MEASURING IMPACTS OF HALOGENATED PHENOLIC COMPOUNDS ON THYROID REGULATING DEIODINASE ACTIVITY USING IN VITRO TECHNIQUES

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Introduction

Thyroid hormones are essential for the regulation of growth and development in humans and wildlife. The major thyroid hormone is thyroxine (3,5,3',5'-tetraiodothyronine, T4) which, in humans, is primarily transported throughout the body by the thyroxine-binding globulin (TBG). In tissues, T4 is converted to the active hormone, 3,3',5-tridothyronine (T3), which is formed by the outer-ring deiodination (ORD) of T4 and is catalyzed by deiodinase enzymes (Figure 1). Inner-ring deiodination (IRD) may also occur, forming the biologically inactive 3,3',5'-tridothyronine (rT3). However, IRD occurs at much lower rates. The rT3 may undergo further outer-ring deiodination, forming 3,3'-diiodothyronine (T2).

Thyroid hormones and deiodinase (DI) activity have traditionally been measured using radiolabeled compounds and/or using radioimmunoassay (RIA) techniques. However, these techniques can lack specificity and thus may be prone to quantification errors. In addition, RIA methods cannot evaluate ORD and IRD activities simultaneously. In contrast, LC-MS/MS approaches are highly specific, less susceptible to interferences, and can measure thyroid hormones and ORD and IRD activities simultaneously. Studies have shown that LC-MS/MS methods are more accurate than RIA techniques for measuring hormones [1]. Recently, our laboratory developed an LC-MS/MS method for the analysis of 5 thyroid hormones (T4, T3, rT3, T2 and rT2) using liquid-liquid extraction, followed by clean-up using solid phase extraction [2]. Using this method, we can measure ORD and IRD activities by incubating tissues containing deiodinases (e.g. liver tissue) with thyroid hormone substrates and measuring changes in thyroid hormone levels.

It is recognized that some halogenated phenolic compounds may disrupt thyroid hormone homeostasis due to their structural similarity to endogenous thyroid hormones. One proposed mechanism is through competition for deiodinase binding by exogenous phenolic compounds. For example, it has been suggested that deiodinase enzymes are involved in the metabolism of PBDEs in fish [3] and thus may act as competitors. In this study we developed *in vitro* techniques for the measurement of deiodinase activity in human liver microsomes. Specifically, we monitored the production of T3 from T4, and T2 from rT3, which is catalyzed by deiodinases. In addition, incubations were performed with the addition of known deiodinase inhibitors, propylthiouracil (PTU) and iodoacetate (IaC). Finally, experiments were performed to investigate the role of chlorinated and brominated phenolic compounds (e.g. triclosan, tetrabromobisphenol a, hydroxylated-PBDEs) on the inhibition of deiodinase activity *in vitro*.

Materials and Methods

Human liver microsomes were purchased from a commercial source (CellzDirect Inc., Durham, NC). Microsomes (final protein concentration = 1 mg/ml) were incubated with the substrate (either rT3 or T4) in a potassium phosphate buffer (pH 7.4), containing 10 mM dithiothreitol (DTT), for 60 minutes. After incubation, 200 µl of a citric acid/ascorbic acid/DTT solution (25 g/L) was added to prevent further degradation of the thyroid hormones. The ^{13}C-T4 internal standard was added (Cambridge Isotope Laboratories, Andover, MA) and the analytes were extracted by liquid-liquid extraction with acetone. The solution was vortexed, centrifuged, the acetone:aqueous was decanted into a clean vial and the extraction was repeated twice with 1:1 acetone:water. The combined supernatants were blown down under a gentle stream of nitrogen to remove the acetone and cleaned using SampliQ solid phase extraction (SPE) cartridges (Agilent Technologies) following adapted from Wang & Stapleton [2]. Extracts were analyzed by LC-MS/MS using methods developed in our laboratory and is thoroughly described by Wang & Stapleton [2].

Initial experiments were performed using T4 and rT3 concentrations of 6.4 μM and 7.7 μM, respectively. Additional experiments were performed using T4 and rT3 concentrations of 100, 10, 1, 0.1 and 0.01 μM to determine the influence of substrate concentration on DI activity. Experiments were also conducted with the DI inhibitors, PTU (0.1 and 1 mM) and IaC (10 mM), and with halogenated phenolic contaminants including 2,4,6-tribromophenol (TBP), tetrabromobisphenol A (TBBPA), and triclosan (10 nM to 10 mM). These latter experiments were conducted to determine if these compounds would reduce DI activity *in vitro* through competitive binding. All data are reported in terms of mass of thyroid hormone produced (e.g. T2) per mg protein per minute.

Results and Discussion

In incubations with T4 (6.4 μM), ORD activity was measured by quantifying the mass of T3 produced. The rate of ORD activity was 1.1 ± 0.04 (standard deviation) pmoles/mg protein/min; whereas microsomes incubated with 7.7 μM of rT3 produced 19.9 ± 0.21 pmoles of T2/mg protein/min. These observations are expected based on the substrate preference for rT3 by DI isoform Type 1, the only DI isoform present in human liver tissues.

In both the T4 and rT3 incubations, the addition of PTU and IaC inhibitors resulted in the complete or partial shut-down of deiodinase activity (Figures 2 & 3). In all treatments, the T3 (T4 incubation) or T2 (rT3 incubation) detected in inhibitor-incubated samples were below MDLs. Regarding the IaC treatments, deiodinase activity was completely shut-down when either the T4 or rT3 were used as substrates (Figure 2). Regarding the PTU treatments, both the T4 and rT3 incubations showed a dose-response relationship with higher concentrations of PTU resulting in a greater inhibition of deiodinase activity (Figure 3). In the T4 + PTU samples, the incubation of 0.1 mM and 1 mM PTU resulted in the inhibition of T3 formation by ~3- and ~7-fold, respectively, relative to the control levels. More dramatically, in the rT3 + PTU samples, the incubation of 0.1 mM PTU resulted in the ~15-fold decrease of T2 formation, whereas, deiodinase activity was completely suppressed in the 1 mM PTU incubation. These trends demonstrate that the PTU and IaC prevented the deiodination of T4 and rT3, respectively.

Further work is now in progress to measure DI activity in human microsomes co-incubated with the flame retardant chemicals TBP, and TBBPA, and with the chlorinated anti-bacterial chemical, triclosan. These compounds are halogenated phenolic chemicals that have structural similarities with thyroid hormones. Our preliminary data indicates that TBBPA decreased DI activity; however, these experiments are currently being repeated for confirmation.

Acknowledgements

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References

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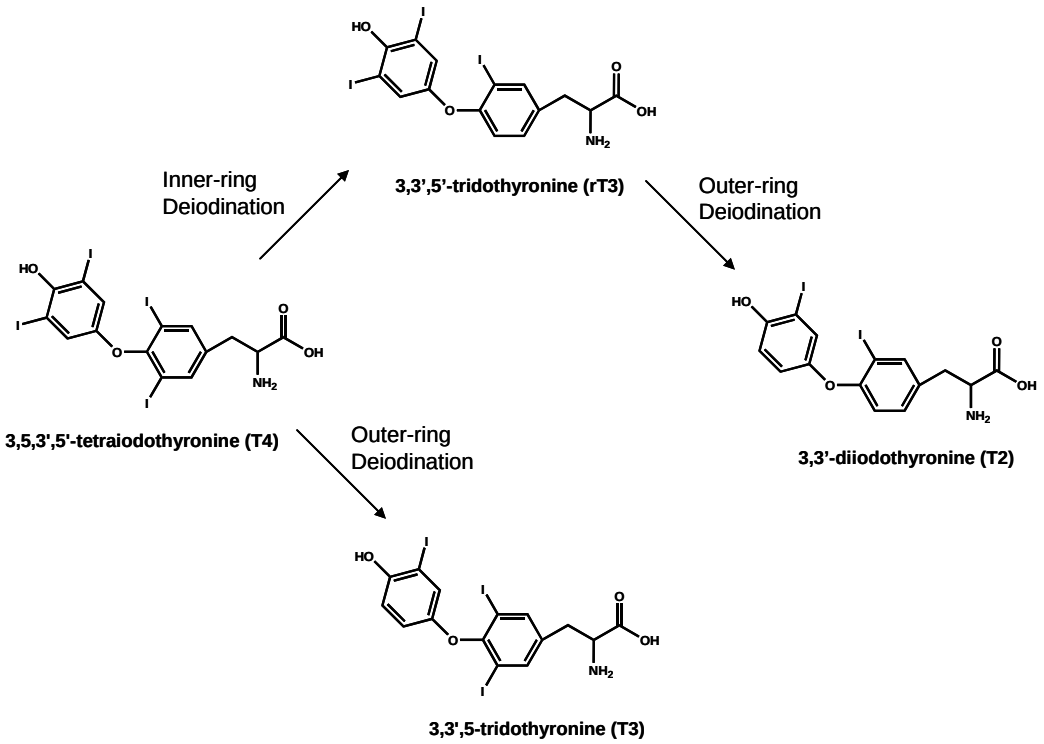

Figure 1. Deiodination reactions investigated in this study.

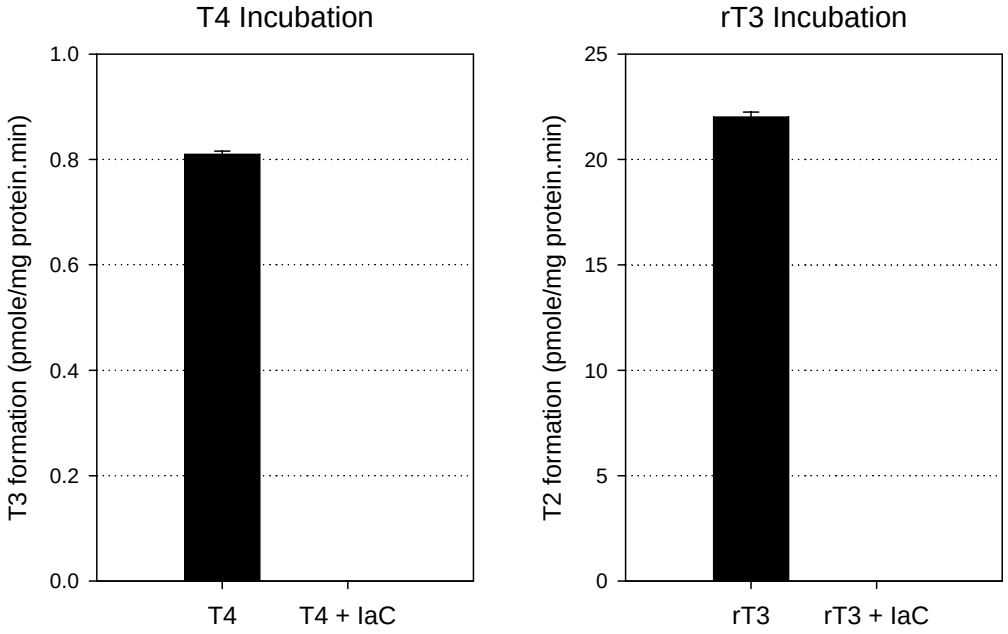

Figure 2. Mean (± standard deviation) formation (pmol/mg protein.min) of T3 from T4 incubation (top panel) and T2 from rT3 incubation (bottom panel) with treatments of 0 and 10 mM IaC,

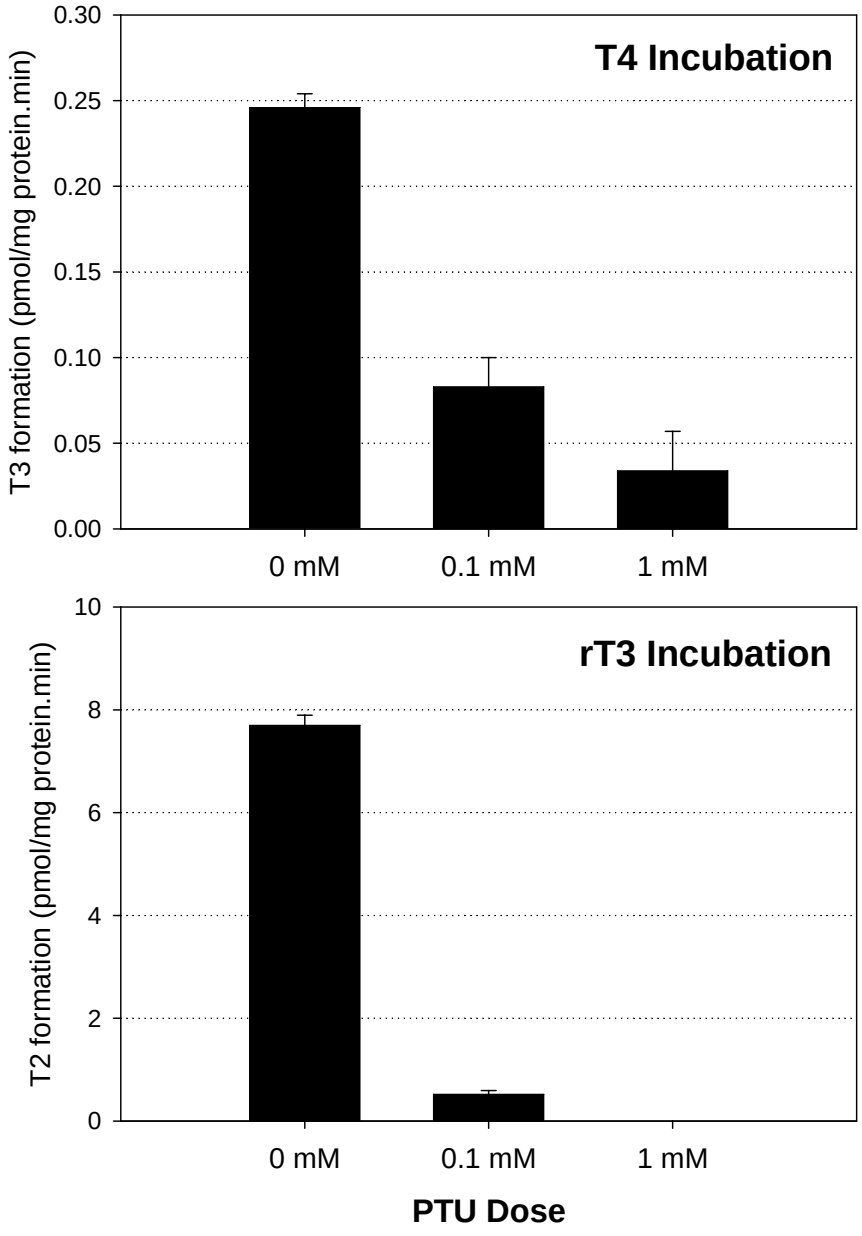

Figure 3. Mean (± standard deviation) formation (pmol/mg protein.min) of T3 from T4 incubation (top panel) and T2 from rT3 incubation (bottom panel) with treatments of 0, 0.1 and 1 mM PTU.