[27] P.A. Schueler, et al., Binding of 3,5,3′-triiodothyronine (T3) and its analogs to the in vitro translational products of c-erbA protooncogenes: differences in the affinity of the alpha- and beta-forms for the acetic acid analog and failure of the human testis and kidney alpha-2 products to bind T3, Mol. Endocrinol. 4 (2) (1990) 227–234.
[28] R.C. Ribeiro, et al., Definition of the surface in the thyroid hormone receptor ligand binding domain for association as homodimers and heterodimers with retinoid X receptor, J. Biol. Chem. 276 (18) (2001) 14987–14995.
[29] M.T. Sonoda, et al., Ligand dissociation from estrogen receptor is mediated by receptor dimerization: evidence from molecular dynamics simulations, Mol. Endocrinol. 22 (7) (2008) 1565–1578.