ApoB-100-containing Lipoproteins Are Major Carriers of 3-Iodothyronamine in Circulation*

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3-Iodothyronamine (T\textsubscript{1}AM)\textsuperscript{2} is a naturally occurring derivative of thyroid hormone that has biological actions distinct from those of the predominant secreted form of thyroid hormone, thyroxine (T\textsubscript{4}), and its peripheral diverted deiodination product 3,5,3\textsuperscript{-}triiodothyronine (T\textsubscript{3}). T\textsubscript{1}AM appears as the endogenous ligand for thyroid hormone receptors (1). T\textsubscript{1}AM has rapid short term effects, including induction of hypothermia, bradycardia, and hyperglycemia in mice (2–5). In addition, T\textsubscript{1}AM administration rapidly triggers a shift in fuel usage toward lipids and away from carbohydrates in both mice and Siberian hamsters (6). It has been suggested that T\textsubscript{1}AM may be generated in vivo by enzymatic deiodination and decarboxylation of T\textsubscript{4} but this has not yet been demonstrated experimentally (7). T\textsubscript{1}AM has been detected in rodent brain, heart, and liver tissues and also in circulation of mice, guinea pigs, and Siberian hamsters (2, 6, 8). Quantitative analysis of T\textsubscript{1}AM levels in rat using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) revealed that tissue concentrations of T\textsubscript{1}AM are substantially higher than serum concentrations, and in certain tissues, such as the liver, T\textsubscript{1}AM is present at significantly higher levels than T\textsubscript{4} and T\textsubscript{3} (9). However, human sera analyzed with a recently developed highly selective T\textsubscript{1}AM immunoassay demonstrated T\textsubscript{1}AM levels comparable with those of total circulating T\textsubscript{4} (10).

Thyroid hormones are present in circulation largely bound to carrier proteins. More than 99% of circulating T\textsubscript{4} is bound to serum proteins, including thyroxine-binding protein, and transthyretin (11–13). T\textsubscript{3} binds to the same proteins, although with lower affinity. Because of the chemical similarities and potential biosynthetic origins of T\textsubscript{1}AM from thyroid hormones, and because of the presence of T\textsubscript{1}AM in both serum and tissues, we investigated whether T\textsubscript{1}AM, like thyroid hormones, was also bound to carrier proteins in serum.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-Thyroxine (T\textsubscript{4}), 3,3\textsuperscript{-},5\textsuperscript{-}triiodothyronine (T\textsubscript{3}), 3,3\textsuperscript{-},5\textsuperscript{-} triiodothyronamine (rT\textsubscript{3}AM), 3,5-diiodothyronamine (3,5-T\textsubscript{2}AM), and 3-(3,5-diiodo-1-thyronine (rT\textsubscript{3}) were obtained from Sigma, and 3-iodo-1-thyronine (T\textsubscript{1}) was from Toronto Research Chemical Inc. Canada. T\textsubscript{1}AM and other tyronamines such as 3,3\textsuperscript{-},5\textsuperscript{-} tetraiodothyronamine (T\textsubscript{4}AM), 3,3\textsuperscript{-},5\textsuperscript{-} triiodothyronamine (T\textsubscript{3}AM), 3,5-diiodothyronamine (3,5-T\textsubscript{2}AM), 3,3\textsuperscript{-},3\textsuperscript{-},5\textsuperscript{-} diiodothyronamine (3,3\textsuperscript{-},3\textsuperscript{-},5\textsuperscript{-} T\textsubscript{3}AM), and thyronamine (T\textsubscript{0}AM) were synthesized according to the literature (14). Anhydrous dimethylformamide (DMF) was obtained by passing through two columns of activated molecular sieves. Final compounds were characterized by \textsuperscript{1}H NMR and mass spectrometry.

**Determination of T\textsubscript{1}AM Binding to Serum Protein**—500 μl of normal pooled serum (human and rat serum from Innovative Research, Novi, MI; mouse serum from Millipore, Billerica, MA) was incubated with tracer quantities of [\textsuperscript{125}I]T\textsubscript{1}AM for 24 h at 4 °C in the presence or absence of excess unlabeled T\textsubscript{1}AM (50 μM). Bound and free [\textsuperscript{125}I]T\textsubscript{1}AM was separated by filtering through 3K Amicon ultracentrifugal filters (Fisher) at 3000 rpm on a table top centrifuge (Beckman, GS-6R centri-
Preparation of Affinity Chromatography Supports—All solvents (distilled water, buffer) used in this synthesis were degassed with argon. Synthesis of T₁AM containing activated disulfide (compound 3) and tyramine containing activated disulfide (compound 7, supplemental Fig. S1) are described in the supplemental material. Compounds 3 and 7 were both immobilized at thiol-Sepharose 4B (Sigma) according to the following procedure: 1 g of freeze-dried activated thiol-Sepharose 4B was suspended in distilled water, and the slurry was poured into a 25 × 1.2-cm column and washed for 15 min with distilled water. The free thiol form of Sepharose 4B was prepared according to the manufacturer’s protocol. The thiol-Sepharose 4B column was then equilibrated with immobilization buffer (0.5 M NaCl, 1 mM EDTA, 10 mM sodium acetate (pH 5.0)). The coupling reaction was performed by incubating activated disulfide containing either T₁AM (compound 3) or tyramine with the thiol-Sepharose matrix in buffer (DMF/buffer = 1:1, 10 mM sodium acetate, 0.5 M NaCl, and 1 mM EDTA) by gentle swirling for 6 h at 4 °C. The support was then washed with 10 mM sodium acetate (pH 6.0), and the amount of immobilized compound 4 was determined by UV-spectroscopic quantification of the released 2-thiopyridone at 343 nm (15). An alternative procedure was also found to be successful, and this is described in the supplemental material, method B.

Affinity Chromatography—Normal pooled human serum (1 ml) was incubated with the T₁AM-attached thiol-Sepharose matrix (4 ml) in 0.01 M PBS buffer containing 1 mM EDTA, overnight at 4 °C. After incubation, the column was washed exhaustively with PBS buffer (8 times, 50 ml) containing different concentrations of salt (NaCl, 137–500 mM) until A₂₈₀ <0.02. Bound proteins were eluted by cleavage of the disulfide bond with 1% DTT in 4 ml of PBS buffer (1 h at room temperature), and the resulting eluates were collected separately. Identification of T₁AM-binding protein was determined by gel electrophoresis (4–20% Tris–HCl gel) of the DTT eluates obtained from three affinity columns, namely T₁AM-attached Sepharose, tyramine-attached Sepharose, and activated thio-Sepharose. Coomassie stain was used to visualize the gel for both human and mouse sera, and silver stain was used for rat. Extra protein bands (circled in Fig. 3A), observed in lane 4, correspond to the eluate from T₁AM-attached thiol-Sepharose matrix and were analyzed by LC/MS (supplemental material) using an Agilent 1100 series capillary LC system (Agilent Technologies Inc, Santa Clara, CA) and an LTQ linear ion trap mass spectrometer (ThermoFisher, San Jose, CA).

Effect of LDL on the Entry of T₁AM into HepG2 Cells—HepG2 cells were washed with Dulbecco’s phosphate-buffered saline (supplemented with CaCl₂ and MgCl₂ (pH 7.2)), harvested by
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trypsinization (0.05% trypsin and 0.5 mM EDTA), and then diluted with cell culture medium (DMEM/FBS) so as to seed them at about 5 × 10\textsuperscript{4} cells/well (2 ml) in 6-well plates (3.5 × 1.0 cm; Labware). To induce maximal expression of LDL receptors, the usual culture medium (DMEM/FBS) was changed 48 – 60 h before the experiment with DMEM supplemented with 10% (v/v) human lipoprotein-deficient serum (LDS; Sigma; product number S5519) (24).

**Incubation Procedure**—A detailed procedure is described in the literature (24). In brief, HepG2 cells were washed with 2 ml of Hanks’ balanced salt solution (Thermo Scientific) buffered with 15 mM HEPES and preincubated with 2 ml of Hanks’ balanced salt solution for 45 min at 37 °C under 5% CO\textsubscript{2}. For analysis of saturable uptake of T\textsubscript{1}AM in cells, 0.5 ml of the incubation mixture was added to each well. Typically, in each 6-well plate, three wells were used for the total cell uptake of T\textsubscript{1}AM, whereas the other three wells were used for the nonsaturable cell uptake of T\textsubscript{1}AM. The incubation mixture for test experiments contained 0.5 nM \textsuperscript{125}I-T\textsubscript{1}AM plus LDL (10 μg/ml) in the presence and absence of 10 μM unlabeled T\textsubscript{1}AM. However, the incubation mixture for control experiments contain only 0.5 nM \textsuperscript{125}I-T\textsubscript{1}AM in the presence and absence of 10 μM unlabeled T\textsubscript{1}AM (24). After incubation for the specified time at 37 °C under 5% CO\textsubscript{2}, the plates were washed three times in rapid succession with 2 ml of ice-cold Dulbecco’s phosphate-buffered saline containing 0.1% BSA. Cells were solubilized in 1 ml of 0.1 N NaOH with gentle shaking, and a 750-μl aliquot was used for measurement of radioactivity. Protein concentration was determined with a BCA protein assay (Thermo Scientific). Results (means ± S.D.) are expressed in terms of specific (saturable) uptake of T\textsubscript{1}AM per mg of cell protein. For determination of the amount of intracellular and cell surface T\textsubscript{1}AM uptake, a similar procedure was used with an incubation time of 60 min. After incubation, fractionation was performed using a subcellular protein fractionation kit (Thermo Scientific), and the saturable T\textsubscript{1}AM uptake in each fraction was determined by gamma counting.

**Uptake of \textsuperscript{125}I-LDL to Fibroblasts**—To induce the maximal expression of LDL receptors, the usual culture medium (DMEM/FBS) was changed 48 – 60 h before the experiment with DMEM supplemented with 10% (v/v) human lipoprotein-deficient serum (DMEM/LDS) (see above). In a separate experiment, 10 μg/ml of \textsuperscript{125}I-LDL (Biomedical Technologies Inc., MA) was preincubated with different concentrations of T\textsubscript{1}AM (0, 10, 40, 80, 160, and 320 pM) for 24 h at 4 °C. Cells were washed with Dulbecco’s phosphate-buffered saline, harvested by trypsinization, and suspended in incubation medium (DMEM supplemented with 10% LDS and 24 mM bicarbonate at pH 7.4) to determine binding and uptake of \textsuperscript{125}I-LDL. Fibroblasts were incubated with \textsuperscript{125}I-LDL in the presence and absence of a 50-fold excess of unlabeled LDL in DMEM supplemented with 24 mM bicarbonate and 10% human LDS at pH 7.4 for 2 h (25). Intracellular uptake and surface binding of \textsuperscript{125}I-LDL to the fibroblasts at 37 °C were determined as described previously (25).

**Effect of T\textsubscript{1}AM on Secretion of ApoB Protein in HepG2 Cells**—HepG2 cells were grown in 75-cm\textsuperscript{2} flasks in culture medium for 24 h at 37 °C with 5% CO\textsubscript{2}. The medium was then removed, and the cells were washed with Hanks’ balanced salt solution and incubated with DMEM supplemented with 10% LDS. Cells were then treated with different doses of T\textsubscript{1}AM (dissolved in DMSO), and the same volume of DMSO was used for the control experiment. ApoB secretion into the media after 48 h was measured as described previously (26–29). ApoB secretion into media was determined by ELISA (“CardioCHEK,” ALerCHEK) according to the manufacturer’s instructions. ApoB secretion was normalized to total cell protein as determined by BCA protein assay.

**Animals**—The experimental protocol was in compliance with the Federal guidelines for care and handling of small rodents and approved by the Institutional Animal Care and Use Committee (IACUC) of Oregon Health and Science University. Animals were housed in a temperature-controlled room with alternating 12-h periods of light and dark and had free access to food and water. All animals were allowed to adapt to the environment for at least 2 weeks prior to treatment. Wild type mice (C57BL/6; male; 8 – 10 weeks old; The Jackson Laboratory, Bar Harbor, ME) were given high fat diet (Rodent Diet 60% kcal fat, Research Diet Inc; Item D12492) for 15 days prior to the administration of T\textsubscript{1}AM and continued the same high fat diet during 15 days administration of T\textsubscript{1}AM. Conversely, apoB-100 transgenic mice (B6.SJL-Tg(APOB)1102Sgy N20; female; 9 – 11 weeks old; Taconic Laboratories, Hudson, NY) were given only normal rodent chow diet throughout the experiment. Animals were injected intraperitoneally once daily with different doses of T\textsubscript{1}AM (0.4, 0.25, and 0.01 mg/kg), and the control mice were treated with same volume of saline. Each group contained five individual mice. Weight gain of all mice was monitored every day during the period of administration. After 15 days administration of T\textsubscript{1}AM, mice were euthanized with CO\textsubscript{2} and blood was collected via cardiac puncture into tubes containing EDTA (BD Microtainer). Food was removed from the mice 4 h before the collection of blood. Lipid profile from the mice 4 h before the collection of blood. Lipid profile of all mice was determined by using an ELISA kit (see above).

**Statistical Analysis**—Values are reported as mean ± S.D. Statistical analysis was performed with Student’s t test. p > 0.05 was considered not to be significant.

**RESULTS**

To test whether T\textsubscript{1}AM was protein-bound in serum, we incubated a tracer quantity [\textsuperscript{125}I]T\textsubscript{1}AM with serum from mouse, rat, and human, separated bound T\textsubscript{1}AM from free by centrifugal dialysis filtration, and measured the amount of bound T\textsubscript{1}AM by gamma counting compared with a buffer control. For each sample (and all subsequent binding experiments) specific binding of T\textsubscript{1}AM was assessed by adding a large excess of unlabeled T\textsubscript{1}AM to determine the component of nonspecific T\textsubscript{1}AM binding. A comparable amount of specifically bound T\textsubscript{1}AM was observed in serum from all three species (Fig. 1A).

Using human serum, we next assessed the concentration dependence of T\textsubscript{1}AM-specific binding by incubating a range of T\textsubscript{1}AM concentrations (0.1 nM to 10 μM) supplemented with tracer [\textsuperscript{125}I]T\textsubscript{1}AM (Fig. 1B). In this case, free T\textsubscript{1}AM was separated from bound by charcoal filtration. Analysis of the specifically bound radioactivity revealed saturation binding of T\textsubscript{1}AM to a macromolecular serum component.
We devised an affinity chromatography strategy for isolating the putative T\textsubscript{1}AM serum-binding protein(s) that involved the chemical synthesis of T\textsubscript{1}AM-immobilized to a solid support (Fig. 2). The amine group of T\textsubscript{1}AM was first protected with a tert-butylcarbonyl (BOC) group, and then the BOC-protected T\textsubscript{1}AM was reacted with propargyl bromide in DMF under basic conditions to afford alkyne \textit{2}. Activated disulfide \textit{N-[4-(p-azidosalicylamido)butyl]-3′-(2′-pyridyldithio) propionamide} was used as a cross-linker, which contains the azide functional group. The azide was then reacted with the alkyne group of \textit{2} in the presence of 5 mol \% of sodium ascorbate and 1 mol \% of copper(II) sulfate in DMF providing a white solid fluorescent compound. Deprotection of the BOC group with dry HCl/ethyl acetate gave T\textsubscript{1}AM-containing activated disulfide \textit{3}. For a control column, we attached tyramine to Sepharose beads through its phenolic-OH group using similar chemistry. Both T\textsubscript{1}AM and tyramine containing activated disulfides were covalently attached to Sepharose 4B beads via a disulfide exchange reaction, the progress of which was monitored by following the generation of 2-mercaptopyridine by UV-visible spectrophotometry.

Pooled normal human serum was incubated with T\textsubscript{1}AM-de-derivatized Sepharose beads (compound \textit{4}), washed extensively with a PBS/NaCl gradient, and the flow-through (eluate) obtained during washing was collected until an \textit{A\textsubscript{280}} <0.02 in the eluent was observed. Bound protein was eluted by cleaving the disulfide bond connecting T\textsubscript{1}AM to the Sepharose bead with 1\% DTT. The DTT eluate was analyzed by SDS-PAGE. SDS-PAGE results show that the DTT eluate obtained from the T\textsubscript{1}AM-derivatized column contained two unique high molecular weight bands compared with the thiol-Sepharose and tyramine control columns (Fig. 3A). The protein contained in these two bands (Fig. 3A, \textit{lane 4}) was extracted from the gel and sequenced using standard proteomic mass spectrometry techniques. Sequence analysis showed that both bands corresponded to the same protein-apolipoprotein B-100 (apoB-100), the major protein component of very low density lipoprotein (VLDL), and the sole protein component of low density lipoprotein (LDL) particles (30). Mass spectrometry analysis of these two bands (Fig. 3A, \textit{lane 4}) showed 25\% protein sequence coverage for apoB-100 (supplemental Tables S1–S3).
We next examined whether T1AM could bind to intact lipoprotein particles containing apoB-100 such as VLDL and LDL. A mixture of VLDL and LDL was isolated from pooled normal human serum by sequential density ultracentrifugation (16, 31). SDS-PAGE of this preparation revealed the presence of apoB-100 (500 kDa) as the major protein present in this lipoprotein fraction (Fig. 3B and supplemental Fig. S6). This apoB-100-enriched fraction was incubated with [125I]T1AM (32), and free [125I]T1AM was separated from bound [125I]T1AM by gel filtration. Native-PAGE of the bound fraction revealed that T1AM co-migrates with apoB-100-containing particles (Fig. 3C and supplemental Fig. S7), whereas no radioactivity was observed in the SDS-PAGE (Fig. 3B). Furthermore, mass spectrometry analysis of this particular band in the native-polyacrylamide gel (circled in Fig. 3C) revealed that apoB-100 is the major protein present (supplemental Tables S4–S6). These results indicate that T1AM binds reversibly to VLDL and LDL particles, most likely through association with apoB-100. We also used a similar affinity chromatography strategy to identify T1AM-specific binding protein(s) from mouse and rat serum. SDS-PAGE results show that the DTT eluate obtained from the T1AM-derivatized columns incubated with rodent sera also contained apoB-100 (Fig. 3D).

We next examined the distribution of bound T1AM in human serum to determine what fraction of bound T1AM was associated with apoB-100-containing lipoprotein particles. [125I]T1AM was incubated with human serum followed by sequential fractionation of the serum by density ultracentrifugation and analysis of bound T1AM by gamma counting. More than 95% of the specifically bound T1AM was found associated with lipoproteins, and the remaining <5% was bound to other unidentified serum components (Fig. 4A). The apoB-100-containing lipoprotein fraction was further fractionated by density ultracentrifugation into VLDL, LDL, and high density lipoprotein (HDL) fractions, and the amount of bound [125I]T1AM was determined by gamma counting. More than 90% of the specifically bound T1AM was found associated with the apoB-100-containing lipoprotein particles such as VLDL and LDL, with roughly equal distribution between VLDL and LDL; less than 10% of the labeled T1AM was contained in the HDL fraction (Fig. 4B).

We next examined the relationship between the bound T1AM and the amount of apoB-100 in the context of both VLDL and LDL (Fig. 5, A and B). Different amounts of purified VLDL and LDL (5–25 μg) were incubated with [125I]T1AM, and the bound [125I]T1AM was quantified. The amount of [125I]T1AM specifically bound was found to be proportional to the amount of apoB-100-containing lipoprotein particle added to the incubation mixture. The time dependence of [125I]T1AM binding to LDL was also examined with the finding that 90% of
the specific binding occurred within 60 min at 4 °C (pH 7.4) (Fig. 5C). We next studied the equilibrium binding properties of T1AM with apoB-100 in the context of a human serum fraction enriched in VLDL and LDL such that apoB-100 was the major protein component present as discussed previously (Fig. 3B and supplemental material). T1AM binding to this preparation was found to be concentration-dependent with saturation of specific binding at high T1AM concentrations. The data showed best fit to a one-site model, suggesting a single apoB-100-binding site for T1AM (Fig. 6A). Scatchard analysis of these data indicated single site binding with an equilibrium dissociation constant (KD) of 17 nM and a ligand/protein stoichiometry of 1:1(Fig. 6A, inset). We also examined equilibrium binding of T1AM to a highly purified LDL fraction (i.e. no VLDL such as that used in Fig. 4B) and observed a similar saturation binding curve with a similar KD of 48 nM (supplemental Fig. S8). To evaluate the specificity of T1AM binding to apoB-100, a competition binding assay was performed between [125I]T1AM and similar analogues, including iodothyronines (T0, T1, 3,3′-T2, 3,5-T2, T3, rT3, and T4), iodothyronamines (T0AM, T1AM, 3,3′-T2AM, 3,5-T2AM, T3AM, rT3AM, and T4AM), and other biogenic amines (tyramine and serotonin). The apoB-100-containing lipoprotein was incubated with a tracer quantity of [125I]T1AM in the presence of different concentrations of unlabeled competitor compounds for 24 h at 4 °C. Free [125I]T1AM was removed using charcoal-dextran solution. Different lipoprotein fractions (VLDL, LDL and HDL) were separated by a standard micro-ultracentrifugation technique. *, p ≤ 0.05; **, p ≤ 0.01.
values from these competition experiments are provided in Table 1. These results indicate that the apoB-100 binding of T$_1$AM is highly selective.

We studied the effect of LDL on the time course of T$_1$AM uptake in HepG2 cells in which LDL receptors are expressed maximally (33). Equilibrium uptake of T$_1$AM increased by 50% over 60 min in the presence of exogenously added LDL (100 mg/ml) compared with that in LDL-free media (Fig. 7A). When we examined where the T$_1$AM taken up by HepG2 resided, we found that 60% was membrane-associated, whereas 40% resided in the cytoplasm (Fig. 7B). We next evaluated whether T$_1$AM affected LDL uptake into fibroblasts in which LDL receptors are overexpressed (24, 33). A dose-dependent 20% increase in the saturable uptake of LDL in fibroblasts was observed (Fig. 7C). About 20% of this was surface-associated and could be released with heparin treatment, whereas about 80% was intracellular and heparin-resistant (Fig. 7D). Motivated by reports of T$_3$ stimulation of apoB synthesis and secretion in HepG2 cells (28, 34), we next examined whether T$_1$AM had any effect on this process in vitro. We found that T$_1$AM stimulated apoB secretion in a dose-dependent manner with a maximal increase in apoB secretion of 2.4-fold (Fig. 7E). We have shown previously that T$_1$AM is oxidatively deaminated to 3-iodothyroacetic acid (TA$_1$) in HepG2 cells, and the question arises whether T$_1$AM or the metabolite TA$_1$ is mediating the observed increase in apoB secretion (35). As such, HepG2 cells were treated with T$_1$AM and iproniazid, a broad spectrum amine oxidase inhibitor that blocks the T$_1$AM to TA$_1$ oxidative deamination, and apoB secretion was assessed. We found that T$_1$AM increased the secretion of apoB equally in the presence and absence of iproniazid indicating that this effect is specific to T$_1$AM and not TA$_1$ (Fig. 7F).

Given these in vitro results, we were next interested to find out whether T$_1$AM elicited similar effects in vivo. For these studies, we used wild type mice (C57BL/6J) fed a high cholesterol diet as well as human apoB-100 transgenic mice that have substantially elevated levels of circulating human apoB-100 (36–38). Mice were treated once daily (intraperitoneally) with 0, 0.025, 0.1, or 0.4 mg/kg T$_1$AM for 15 days; doses of T$_1$AM...
higher than ~0.5 mg/kg induce anorexia, which would interfere with the circulating lipid levels. In addition, these T$_3$AM doses do not induce hypothermia, hyperglycemia, or any of the other previously reported actions of T$_3$AM. After 15 days of dosing, serum was collected and evaluated for VLDL, LDL, HDL, apoB (in the transgenic mice only), triglyceride, and total cholesterol content (supplemental Fig. S3A–E). As expected, the human apoB-100 transgenic mice had elevated levels of apoB-100-containing LDL and VLDL (supplemental Fig. S3A, A and B) but reduced levels of HDL (supplemental Fig. S3C) compared with WT/high cholesterol-fed mice. This also corresponded to elevated triglycerides (supplemental Fig. S3D) and unchanged total cholesterol (supplemental Fig. S3E). T$_3$AM treatment at all doses showed no effect on any of these serum markers and also did not change circulating apoB levels in the apoB-100 transgenic mice relative to vehicle control.

**DISCUSSION**

We demonstrate here that T$_3$AM, like the thyroid hormones T$_4$ and T$_3$, is largely protein bound in circulation. Using affinity chromatography, we isolated the predominant T$_3$AM-binding protein from human and rodent serum, which turned out to be
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apoB-100, the unique protein component of low density lipoprotein particles. More than 95% of specifically bound T₁AM was found to be associated with the lipoprotein fraction in human serum, and more than 90% of the lipoprotein-bound T₁AM was distributed approximately equally between apoB-100-containing LDL and VLDL particles. T₁AM binds reversibly to apoB-100 with a $K_a$ of 17 nM and a T₁AM/apoB-100 stoichiometry of 1:1. This binding site is highly selective for T₁AM as none of the idothyronamines or idothyronines competed effectively with T₁AM in ligand competition binding assays and had IC₅₀ values 50–1000 times higher than that of T₁AM.

ApoB-100 is a >500-kDa polypeptide that functions as the major protein component of LDL, VLDL, IDL, and Lp(a) lipoprotein particles (30). Circulating apoB-100 polypeptide is never free but is part of a large particle composed of lipids and protein. Depending on the circulating lipid inventory, which is a function of life style and genetics in humans, the concentration of circulating apoB-100 in humans is 1.5–3.0 μM (77–153 mg/dl) (39).

Although the majority of circulating thyronine-based thyroid hormones is bound by nonlipoprotein carriers, small amounts of T₄ and T₃ (3% of T₄ and 6% of T₃) are bound to VLDL, LDL, and HDL lipoprotein particles (40). Thyronine binding to LDL and VLDL involves apoB-100, whereas binding to HDL occurs at the HDL-specific apolipoprotein A-I (apoA-I) (41, 42). ApoB-100 reportedly contains a binding site for T₄ with a $K_a$ of 1 μM, and apoB-100-bound T₄ may facilitate entry of T₄ into cells via LDL/LDL receptor-mediated endocytosis. We found a higher affinity interaction between T₁AM and apoB-100, and T₄ was unable to compete against T₁AM for binding to apoB-100. We conclude from this that the T₁AM-binding site on apoB-100 is distinct from the previously reported T₄-binding site and that the T₄-binding site does not allosterically influence the T₁AM-binding site.

The finding that circulating T₁AM is largely bound to apoB-100 as part of a lipoprotein particle may explain the discrepancy in the reported serum levels of T₁AM using different biochemical methods (43). Using an LC/MS/MS approach, Saba et al. (9) reported high tissue concentrations of T₁AM but very low serum concentrations, on the order of 0.3 nM. In contrast, Hoefig et al. (10) measured substantially higher serum T₁AM concentrations averaging ~66 nM using an immunoassay based on a monoclonal antibody that selectively binds T₁AM. This immunoassay did not involve the use of an extraction procedure for isolating biogenic amines from the serum matrix, whereas all LC/MS/MS-based approaches involve some kind of a chemical extraction step. It is therefore possible that the typical biogenic amine extraction procedures do not quantitatively liberate T₁AM from its unique binding site residing within a lipoprotein particle and that extraction-based approaches quantitify free and not total circulating T₁AM. Additionally, if total T₁AM plasma concentration in humans is on the order of 70 nM, and as discussed previously circulating apoB-100 concentrations range from 1.5–3.0 μM, then the T₁AM serum-binding sites are normally in excess compared with the total T₁AM serum concentration suggesting that is largely bound and not free in circulation.

The obvious question that arises concerns the functional role of specific, high affinity binding of T₁AM to apoB-100-containing lipoprotein particles. In experiments aimed to address this, we observed modest effects by T₁AM in vitro on LDL uptake and apoB-100 secretion; however, none of these effects were apparent in vivo. For this we used multiple dose T₁AM treatment on diet-induced hypercholesterolemic wild type or transgenic human apoB-100 mice and observed no change in circulating lipid or lipoprotein inventory, suggesting that T₁AM had no measurable effect on either synthesis or clearance of apoB-100. One caveat to these in vivo studies is that the maximum dose of T₁AM was limited to 0.4 mg/kg due to a dose-limiting side effect of appetite suppression at doses higher than 0.5 mg/kg, which would indirectly result in lipid lowering; nevertheless, T₁AM did not induce a significant dose-dependent change to VLDL, LDL, HDL, apoB-100, triglyceride, or total cholesterol levels.

However, the addition of exogenous LDL to cultured cells incubated with radiolabeled T₁AM resulted in a 50% enhancement in the cellular uptake of T₁AM, suggesting that the physiological role of T₁AM association with apoB-100 may be to provide a mechanism for transportation and entry of T₁AM into target cells via the LDL receptor (LDLr)-mediated endocytosis or some other LDLr-independent pathway. Essentially all cell types express LDLrS for the purpose of accessing cholesterol and other lipids, and LDLrS are especially prevalent in hepatocytes where they mediate the first step in cholesterol clearance (33). Consistent with this is the fact that endogenous T₁AM is also most abundant in the liver (9).

The robust, specific uptake of T₁AM into a variety of cell types has been previously reported with the mechanism of this transport process remaining unclear (44). Transport by a biogenic amine plasma membrane transporter related to dopamine, serotonin, or norepinephrine reuptake transporters was ruled out based on the lack of an observed ion or pH dependence. In addition, a systematic functional screen of most members of the solute carrier (SLC) transporter family did not reveal any orphan family members specific for T₁AM transport. It has been further demonstrated that T₁AM is not a substrate for monocarboxylate transporter (MCT)-8 or MCT-10, both established specific transporters of T₄ and T₃ (45). A T₁AM uptake mechanism based on receptor-mediated endocytosis could not be ruled out, and the prospect of this as the uptake route of T₁AM is clearly strengthened by this study. Indeed, receptor-mediated endocytosis involving apoB-100 is a well established cellular uptake mechanism for small molecule lipids such as cholesterol and triglycerides. If this proves also to be the case for T₁AM, then it suggests that a target of the biological action of T₁AM resides within the cell and not on the plasma membrane. This situation further suggests that the standard theories regarding free and bound hormone fractions where only the free fraction is considered to be biologically active may not be relevant for T₁AM.

REFERENCES


