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Iodothyronamines are oxidatively deaminated to iodothyroacetic acids *in vivo*

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Abstract

3-Iodothyronamine (T₁AM) and 3,5,3'-triiodothyroacetic acid (Triac) are bio-active metabolites of the hormone thyroxine (T₄). In the present study the ability of T₁AM and T₃AM to be metabolized to 3-iodothyroacetic acid (TA₁) and 3,5,3'-triiodothyroacetic acid (Triac), respectively, were investigated. Both T₁AM and T₃AM were converted to their respective iodinated thyroacetic acid analogues in both cell and tissue extracts. This conversion could be significantly inhibited with the monamine oxidase (MAO) and semicarbazide-sensitive amine oxidase (SSAO) inhibitor, iproniazid. TA₁ was found to be present in trace quantity in human serum, and was present in substantial levels in serum from T₁AM treated rats. These results demonstrate that iodothyronamines are substrates for amine oxidases, and that this metabolism may be the source of the corresponding endogenous arylacetic acid products Triac and TA₁.

Keywords

Iodothyronamines; iodothyroacetic acids; metabolism; biotransformations; biosynthesis

Introduction

3-Iodothyronamine (T₁AM) is a novel metabolite of thyroid hormone with unique biological effects. This compound was discovered based upon the hypothesis that analogous to the formation of dopamine from 3,4(OH)₂-phenylalanine (L-DOPA), thyroxine (T₄) or a lower iodinated thyronine could be decarboxylated to form various iodothyronamines.^[1] From these initial studies 3-iodothyronamine was found to be an endogenous compound present in specific thyroid hormone target tissues and circulation, and when administered pharmacologically, induced a hypometabolic state opposite to that which is characteristic of excess thyroid hormone. Animals treated with T₁AM display behavioral inactivity, hypothermia, bradycardia and reduced cardiac output,^[2] hyperglycemia,^[3] and a shift in

fueling away from carbohydrates and toward fat.^[4] In addition, T₁AM has also been shown to induce marked neuroprotection against stroke injury.^[5]

At the molecular level T₁AM is a potent agonist of the orphan GPCR TAAR1,^[11] and also is an agonist ligand against the alpha-2A-adrenergic receptor GPCR for which epinephrine is thought to be the usual endogenous ligand.^[3] T₁AM also functions as an inhibitor of plasma membrane and vesicular catecholamine transport via direct inhibitory action at NET, DAT, and VMAT.^[6] As for T₁AM metabolism, both sulfation via sulfotransferases and deiodination via the type-III deiodinases (Dio3) have been reported.^[7, 8]

We were interested in metabolism of the ethylamine side chain of thyronamines, and specifically whether oxidative deamination could give rise to thyroacetic acids. Triac is a known biologically active T₄ metabolite that is generated from 3,5,3'-triiodothyronine (T₃) both *in vitro*^[9] and *in vivo*.^[10] In addition, other thyroacetic acid derivatives of thyronines were synthesized and have been tested for biological activity, including TA₁.^[11–13] Two different enzymes, L-aminoacid oxidase or transaminase have been postulated to perform the T₃ to Triac conversion involving a keto-acid intermediate.^[14] We envisioned an alternative metabolic pathway involving thyronamines, in which an amine oxidase, such as a MAO or SSAO, converts the thyronamine into an aldehyde that can be further oxidized to a carboxylic acid by another enzyme, such as aldehyde dehydrogenase (Scheme 1). MAO and SSAO are promiscuous enzymes that prefers primary amine substrates, which can catalyze the oxidation of phenylethylamines.^[15,16] After the conversion of the thyronamines to aldehydes, aldehyde dehydrogenase (ALDH) could be responsible for the aldehyde to acid oxidation to yield thyroacetic acids. ALDH is a NAD-dependent enzyme that is universally distributed in mammalian tissue and can oxidize a wide range of aldehydes. Two of this enzyme's substrates are the aldehyde metabolites of the phenylethylamines 5-hydroxytryptamine and dopamine.^[17] Based on substrate preferences of amine oxidases and ALDH it seemed plausible that thyronamines could serve as substrates for these enzymes and be converted to thyroacetic acids, and testing this hypothesis motivated this study.

Results

T₁AM and T₃AM are metabolized to their thyroacetic acid analogs

The expected oxidized products of T₁AM and T₃AM are the thyroacetic acids, TA₁ and Triac, respectively. To determine if formation of these thyroacetic acids occurs, [3-¹²⁵I]-iodothyronamine and 3,5,[3'-¹²⁵I]-triiodothyronamine were synthesized. These labeled compounds were incubated with HepG2 cells (human hepatocellular carcinoma cell line) because this cell line contains MAO A and B^[18] and the liver has the greatest activity of ALDH.^[17] Media (no cells) or HepG2 cells were preincubated with either DMSO or 200 μM iproniazid, a known MAO and SSAO inhibitor, for 30 minutes. Next either [3-¹²⁵I]-iodothyronamine or 3,5,[3'-¹²⁵I]-triiodothyronamine were added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of 1:1 hexanes:ethyl acetate + 0.5% acetic acid and the plate was exposed to film. The autoradiogram for one of the three experiments with [3-¹²⁵I]-iodothyronamine or 3,5,[3'-¹²⁵I]-triiodothyronamine are shown in Figures 2 and 3, respectively. In Figure 1, a radioactive spot with the same retention factor as TA₁ (indicated

by the arrow) was observed only when HepG2 cells are present (lanes 4–6 in triplicate) and not in the absence of cells (lanes 1–3 in triplicate). This HepG2-dependent conversion was significantly inhibited by iproniazid (lanes 7–9 in triplicate) when compared to the DMSO control (lanes 4–6 in triplicate). In Figure 2, a radioactive spot with the same retention factor as Triac (indicated by the arrow) was observed only when HepG2 cells were present (lanes 4–6 in triplicate) and not in the absence of cells (lanes 1–3 in triplicate). This HepG2-dependent conversion was significantly inhibited by iproniazid (lanes 7–9 in triplicate) when compared to the DMSO control (lanes 4–6 in triplicate). A Cell-Titer-Glo Luminescent Cell Viability Assay kit demonstrated that cells exposed to iproniazid under the assay conditions were still viable (data not shown).

Next, similar experiments were conducted with human thyroid tissue to determine whether the thyroid gland can convert T₁AM to TA₁. Buffer or homogenized human thyroid gland (1g/15mL) were preincubated with either DMSO or 200 μ M iproniazid for 30 minutes. Next [3',-¹²⁵I]-iodothyronamine was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of 1:1 hexanes:ethyl acetate + 0.5% acetic acid and the plate was exposed to film. The autoradiogram for one of the three experiments with [3',-¹²⁵I]-iodothyronamine are shown in Figure 3. In Figure 3, a radioactive spot with the same retention factor as TA₁ (indicated by the arrow) was observed only when homogenized thyroid gland was present (lanes 4–6 in triplicate) and not in the absence of the homogenate (lanes 1–3 in triplicate). This homogenized thyroid gland-dependent conversion was significantly inhibited by iproniazid (lanes 7–9 in triplicate) when compared to the DMSO control (lanes 4–6 in triplicate).

TA₁ is produced *in vivo*

We next examined whether TA₁ production occurred *in vivo*. A variety of rodent and human serum and tissue samples were extracted and analyzed for TA₁ content by LC-MS/MS. A deuterated TA₁ analog (d₄-TA₁) was synthesized to facilitate quantitative analysis. Trace amounts of TA₁ were detected in human serum with approximate serum levels determined to be less than 0.1 nM (data not shown). We then extracted serum taken from rats 60, 90, and 120 minutes after treatment with 20 mg/kg T₁AM i.p. Clear LC-MS/MS peaks of serum TA₁ were detected at each of these time points (Figure 4). Serum levels of TA₁ ranged from 3–12 nM, and the level decreased with time via pharmacokinetic elimination (Figure 5).

Discussion

3-Iodothyronamine (T₁AM) is a newly discovered thyroid hormone metabolite that has unique biological activity and the catabolism of T₁AM is therefore an important determinant of T₁AM levels in tissue and circulation. Enzyme-catalyzed deiodination^[8] and sulfation^[7] of T₁AM have been previously reported, and phenylethylamine side chain catabolism was the focus of the present study. In analogy to other biogenic amines, we postulated that T₁AM would be subject to oxidative deamination by amine oxidases enzymes (MAO or SSAO). The expected products of such catabolism would be thyroacetic acids, which interestingly, are already known as metabolites of thyroxine. Our results demonstrate that

conversion of iodothyronamines to iodothyroacetic acids occurs in cultured cells, *ex-vivo* tissue homogenates, and *in vivo*.

Endogenous TA₁, the thyroacetic acid corresponding to amine oxidase action on T₁AM, was found in serum in only trace quantities (<0.1 nM); however, 3–12 mM concentrations of TA₁ were measured in serum from rats injected with 20 mg/kg T₁AM. This indicates that TA₁ is a major metabolite of T₁AM, and that like deiodination and sulfation, oxidative deamination is a significant pathway for T₁AM catabolism. It is interesting to note that the more highly iodinated thyroacetic acids Triac and Tetrac (3,5,3',5'-tetrathyroacetic acid), which are biologically active oxidative deamination metabolites of T₃ and T₄, respectively, have been known for several decades and the biosynthetic origins of these compounds and the enzymes involved in the conversion is not completely clear.^[9,10] We found that T₃AM is converted into Triac in the presence of HepG2 cells, suggesting a new or alternative biosynthetic sequence for the production of Triac, and by extension, Tetrac. The iodothyronines T₄ and T₃ could be decarboxylated to the corresponding iodothyronamines followed by oxidative deamination to Tetrac and Triac.

As metabolites of thyroid hormone, Triac and Tetrac are not overly abundant in blood. The blood concentration of the more abundant thyroacetic acid Tetrac is less than 1 nM.^[19] Despite low iodothyroacetic acid blood levels, a high quantity of thyroacetic acid (TA₀) is excreted in urine indicating that a much larger quantity of T₄ is converted into iodothyroacetic acids than can be accounted for by blood levels of these substances.^[20] The potential implication of iodothyronamines in this process also suggests that the fraction of iodothyronamines presumably derived from circulating T₄, is much larger than previous estimates.^[1]

Conclusion

Our demonstration here that iodothyronamines can be converted to iodothyroacetic acids by robust and established catabolic enzymology suggests an intriguing explanation for this large excreted pool of TA₀ which is difficult to account for on the basis of iodothyronine conversion to iodothyroacetic acids alone. At present, T₁AM and T₀AM are the only thyronamines that have been detected *in vivo*; however, T₄AM, rT₃AM, and 3,3'-T₂AM are good substrates for the deiodinase enzymes *in vitro*.^[8] T₄AM is efficiently converted to rT₃AM by Dio3 and rT₃AM is efficiently converted to 3,3'-T₂AM by Dio1 and Dio2. If these additional iodothyronamines are endogenous compounds, they are likely also to be amine oxidase substrates and converted to their corresponding iodothyroacetic acid derivatives. Each of these compounds would converge on TA₀ after exhaustive deiodination. Thus, some fraction of the large excreted TA₀ pool may ultimately derive from the conversion of thyroxine into iodothyronamines, suggesting that iodothyronamines may constitute a larger fraction of the thyroid hormone metabolite pool than has been previously appreciated.

Experimental Section

Materials

A Cell-Titer-Glo Luminescent Cell Viability Assay kit was obtained from Promega. Cell culture media and supplements were obtained from Gibco. Fetal bovine serum was obtained from Gemini BioProducts. Residual thyroid tissue remaining after standard of care procedures was received anonymously from the OHSU Pathology department under the determination of the OHSU IRB as nonhuman subjects research.

Iproniazid was obtained from Sigma and [3-¹²⁵I]-iodothyronamine was synthesized according to literature procedure.^[21] The synthesis of 3,5,[3'-¹²⁵I]-triiodothyronamine, 3-iodothyroacetic acid, and deuterated 3-iodothyroacetic acid are described in the supporting information.

Cell Culture

HepG2 cells were cultured in minimum essential medium with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L of sodium carbonate, 0.10 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100unit/mL penicillin, 100 ug/mL streptomycin, and 10% heat inactivated fetal bovine serum. Cells were grown in a humidified atmosphere of 5% CO₂ at 37°C.

HepG2 assays

Cells were seeded into wells of 24-well plates and grown to 80–95% confluence. Prior to the start of each experiment, 500 µL of media was placed into wells with or without cell monolayers. To each well was added either 1 µL of DMSO or 1µL of a 100 mM iproniazid DMSO solution. The plate was placed in a humidified atmosphere of 5% CO₂ at 37°C for 30 minutes. Next 1.0 µL of a 0.19 mCi/µL solution of [3-¹²⁵I]-iodothyronamine was added to each well and the plate was returned to a humidified atmosphere of 5% CO₂ at 37°C. After 3 h the media was removed and transferred to a 1.7 mL eppendorf tube containing 50 µL of a 3M HCl solution. This acidified solution was extracted three times with 300 µL of dichloromethane. For each extraction the solution was vortexed vigorously and centrifuged to facilitate the separation of the organic and aqueous phases. The combined organic washes were concentrated and the residue was dissolved in 10 µL of methanol and then spotted onto a TLC plate. The vessel with residue was washed with another 10 µL of methanol and was spotted in the same location on the TLC plate. The methanol was allowed to evaporate from the TLC plate before the plate was developed with a solution of 1:1 hexanes:ethyl acetate containing 0.5% acetic acid. After the plate had dried, it was exposed to film and then the film was developed. Triplicates of each condition were performed and experiments were repeated three times. The same assay procedure was also conducted with [3'-¹²⁵I]-3,3',5-triiodothyronamine.

Thyroid tissue homogenate assays

Thyroid tissue was homogenized on ice in homogenization buffer (0.12 M NaCl, 0.4 mM KCl, 0.4 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.5 mM CaCl₂, pH = 7.4) to provide a homogenate of 1 g of tissue per 15 mL. Next, 500 µL of thyroid homogenate or

buffer was placed into wells of a 24 well plate. To each well was added either 1 μ L of DMSO or 1 μ L of a 100 mM iproniazid DMSO solution. The plate was placed in a humidified atmosphere of 5% CO₂ at 37°C and rocked for 30 minutes. Next 1.0 μ L of a 0.19 uCi/ μ L solution of [3-¹²⁵I]-iodothyronamine was added to each well and the plate was returned to a humidified atmosphere of 5% CO₂ at 37°C and rocked. After 3 h the buffer or homogenate was removed and transferred to a 1.7 mL eppendorf tube containing 50 μ L of a 3.0 M HCl solution. This acidified solution was extracted three times with 300 μ L of dichloromethane. For each extraction the solution was vortexed vigorously and centrifuged to facilitate the separation of the organic and aqueous phases. The combined organic washes were concentrated and the residue was dissolved in 10 μ L of methanol and then spotted onto a TLC plate. The vessel with residue was washed with another 10 μ L of methanol and was spotted in the same location on the TLC plate. The methanol was allowed to evaporate from the TLC plate before the plate was developed with a solution of 1:1 hexanes:ethyl acetate containing 0.5% acetic acid. After the plate had dried, it was exposed to film and then the film was developed. Triplicates of each condition were performed and experiments were repeated three times.

Extraction procedure for endogenous 3-iodothyroacetic acid

For isolation of TA₁ in rat serum dosed with pharmacological levels of T₁AM, samples were thawed on ice from stores frozen at -80 °C. Triplicates of 50 μ L serum from two rats at three sequential dose time points—60, 90 and 120 minutes post injection—were used in sample work-up. To the aliquots, was added 1,000 pmol synthetic deuterated 3-iodothyroacetic acid (d₄-TA₁) as an internal standard. The samples were brought to a final volume adjusted to 1 mM concentration iopanoic acid, used to inhibit deiodination during processing. Proteins were precipitated by the addition of acidified acetone (pH adjusted to 4–5 with concentrated hydrochloric acid) in a 2:1 ratio by volume. The samples were then vortexed and centrifuged at 14,000 rpm for 5 minutes. The supernatant was transferred to a glass culture tube and evaporated to dryness using a SPD 121P Thermo Savant model speedvac concentrator at 35 °C (Thermo Scientific, Waltham, MA). Residues were dissolved in 1% aqueous formic acid (2 mL) and processed using polymeric SPE chromatography. Bond Elut Plexa cartridges (6 mL, 200 mg) were preconditioned with methanol (2 mL) and deionized water (2 mL) using positive pressure argon. The acidified sample dilutions were then loaded onto the columns under gravity and washed with 5% methanol in deionized water (2 mL). TA₁ was eluted with methanol (1 mL, then 0.5 mL twice) using positive pressure argon. The methanol solvent was removed using a speedvac at 35 °C and the dried organic extracts were reconstituted in 80 μ L of 1:1 acetonitrile and deionized water. The samples were centrifuged and the supernatants were transferred to vials and stored at -80° C prior to analysis with LC-MS/MS.

LC-MS/MS analysis procedure

The method was validated using a Thermo TSQ Quantum Discovery triple-quadrupole mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The ionization interface was operated in the negative ion mode using the following settings: spray voltage, 1.8 kV; sheath and aux gas flow rates, 25 and 10 respectively; tube lens voltage, 80 V; capillary voltage, 35 V; and a capillary temperature of 275 °C. An instrument

method was created to monitor for the TA₁ transition from m/z 369 precursor to m/z 127 product ion and the d₄-TA₁ transition from precursor m/z 373 to product m/z 127 ion (each transition expressing a ratio of parent ion to molecular iodine). The collision energy was 10 with a collision gas pressure of 0.8 mTorr. Scan event settings were: scan width 1.5 m/z , scan time 0.5 seconds, a Q1 peak width of 0.7 and Q3 peak width of 0.8. The LC-MS system was composed of an in-line Thermo Surveyor auto-sampler and HPLC pump. TA₁ was resolved from closely eluting interfering analytes using a Waters Xterra Phenyl 3.5 μ m, 3.0 $\times$ 50 mm, HPLC column (Milford, MA). The HPLC column temperature was 30 °C. The mobile phase was delivered in a three-phase gradient and consisted of two solvents: A, water and 0.002% acetic acid, and B, acetonitrile and 0.002% acetic acid (phase I: 0–5 minutes 80% A, 20% B, in a positive linear slope to phase II: 5–15 minutes 5% A, 95% B; and phase III: 15.50–25 minutes 80% A, 20% B). The mobile phase was delivered at a flow rate of 250 μ L/ minute and a three-minute column wash was included between sample runs. The injection volume was 20 μ L of 80 μ L of sample work-up consisting of 1:1 acetonitrile and deionized water.

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Figure 1.

Autoradiogram for the conversion of T₁AM to TA₁ with HepG2 cells. Media (no cells) or HepG2 cells were preincubated with either DMSO or 200 μM iproniazid for 30 minutes. Next [3'-¹²⁵I]-iodothyronamine was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of 1:1 hexanes:ethyl acetate + 0.5% acetic acid and the plate was exposed to film. The arrow indicates the retention factor for authentic TA₁. Lanes 1–3: wells containing media that were preincubated with DMSO. Lanes 4–6: wells containing HepG2 cells that were preincubated with DMSO. Lanes 7–9: wells containing HepG2 cells that were preincubated with 200 μM iproniazid. Lanes 10–12: wells containing media that were preincubated with 200 μM iproniazid.

Figure 2.

Autoradiogram for the conversion of T₃AM to Triac with HepG2 cells. Media (no cells) or HepG2 cells were preincubated with either DMSO or 200 μM iproniazid for 30 minutes. Next 3, 5, [3'-¹²⁵I]-triiodothyronamine was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of 1:1 hexanes:ethyl acetate + 0.5% acetic acid and the plate was exposed to film. The arrow indicates the retention factor for authentic Triac. Lanes 1–3: wells containing media that were preincubated with DMSO. Lanes 4–6: wells containing HepG2 cells that were preincubated with DMSO. Lanes 7–9: wells containing HepG2 cells that were preincubated with 200 μM iproniazid. Lanes 10–12: wells containing media that were preincubated with 200 μM iproniazid.

Figure 3.

Autoradiogram for the conversion of T₁AM to TA₁ with homogenized human thyroid gland. Buffer or homogenized human thyroid gland (1g/15mL) were preincubated with either DMSO or 200 μM iproniazid for 30 minutes. Next [3'-¹²⁵I]-iodothyronamine was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of 1:1 hexanes:ethyl acetate + 0.5% acetic acid and the plate was exposed to film. The arrow indicates the retention factor for authentic TA₁. Lanes 1–3: wells containing buffer that were preincubated with DMSO. Lanes 4–6: wells containing human thyroid gland homogenate that were preincubated with DMSO. Lanes 7–9: wells containing human thyroid gland homogenate that were preincubated with 200 μM iproniazid. Lanes 10–12: wells containing buffer that were preincubated with 200 μM iproniazid.

Figure 4.

TA₁ detected in 50 μ L of rat serum (60 minutes post T₁AM injection) by LC-tandem mass spectrometry (LC-MS/MS). The upper extracted ion chromatogram demonstrates detection of TA₁ (m/z 369 > 127). The lower chromatogram demonstrates the detection of synthetic deuterated standard, d₄-TA₁ (m/z 373 > 127), spiked into the rat serum.

Figure 5.

Rat serum concentration TA₁ (μM) for each sample set analyzed. TA₁ was quantified from serum samples collected from rats (n=2) for 60, 90, and 120 minutes after injection with 20 mg/kg T₁AM.

Scheme 1.

Proposed pathway for the metabolism of T₃AM and T₁AM to Triac and TA₁, respectively.