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Iodothyronamines are Oxidatively Deaminated to Iodothyroacetic Acids in vivo

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3-Iodothyronamine (T_1AM) and 3,3′,5-triiodothyroacetic acid (Triac) are bioactive metabolites of the hormone thyroxine (T_4). In the present study, the ability of T_1AM and 3,3′,5-triiodothyronamine (T_3AM) to be metabolized to 3-iodothyroacetic acid (TA_1) and Triac, respectively, was investigated. Both T_1AM and T_3AM 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. TA_1 was found to be present in trace quantities in human serum and in substantial levels in serum from T_1AM-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 TA_1.

Introduction

3-Iodothyronamine (T_1AM) 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 (T_4) or a lower iodinated thyronine could be decarboxylated to form various iodothyronamines.[1] From these initial studies, T_1AM was found to be an endogenous compound present in specific thyroid hormone target tissues and circulation, and when administered pharmacologically, it induced a hypometabolic state opposite to that which is characteristic of excess thyroid hormone. Animals treated with T_1AM 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, T_1AM has also been shown to induce marked neuroprotection against stroke injury.[5]

At the molecular level T_1AM is a potent agonist of the orphan G protein-coupled receptor (GPCR) TAAR1,[1] 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] T_1AM also functions as an inhibitor of plasma membrane and vesicular catecholamine transport through direct inhibitory action at the norepinephrine, dopamine, and vesicular monoamine transporters (NET, DAT, and VMAT, respectively).[6] As for T_1AM metabolism, both sulfation through sulfotransferases and deiodination through the type-III deiodinases (Dio3) have been reported.[7,8]

We were interested in the metabolism of the ethylamine side chain of thyronamines, and specifically whether oxidative deamination could give rise to thyroacetic acids. 3,3′,5-triiodothyroacetic acid (Triac) is a known biologically active T_4 metabolite that is generated from 3,3′,5-triiodothyronine (T_3) 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 3-iodothyroacetic acid (TA_1).[11–13] Two different enzymes, L-amino acid oxidase or transaminase, have been postulated to perform the T_3-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 monamine oxidase (MAO) or semicarbazide-sensitive amine oxidase (SSAO), converts the thyronamine into an aldehyde (**1**) 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 prefer primary amine substrates, and 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 an 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

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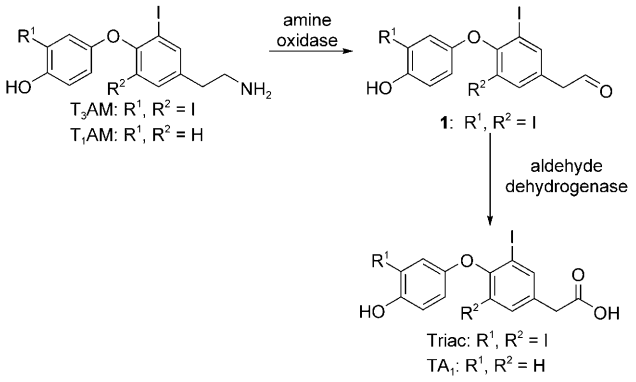

Scheme 1. Proposed pathway for the metabolism of T_3AM and T_1AM to Triac and TA_1, respectively.

and be converted to thyroacetic acids, and testing this hypothesis motivated this study.

Results

T_1AM and T_3AM are metabolized to their thyroacetic acid analogues

The expected oxidized products of T_1AM and 3,3′,5-triiodothyronamine (T_3AM) are the thyroacetic acids TA_1 and Triac, respectively. To determine if formation of these thyroacetic acids occurs, [3-^{125}I]-T_1AM and [3′-^{125}I]-T_3AM were synthesized. These labeled compounds were incubated with HepG2 cells (a 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 min. Next, either [3-^{125}I]-T_1AM or [3′-^{125}I]-T_3AM 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 hexanes/ethyl acetate (1:1) containing 0.5% acetic acid and the plate was exposed to film. The autoradiograms for one of the three experiments with [3-^{125}I]-T_1AM or [3′-^{125}I]-T_3AM are shown in Figures 2 and 3, respectively. In Figure 1, a radioactive spot with the same retention factor as TA_1 (indicated by the arrow) was observed only in the presence of HepG2 cells (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 in the presence of HepG2 cells (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) as 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).

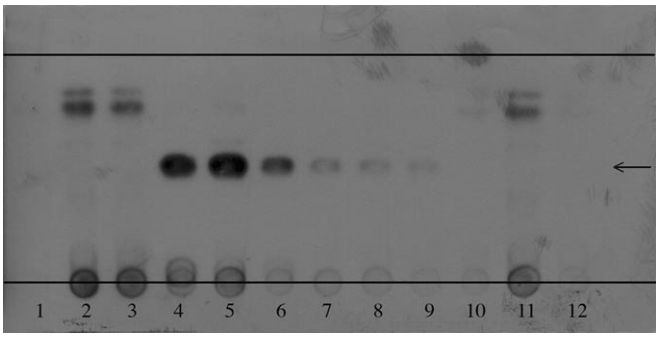

Figure 1. Autoradiogram for the conversion of T_1AM to TA_1 with HepG2 cells. Media (no cells) or HepG2 cells were preincubated with either DMSO or iproniazid (200 μM) for 30 min. Next, [3-^{125}I]-T_1AM was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of hexane/ethyl acetate (1:1) containing 0.5% acetic acid and the plate was exposed to film. The arrow indicates the retention factor for authentic TA_1. 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 iproniazid (200 μM). Lanes 10–12: wells containing media that were preincubated with iproniazid (200 μM).

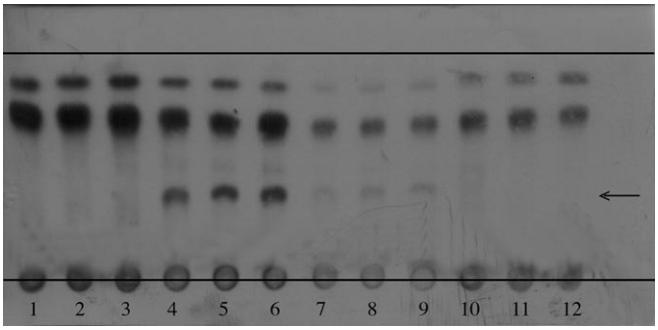

Figure 2. Autoradiogram for the conversion of T_3AM to Triac with HepG2 cells. Media (no cells) or HepG2 cells were preincubated with either DMSO or iproniazid (200 μM) for 30 min. Next, [3′-^{125}I]-T_3AM was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of hexane/ethyl acetate (1:1) containing 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 iproniazid (200 μM). Lanes 10–12: wells containing media that were preincubated with iproniazid (200 μM).

Next, similar experiments were conducted with human thyroid tissue to determine whether the thyroid gland can convert T_1AM to TA_1 (Figure 3). Buffer or homogenized human thyroid gland (1 g/15 mL) were preincubated with either DMSO or 200 μM iproniazid for 30 min. Next [3-^{125}I]-T_1AM 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 hexanes:ethyl acetate (1:1) containing 0.5% acetic acid and the plate was exposed to film. The autoradiogram for one of the three experiments with [3-^{125}I]-T_1AM is shown in Figure 3. A radioactive spot with the same retention factor as TA_1 (indicated by the arrow) was ob-

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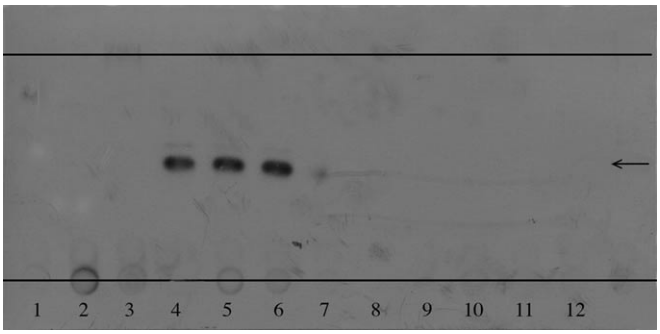

Figure 3. Autoradiogram for the conversion of T_1AM to TA_1 with homogenized human thyroid gland. Buffer or homogenized human thyroid gland (1 g, 15 mL) were preincubated with either DMSO or iproniazid (200 μM) for 30 min. Next, [3-^{125}I]-iodothyronamine was added and after a 3 h incubation, the solution was acidified, extracted, concentrated and spotted onto a TLC plate. The TLC plate was developed with a solution of hexane/ethyl acetate (1:1) containing 0.5% acetic acid and the plate was exposed to film. The arrow indicates the retention factor for authentic TA_1. 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 iproniazid (200 μM). Lanes 10–12: wells containing buffer that were preincubated with iproniazid (200 μM).

served only in the presence of homogenized thyroid gland (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) as compared to the DMSO control (lanes 4–6, in triplicate).

TA_1 is produced in vivo

We next examined whether TA_1 production occurred in vivo. A variety of rodent and human serum and tissue samples were extracted and analyzed for TA_1 content by LC-MS/MS. A deuterated TA_1 analogue (d_4-TA_1) was synthesized to facilitate quantitative analysis. Trace amounts of TA_1 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 min after treatment with 20 mg kg^{-1} T_1AM (i.p.). Clear LC-MS/MS peaks of serum TA_1 were detected at each of these time points (Figure 4). Serum levels of TA_1 ranged from 3–12 μM, and the level decreased with time through pharmacokinetic elimination (Figure 5).

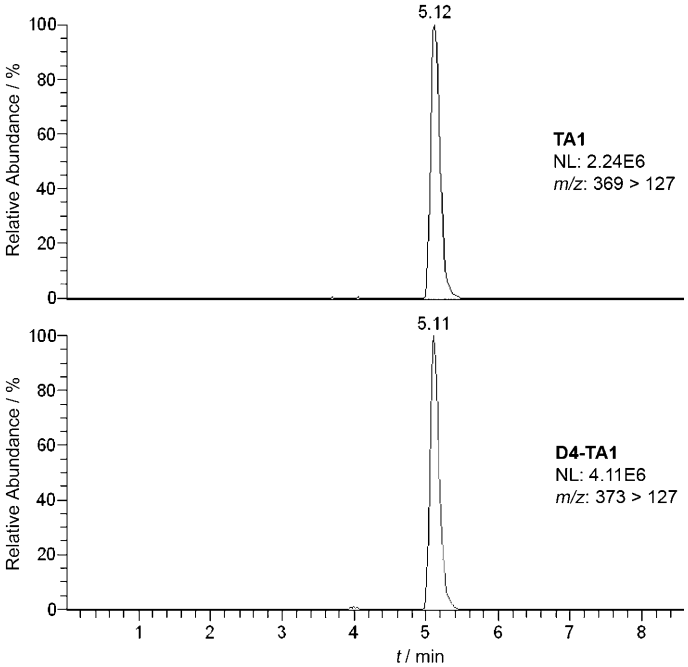

Figure 4. TA_1 detected in 50 μL of rat serum (60 min post T_1AM injection) by LC-tandem mass spectrometry (LC-MS/MS). The upper extracted ion chromatogram demonstrates detection of TA_1 (m/z 369 > 127). The lower chromatogram demonstrates the detection of synthetic deuterated standard, d_4-TA_1 (m/z 373 > 127), spiked into the rat serum.

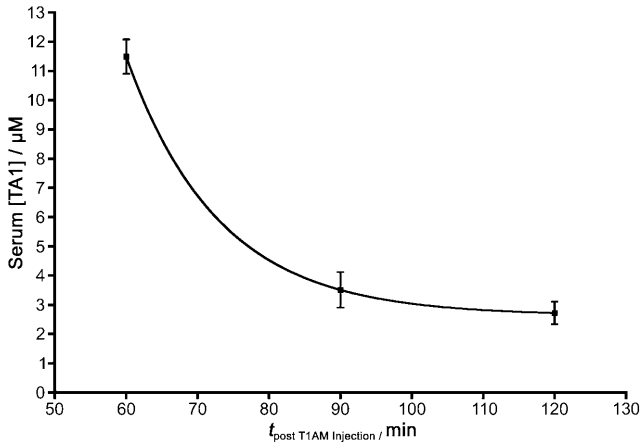

Figure 5. Rat serum concentration TA_1 (μM) for each sample set analyzed. TA_1 was quantified from serum samples collected from rats ($n = 2$) 60, 90, and 120 min after injection with 20 mg kg^{-1} T_1AM.

Discussion

T_1AM is a newly discovered thyroid hormone metabolite that has unique biological activity, and the catabolism of T_1AM is therefore an important determinant of T_1AM levels in tissue and circulation. Enzyme-catalyzed deiodination[8] and sulfation[7] of T_1AM 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 T_1AM would be subject to oxidative deamination by amine oxidase enzymes (MAO or SSAO). The expected products of such catabolism would be thyroacetic acids, which interestingly, are already known as metabolites of T_4. Our results demonstrate that conversion of iodothyronamines to iodothyroacetic acids occurs in cultured cells, ex-vivo tissue homogenates, and in vivo.

Endogenous TA_1, the thyroacetic acid corresponding to amine oxidase action on T_1AM, was found in serum in only trace quantities (< 0.1 nM); however, 3–12 mM concentrations of TA_1 were measured in serum from rats injected with 20 mg kg^{-1} T_1AM. This indicates that TA_1 is a major metabolite of T_1AM, and that like deiodination and sulfation, oxidative deamination is a significant pathway for T_1AM catabolism. It is in-

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teresting to note that the more highly iodinated thyroacetic acids Triac and 3,3′,5,5′-tetrathyroacetic acid (Tetrac), which are biologically active oxidative deamination metabolites of T_3 and T_4, respectively, have been known for several decades, and the biosynthetic origin of these compounds and the enzymes involved in the conversion are not completely clear.[9,10] We found that T_3AM is converted into Triac in the presence of HepG2 cells; this suggests a new or alternative biosynthetic sequence for the production of Triac, and by extension, Tetrac. The iodothyronines T_4 and T_3 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 (TA_0) is excreted in urine; this indicates that a much larger quantity of T_4 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 T_4, is much larger than previous estimates.[1]

Conclusions

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 TA_0, which is difficult to account for on the basis of iodothyronine conversion to iodothyroacetic acids alone. At present, T_1AM and thyronamine (T_0AM) are the only thyronamines that have been detected in vivo; however, 3,3′,5,5′-tetraiodothyronamine (T_4AM), phenolic ring deiodinated-T_3AM (rT_3AM), and 3,3′-diiodothyronamine (T_2AM) are good substrates for the deiodinase enzymes in vitro.[8] T_4AM is efficiently converted to rT_3AM by Dio3, and rT_3AM is efficiently converted to 3,3′-T_2AM by Dio1 and Dio2. If these additional iodothyronamines are endogenous compounds, they are likely to be also amine oxidase substrates and converted to their corresponding iodothyroacetic acid derivatives. Each of these compounds would converge on TA_0 after exhaustive deiodination. Thus, some fraction of the large excreted TA_0 pool may ultimately derive from the conversion of T_4 into iodothyronamines; this suggests 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-^{125}I]-T_1AM was synthesized according to literature procedure.[21] The synthesis of [3′-^{125}I]-T_3AM, TA_1, and deuterated TA_1 are described in the Supporting Information.

Cell culture: HepG2 cells were cultured in minimum essential medium with glutamine (2 mM L^{-1}) and Earle's balanced salt solution (BSS) adjusted to contain sodium carbonate (1.5 g L^{-1}), nonessential amino acids (0.10 mM), sodium pyruvate (1.0 mM), penicillin (100 units per mL), streptomycin (100 μg mL^{-1}), and heat-inactivated fetal bovine serum (10%). Cells were grown in a humidified atmosphere of 5% CO_2 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 medium was placed into wells with or without cell monolayers. To each well was added either DMSO (1 μL) or iproniazid DMSO solution (1 μL, 100 mM). The plate was placed in a humidified atmosphere of 5% CO_2 at 37 °C for 30 min. Next, a solution of [3-^{125}I]-T_1AM (1 μL, 0.19 mCi μL^{-1}) was added to each well, and the plate was returned to a humidified atmosphere of 5% CO_2 at 37 °C. After 3 h the medium was removed and transferred to a 1.7 mL eppendorf tube containing HCl solution (50 μL, 3 M). This acidified solution was extracted three times with dichloromethane (300 μL). 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 methanol (10 μL) and then spotted onto a TLC plate. The vessel with residue was washed with methanol (10 μL) 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 hexanes/ethyl acetate (1:1) containing acetic acid (0.5%). 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′-^{125}I]-T_3AM.

Thyroid tissue homogenate assays: Thyroid tissue was homogenized on ice in homogenization buffer (0.12 M NaCl, 0.4 mM KCl, 0.4 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.4) to provide a homogenate of 1 g of tissue per 15 mL. Next, thyroid homogenate or buffer (500 μL) was placed into wells of a 24-well plate. To each well was added either DMSO (1 μL) or iproniazid DMSO solution (1 μL, 100 mM). The plate was placed in a humidified atmosphere of 5% CO_2 at 37 °C and rocked for 30 min. Next a solution of [3-^{125}I]-T_1AM (1.0 μL, 0.19 μCi μL^{-1}) was added to each well and the plate was returned to a humidified atmosphere of 5% CO_2 at 37 °C and rocked. After 3 h the buffer or homogenate was removed and transferred to a 1.7 mL eppendorf tube containing a HCl solution (50 μL, 3.0 M). This acidified solution was extracted three times with dichloromethane (300 μL). 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 methanol (10 μL) and then spotted onto a TLC plate. The vessel with residue was washed with methanol (10 μL) 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 hexanes/ethyl acetate (1:1) containing acetic acid (0.5%). 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.

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Extraction procedure for endogenous TA_1: For isolation of TA_1 in rat serum dosed with pharmacological levels of T_1AM, samples were thawed on ice from stores frozen at −80 °C. Triplicates of 50 μL serum from two rats at three sequential dose time points—60, 90 and 120 min post injection—were used in sample work-up. To the aliquots, synthetic deuterated TA_1 (d_4-TA_1, 1000 pmol) was added as an internal standard. The samples were brought to a final volume of 200 μL with water, and iopanoic acid (final concentration 1 mM), which is used to inhibit deiodination during processing, was added. 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 14000 rpm for 5 min. The supernatant was transferred to a glass culture tube and evaporated to dryness with a SPD 121P Thermosavant model speedvac concentrator at 35 °C (Thermo Scientific, Waltham, MA). Residues were dissolved in aqueous formic acid (1%, 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). TA_1 was eluted with methanol (1 mL, then 0.5 mL twice) using positive pressure argon. The methanol solvent was removed with a speedvac at 35 °C and the dried organic extracts were reconstituted in acetonitrile/deionized water (1:1, 80 μL). 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 with 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 auxiliary gas flow rates, 25 and 10 (arbitrary units), 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 TA_1 transition from the *m/z* 369 precursor to the *m/z* 127 product ion and the d_4-TA_1 transition from the *m/z* 373 precursor to the *m/z* 127 product ion (each transition expressing a ratio of parent ion to molecular iodine). The collision energy was 10 eV with a collision gas pressure of 0.8 mTorr. Scan event settings were: scan width 1.5 *m/z*, scan time 0.5 s, 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 autosampler and HPLC pump. TA_1 was resolved from closely eluting interfering analytes using a Waters Xterra Phenyl 3.5 μm, 3.0×50 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: solvent A, water and acetic acid (0.002%), and solvent B, acetonitrile and acetic acid (0.002%). Phase I: 0–5 min 80% A, 20% B, in a positive linear slope to phase II: 5–15 min 5% A, 95% B; and phase III: 15.50–25 min 80% A, 20% B. The mobile phase was delivered at a flow rate of 250 μL min^{-1} and a 3 min column wash was included between sample runs. The injection volume was 20 μL of 80 μL of sample work-up consisting of acetonitrile and deionized water (1:1).

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Keywords: biosynthesis · biotransformations · iodothyroacetic acids · iodothyronamines · metabolism

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