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

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3-Iodothyronamine (T_1AM) is a biogenic amine derivative of thyroid hormone present in tissue and blood of vertebrates. Approximately 99% of the circulating thyroid hormones are bound to plasma proteins, including three major thyroid hormone-binding proteins, and the question arises as to whether circulating T_1AM is also bound to serum factors. We report here that T_1AM is largely bound to a single protein component of human serum. Using T_1AM-affinity chromatography, we isolated this protein, and sequence analysis identified it as apolipoprotein B-100 (apoB-100), the protein component of several low density lipoprotein particles. Consistent with this finding, we demonstrate that >90% of specifically bound T_1AM in human serum resides in the apoB-100-containing low density lipoprotein fraction. T_1AM reversibly binds to apoB-100-containing lipoprotein particles with an equilibrium dissociation constant (K_D) of 17 nM and a T_1AM/apoB-100 stoichiometry of 1:1. Competition binding assays demonstrate that this binding site is highly selective for T_1AM. Intracellular T_1AM uptake is significantly enhanced by apoB-100-containing lipoprotein particles. Modest enhancements to apoB-100 cellular uptake and secretion by T_1AM were observed; however, multidose T_1AM treatment did not affect lipid or lipoprotein inventory *in vivo*. Thus, it appears that apoB-100 serves as a carrier of circulating T_1AM and affords a novel mechanism by which T_1AM gains entry to cells.

3-Iodothyronamine (T_1AM)[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_4), and its peripherally derived deiodination product 3,5,3'-triiodothyronine (T_3), the endogenous ligand for thyroid hormone receptors (1). T_1AM has rapid short term effects, including induction of hypothermia, bradycardia, and hyperglycemia in mice (2–5). In addition, T_1AM administra-

tion 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_1AM may be generated *in vivo* by enzymatic deiodination and decarboxylation of T_4, but this has not yet been demonstrated experimentally (7). T_1AM 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_1AM levels in rat using liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) revealed that tissue concentrations of T_1AM are substantially higher than serum concentrations, and in certain tissues, such as the liver, T_1AM is present at significantly higher levels than T_4 and T_3 (9). However, human sera analyzed with a recently developed highly selective T_1AM immunoassay demonstrated T_1AM levels comparable with those of total circulating T_4 (10).

Thyroid hormones are present in circulation largely bound to carrier proteins. More than 99% of circulating T_4 is bound to serum proteins, including thyroxine-binding protein, and transthyretin (11–13). T_3 binds to the same proteins, although with lower affinity. Because of the chemical similarities and potential biosynthetic origins of T_1AM from thyroid hormones, and because of the presence of T_1AM in both serum and tissues, we investigated whether T_1AM, like thyroid hormones, was also bound to carrier proteins in serum.

EXPERIMENTAL PROCEDURES

Materials—L-Thyroxine (T_4), 3,3',5-triiodo-L-thyronine (T_3), 3,3',5'- triiodo-L-thyronine (rT_3), 3,5-diiodo-L-thyronine (3,5-T_2), and L-thyronine (T_0) were obtained from Sigma, and 3-iodo-L-thyronine (T_1) was from Toronto Research Chemicals Inc. (Canada). T_1AM and other thyronamines such as 3,3',5,5'-tetraiodothyronamine (T_4AM), 3,3',5'-triiodothyronamine (rT_3AM), 3,5,3'-triiodothyronamine (T_3AM), 3,5-diiodothyronamine (3,5-T_2AM), 3,3'-diiodothyronamine (3,3'-T_2AM), and thyronamine (T_0AM) 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 ^1H NMR and mass spectrometry.

Determination of T_1AM 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 $[^{125}I]T_1AM$ for 24 h at 4 °C in the presence or absence of excess unlabeled T_1AM (50 μM). Bound and free $[^{125}I]T_1AM$ was separated by filtering through 3K Amicon ultracentrifugal filters (Fisher) at 3000 rpm on a table top centrifuge (Beckman, GS-6R centri-

[2] The abbreviations used are: T_1AM, 3-iodothyronamine; apoB-100, apolipoprotein B-100; T_4, L-thyroxine; T_3, 3,5,3'-triiodo-L-thyronine; rT_3, 3,3',5'-triiodo-L-thyronine; 3,5-T_2, 3,5-diiodo-L-thyronine; T_1, 3-iodo-L-thyronine; T_0, L-thyronine; BOC, *tert*-butyloxycarbonyl; LDS, lipoprotein-deficient serum; DMF, dimethylformamide; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; LDLR, LDL receptor.

fuge) followed by repeated washing with Tris-HCl buffer (0.1 M (pH 7.4)) at 4 °C. After excessive washing, the bound [^{125}I]T$_1$AM was measured by gamma counting (Packard Gamma Cobra II D5005). A control experiment was done in a similar manner by using 500 μl of buffer instead of sera. For the concentration-dependent binding experiment, 80 μl of normal pooled human serum was incubated with different concentrations of [^{125}I]T$_1$AM (from 0.1 nM to 10 μM), in 0.1 M Tris-HCl buffer (pH 7.4) for 24 h at 4 °C in the presence or absence of excess unlabeled T$_1$AM (50 μM). Bound and free T$_1$AM were separated by incubation with 0.5 ml of a charcoal (0.5%)/dextran (0.05%) suspension in Tris-HCl buffer for 15 min at 4 °C with gentle shaking. After centrifugation for 15 min at 4000 $\times$ g the supernatant was used for the measurement of radioactivity by gamma counting. Specific binding of [^{125}I]T$_1$AM in this experiment, and all other experiments reported in this paper, was determined by subtracting nonspecific binding from total binding.

Preparation of Affinity Chromatography Supports—All solvents (distilled water, buffer) used in this synthesis were degassed with argon. Synthesis of T$_1$AM 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 $\times$ 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 T$_1$AM (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 T$_1$AM-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 A_{280} <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 T$_1$AM-binding protein was determined by gel electrophoresis (4–20% Tris-HCl gel) of the DTT eluates obtained from three affinity columns, namely T$_1$AM-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*, corre-

spond to the eluate from T$_1$AM-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).

Serum Fractionation by Density Centrifugation—Very low density lipoprotein (VLDL; <1.006 kg/liter), low density lipoprotein (LDL; 1.019–1.063 kg/liter), and high density lipoprotein (HDL; 1.063–1.21 kg/liter) were separated from normal pooled human serum by a standard micro-ultracentrifugation technique, using KBr for density adjustment (16). Purified lipoprotein fractions were analyzed by running gel electrophoresis, native-PAGE, SDS-PAGE, and agarose gel (supplemental material) (17–20). Coomassie stain was used after running gel electrophoresis to visualize the protein in all cases.

Gel Electrophoresis and Autoradiography—Isolated apoB-100-containing lipoprotein (in 0.1 M Tris-HCl buffer (pH 7.4)) was incubated with tracer quantity of [^{125}I]T$_1$AM for 24 h at 4 °C. Separation of bound from free [^{125}I]T$_1$AM was achieved by Sephadex G-25 chromatography. The reaction mixture was then analyzed by running gel electrophoresis (4–16% BisTris native-PAGE with 4% stacking gel) (21–23) followed by staining with Coomassie Blue. Gels were dried and subjected to autoradiography at −80 °C for 7 days.

ApoB-100 Ligand Binding Experiments—For the saturable binding experiment, LDL was incubated with different concentrations of [^{125}I]T$_1$AM (from 0.25 to 200 nM), in 0.1 M Tris-HCl buffer (pH 7.4) for 24 h at 4 °C in the presence or absence of excess unlabeled T$_1$AM (50 μM). Nonspecific binding was determined by including 50 μM unlabeled T$_1$AM at each concentration of labeled T$_1$AM, [^{125}I]T$_1$AM. Bound [^{125}I]T$_1$AM was separated from free [^{125}I]T$_1$AM by using charcoal/dextran solution (as mentioned above), and the radioactivity of bound [^{125}I]T$_1$AM was measured with a gamma counter. All samples were run in triplicate. The dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were determined by nonlinear least squares curve fit utilizing the ligand depletion method to determine free concentration (Graphpad Prism Software, San Diego). For the competition binding assays, apoB-100-containing lipoprotein was incubated with tracer quantity of [^{125}I]T$_1$AM in the presence or absence of excess (0.1 pM to 100 μM) of unlabeled different structural analogues (thyronines: T$_0$, T$_1$, 3,3'-T$_2$, 3,5-T$_2$, T$_3$, rT$_3$, and T$_4$; thyronamines: T$_0$AM, T$_1$AM, 3,3'-T$_2$AM, 3,5-T$_2$AM, T$_3$AM, rT$_3$AM, and T$_4$AM; serotonin and tyramine) in buffer for 24 h at 4 °C. Free [^{125}I]T$_1$AM was removed from the bound by using charcoal/dextran solution as described above, and the radioactivity in the supernatant was counted.

Cell Cultures—HepG2 cells and normal human skin fibroblasts were obtained from ATCC (Manassas, VA). Cells were cultured in flasks with Dulbecco's modified Eagle's media (DMEM) supplemented with 10% (v/v) fetal calf serum (culture medium, DMEM/FBS) and 1% mg/ml penicillin/streptomycin at 37 °C with 5% CO$_2$. Cells were used for experiments at 80% confluency.

Effect of LDL on the Entry of T$_1$AM into HepG2 Cells—HepG2 cells were washed with Dulbecco's phosphate-buffered saline (supplemented with CaCl$_2$ and MgCl$_2$ (pH 7.2)), harvested by

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 5×10^4 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_2. For analysis of saturable uptake of T₁AM 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 T₁AM, whereas the other three wells were used for the nonsaturable cell uptake of T₁AM. The incubation mixture for test experiments contained 0.5 nM [¹²⁵I]T₁AM plus LDL (10 µg/ml) in the presence and absence of 10 µM unlabeled T₁AM. However, the incubation mixture for control experiments contain only 0.5 nM [¹²⁵I]T₁AM in the presence and absence of 10 µM unlabeled T₁AM (24). After incubation for the specified time at 37 °C under 5% CO_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 T₁AM per mg of cell protein. For determination of the amount of intracellular and cell surface T₁AM 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 T₁AM uptake in each fraction was determined by gamma counting.

Uptake of ¹²⁵I-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 ¹²⁵I-LDL (Biomedical Technologies Inc., MA) was preincubated with different concentrations of T₁AM (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 resuspended in incubation medium (DMEM supplemented with 10% LDS and 24 mM bicarbonate at pH 7.4). To determine binding and uptake of ¹²⁵I-LDL, fibroblasts were incubated with ¹²⁵I-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 ¹²⁵I-LDL to the fibroblasts at 37 °C were determined as described previously (25).

Effect of T₁AM on Secretion of ApoB Protein in HepG2 Cells—HepG2 cells were grown in 75-cm² flasks in culture medium for 24 h at 37 °C with 5% CO_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 T₁AM (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/6J; 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 T₁AM and continued the same high fat diet during 15 days administration of T₁AM. 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 T₁AM (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 T₁AM, mice were euthanized with CO_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 and the amounts of apoB of all mice were examined from serum. The amounts of apoB protein in the transgenic mice were 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 T₁AM was protein-bound in serum, we incubated a tracer quantity [¹²⁵I]T₁AM with serum from mouse, rat, and human, separated bound T₁AM from free by centrifugal dialysis filtration, and measured the amount of bound T₁AM by gamma counting compared with a buffer control. For each sample (and all subsequent binding experiments) specific binding of T₁AM was assessed by adding a large excess of unlabeled T₁AM to determine the component of nonspecific T₁AM binding. A comparable amount of specifically bound T₁AM was observed in serum from all three species (Fig. 1*A*). Using human serum, we next assessed the concentration dependence of T₁AM-specific binding by incubating a range of T₁AM concentrations (0.1 nM to 10 µM) supplemented with tracer [¹²⁵I]T₁AM (Fig. 1*B*). In this case, free T₁AM was separated from bound by charcoal filtration. Analysis of the specifically bound radioactivity revealed saturation binding of T₁AM to a macromolecular serum component.
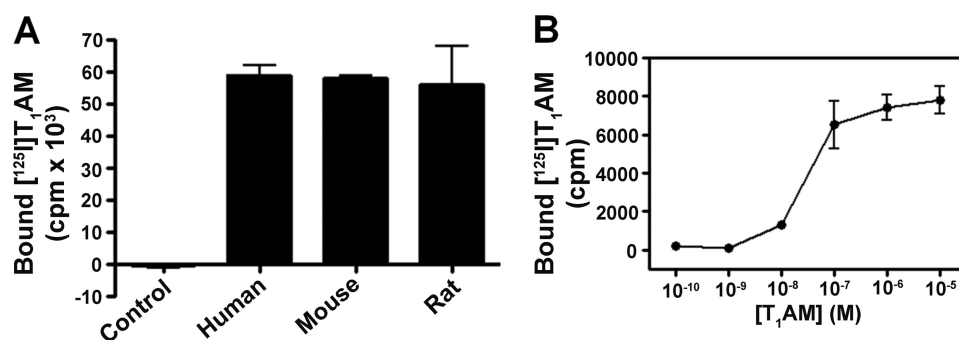
FIGURE 1. *A*, T$_1$AM-specific binding to macromolecular components in serum from human, mouse, and rat. *B*, concentration dependence of specific T$_1$AM binding in human serum.
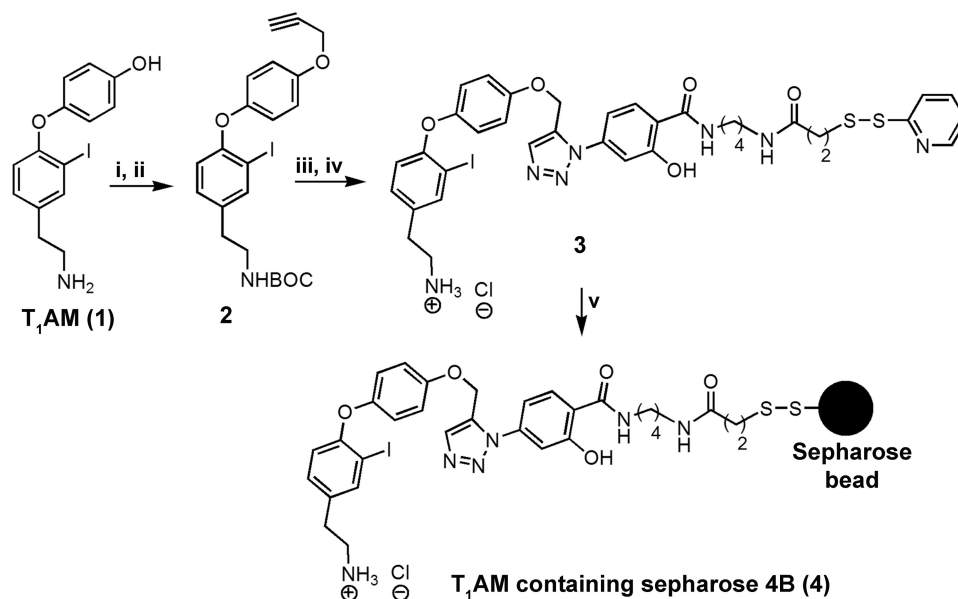

FIGURE 2. **Preparation of affinity chromatography support.** Reagents and conditions are as follows: (i) Boc$_2$O, NaHCO$_3$, THF, and H$_2$O (95%); (ii) propargyl bromide, K$_2$CO$_3$, and *N,N*-dimethylformamide (85%); (iii) cross-linker *N*-[4-(*p*-azidosalicylamido) butyl]-3′-(2′-pyridyldithio) propionamide, CuSO$_4$, 5H$_2$O, sodium ascorbate, and *N,N*-dimethylformamide (80%); (iv) HCl/ethyl acetate (90%); (v) gentle shaking of thiol-Sepharose 4B beads with T$_1$AM containing activated disulfide in 10 mM sodium acetate (pH 5.0) for 6 h.

We devised an affinity chromatography strategy for isolating the putative T$_1$AM serum-binding protein(s) that involved the chemical synthesis of T$_1$AM-immobilized to a solid support (Fig. 2). The amine group of T$_1$AM was first protected with a *tert*-butyloxycarbonyl (BOC) group, and then the BOC-protected T$_1$AM was reacted with propargyl bromide in DMF under basic conditions to afford alkyne **2**. Activated disulfide *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 **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$_1$AM-containing activated disulfide **3**. For a control column, we attached tyramine to Sepharose beads through its phenolic-OH group using similar chemistry. Both T$_1$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$_1$AM-derivatized Sepharose beads (compound **4**), washed extensively with a PBS/NaCl gradient, and the flow-through (eluate) obtained during washing was collected until an A_{280} <0.02 in the eluent was observed. Bound protein was eluted by cleaving the disulfide bond connecting T$_1$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$_1$AM-derivatized column contained two unique high molecular weight bands compared with the thiol-Sepharose and tyramine control columns (Fig. 3*A*). The protein contained in these two bands (Fig. 3*A*, *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. 3*A*, *lane 4*) showed 25% protein sequence coverage for apoB-100 (supplemental Tables S1–S3).
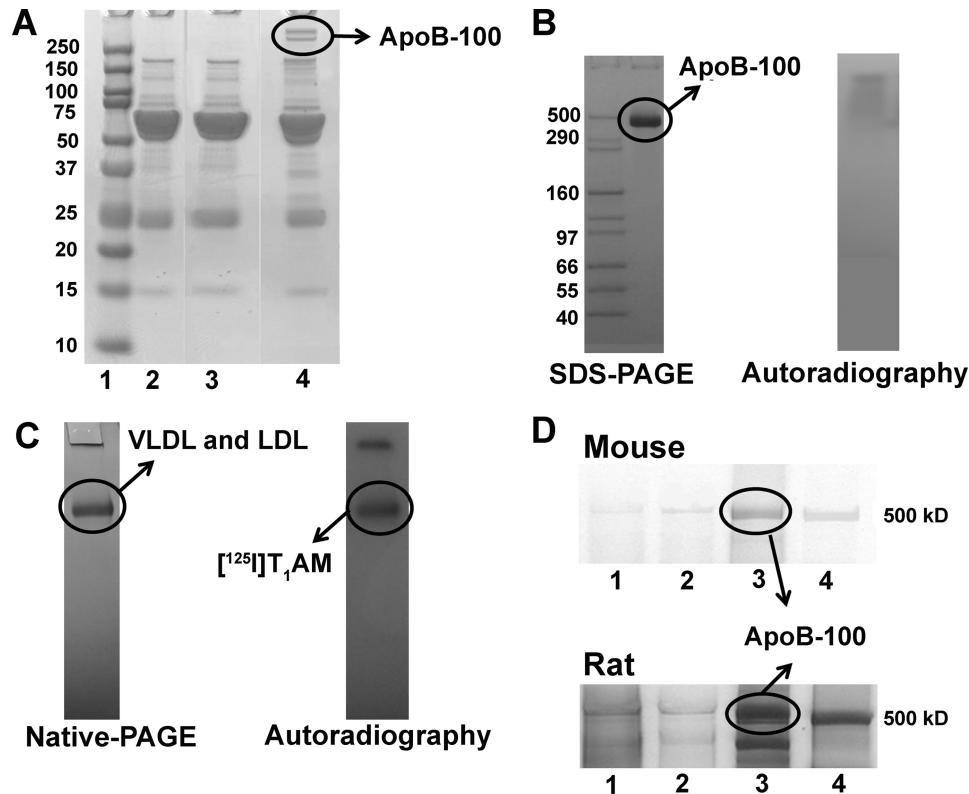
FIGURE 3. **Gel electrophoresis and autoradiography.** *A,* SDS-polyacrylamide gel of eluted fractions from T_1AM affinity chromatography of human serum. SDS-polyacrylamide gel (4–20% Tris-HCl) of standard protein marker (*lane 1*), DTT eluent from activated thiol-Sepharose (control) column (*lane 2*), tyramine-attached Sepharose (control) column (*lane 3*), and T_1AM-attached Sepharose column (*lane 4*) are shown. Protein sequencing revealed that both high molecular weight bands in *lane 4* correspond to apoB-100. *B,* SDS-polyacrylamide gel (3–8% Tris acetate) of isolated lipoprotein showing major protein band corresponding to apoB-100 and the corresponding autoradiography that indicates T_1AM does not covalently bind with apoB-100 protein. *C,* native-polyacrylamide gel (4–16% BisTris with 4% stacking gel) of isolated apoB-100-containing lipoproteins (VLDL and LDL) and the corresponding autoradiography showing $[^{125}I]T_1AM$ binding to apoB-100-containing lipoproteins obtained by exposing dried native-polyacrylamide gel to film for 10 days at -80 °C. *D,* SDS-polyacrylamide gel showing T_1AM-specific binding protein in mouse and rat serum; DTT eluent from activated thio-Sepharose (control) column (*lane 1*), tyramine-attached Sepharose (control) column (*lane 2*), and from T_1AM-attached Sepharose column (*lane 3*), and standard protein marker (*lane 4*).

We next examined whether T_1AM 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 ($\sim$500 kDa) as the major protein present in this lipoprotein fraction (Fig. 3*B* and supplemental Fig. S6). This apoB-100-enriched fraction was incubated with $[^{125}I]T_1AM$ (32), and free $[^{125}I]T_1AM$ was separated from bound $[^{125}I]T_1AM$ by gel filtration. Native-PAGE of the bound fraction revealed that T_1AM co-migrates with apoB-100-containing particles (Fig. 3*C* and supplemental Fig. S7), whereas no radioactivity was observed in the SDS-PAGE (Fig. 3*B*). Furthermore, mass spectrometry analysis of this particular band in the native-polyacrylamide gel (*circled* in Fig. 3*C*) revealed that apoB-100 is the major protein present (supplemental Tables S4–S6). These results indicate that T_1AM binds reversibly to VLDL and LDL particles, most likely through association with apoB-100. We also used a similar affinity chromatography strategy to identify T_1AM-specific binding protein(s) from mouse and rat serum. SDS-PAGE results show that the DTT eluate obtained from the T_1AM-derivatized columns incubated with rodent sera also contained apoB-100 (Fig. 3*D*).

We next examined the distribution of bound T_1AM in human serum to determine what fraction of bound T_1AM was associated with apoB-100-containing lipoprotein particles. $[^{125}I]T_1AM$ was incubated with human serum followed by sequential fractionation of the serum by density ultracentrifugation and analysis of bound T_1AM by gamma counting. More than 95% of the specifically bound T_1AM was found associated with lipoproteins, and the remaining <5% was bound to other unidentified serum components (Fig. 4*A*). 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 $[^{125}I]T_1AM$ was determined by gamma counting. More than 90% of the specifically bound T_1AM 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 T_1AM was contained in the HDL fraction (Fig. 4*B*).

We next examined the relationship between the bound T_1AM 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 $[^{125}I]T_1AM$, and the bound $[^{125}I]T_1AM$ was quantified. The amount of $[^{125}I]T_1AM$ 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 $[^{125}I]T_1AM$ binding to LDL was also examined with the finding that 90% of
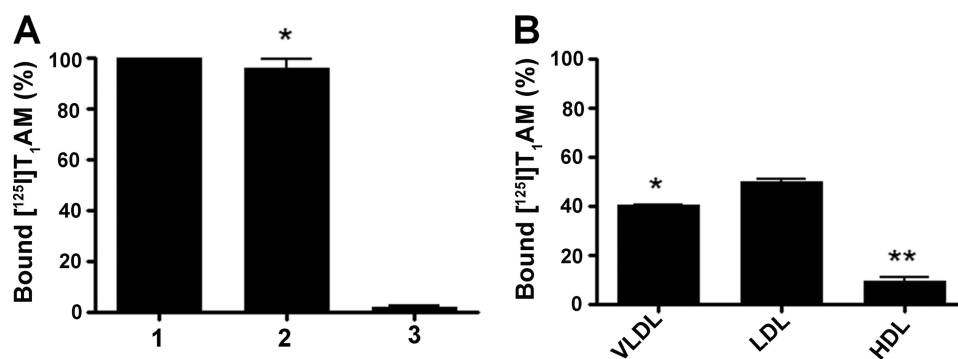
FIGURE 4. *A*, distribution of specifically bound [^{125}I]T$_1$AM in human serum: (*column 1*) unfractionated human serum; (*column 2*) lipoprotein fraction from human serum; (*column 3*) remaining serum minus the lipoprotein fraction. *B*, distribution of specifically bound [^{125}I]T$_1$AM in lipoprotein VLDL, LDL, and HDL fractions from the lipoprotein fraction (*column 2* in (*A*)) derived from human serum. Normal pooled human serum was incubated with tracer quantity of [^{125}I]T$_1$AM in 0.1 M Tris-HCl buffer (pH 7.4) for 24 h at 4 °C in the presence or absence of excess unlabeled T$_1$AM (50 μM). Free [^{125}I]T$_1$AM was removed using charcoal-dextran solution. Different lipoprotein fractions (VLDL, LDL and HDL) were separated by a standard micro-ultracentrifugation technique. *, $p \leq 0.05$; **, $p \leq 0.01$.
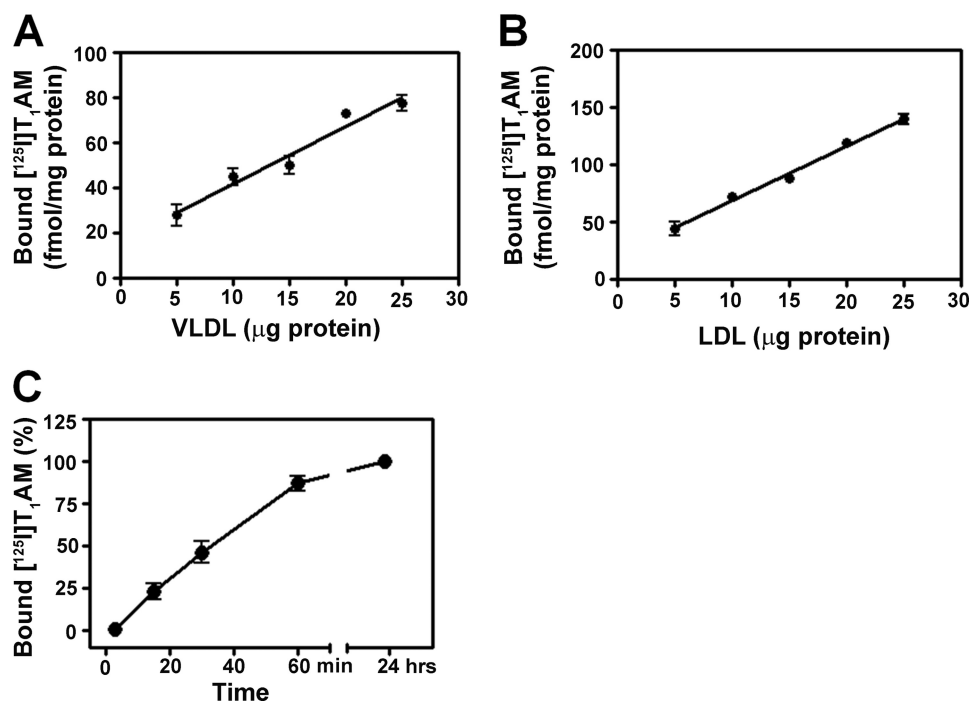

FIGURE 5. Specific binding of [^{125}I]T$_1$AM as a function of VLDL concentration (*A*) and LDL concentration (*B*) is shown. Purified VLDL and LDL fractions were incubated with a tracer quantity of [^{125}I]T$_1$AM in the presence or absence of excess T$_1$AM (50 μM) in 0.1 M Tris-HCl buffer for 24 h at 4 °C. *C*, time course of specific binding of T$_1$AM to LDL. Each data point is the mean $\pm$ S.D. of three experiments.

the specific binding occurred within 60 min at 4 °C (pH 7.4) (Fig. 5*C*). We next studied the equilibrium binding properties of T$_1$AM 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. 3*B* and supplemental material). T$_1$AM binding to this preparation was found to be concentration-dependent with saturation of specific binding at high T$_1$AM concentrations. The data showed best fit to a one-site model, suggesting a single apoB-100-binding site for T$_1$AM (Fig. 6*A*). Scatchard analysis of these data indicated single site binding with an equilibrium dissociation constant (K_D) of 17 nM and a ligand/protein stoichiometry of 1:1(Fig. 6*A*, *inset*). We also examined equilibrium binding of T$_1$AM to a highly purified LDL fraction (*i.e.* no VLDL such as that used in Fig. 4*B*) and observed a similar saturation binding curve with a similar K_D of 48 nM (supplemental Fig. S8). To

evaluate the specificity of T$_1$AM binding to apoB-100, a competition binding assay was performed between [^{125}I]T$_1$AM and similar analogues, including iodothyronines (T$_0$, T$_1$, 3,3′-T$_2$, 3,5-T$_2$, T$_3$, rT$_3$, and T$_4$), iodothyronamines (T$_0$AM, T$_1$AM, 3,3′-T$_2$AM, 3,5-T$_2$AM, T$_3$AM, rT$_3$AM, and T$_4$AM), and other biogenic amines (tyramine and serotonin). The apoB-100-containing lipoprotein was incubated with a tracer quantity of [^{125}I]T$_1$AM in the presence of different concentrations of unlabeled competitor compounds for 24 h at 4 °C. Free [^{125}I]T$_1$AM was separated from bound by charcoal absorption, and bound [^{125}I]T$_1$AM was quantified. As expected, unlabeled T$_1$AM competed successfully with [^{125}I]T$_1$AM for the binding site of apoB-100, and the corresponding IC$_{50}$ value was 5 nM (Fig. 6, *B* and *C*, and Table 1). However, all other competitor compounds were at least 50–1000 times less effective in competition binding against [^{125}I]T$_1$AM (Fig. 6, *B* and *C*). IC$_{50}$ and % inhibition
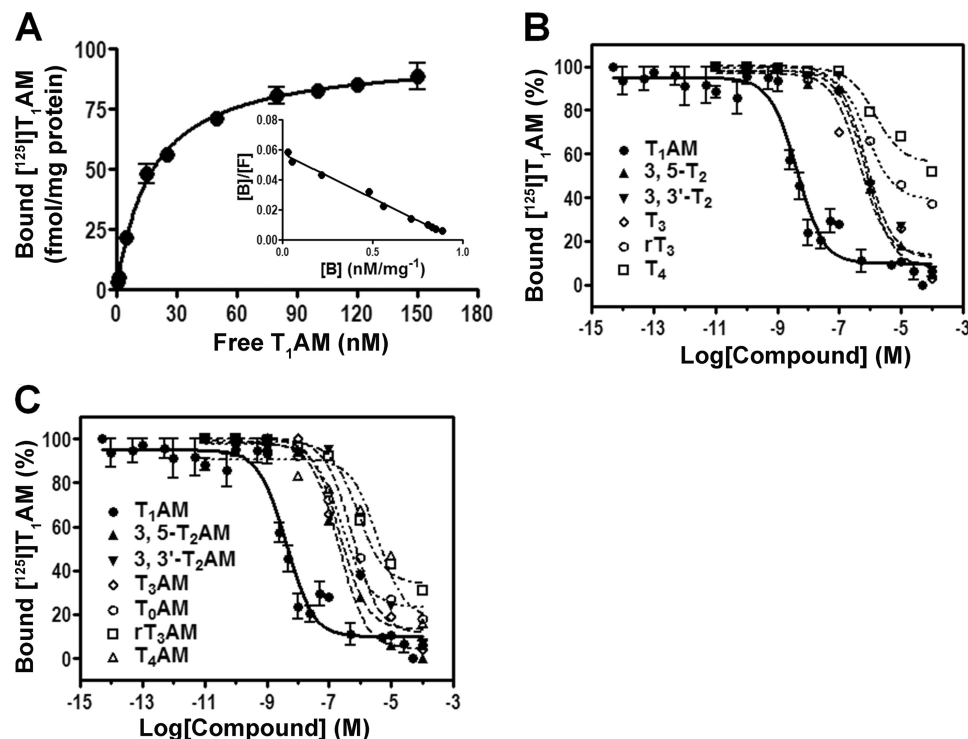
FIGURE 6. **Equilibrium binding of [^{125}I]T$_1$AM to apoB-100-containing lipoprotein particles.** An equilibrium dissociation constant (K_D) of 17 nM was determined from the the fit (*A*). Scatchard analysis reveals a single and saturable binding site with a 1:1 stoichiometry between ligand and protein (*inset* of *A*). Competition of [^{125}I]T$_1$AM binding to apoB-100-containing lipoprotein with unlabeled thyronines (*B*) and thyronamines (*C*) is shown. For competition binding assays, apoB-100-containing lipoprotein was incubated with tracer quantity of [^{125}I]T$_1$AM in the presence or absence of excess T$_1$AM and the indicated concentrations of competitor ligand for 24 h at 4 °C. Plotted data are the mean ± S.D. of three experiments.

TABLE 1

IC$_{50}$ values and % inhibition from competition binding to apoB-100 with [^{125}I]T$_1$AM

Compounds	IC$_{50}$	% of inhibition at 1 μM concentration of inhibitors[a]
	nM	
T$_1$AM	5	91
3′-T$_1$AM	259	48
T$_0$AM	268	54
3,5-T$_2$AM	209	72
3,3′-T$_2$AM	570	62
T$_3$AM	224	62
rT$_3$AM	865	37
T$_4$AM	3834	32
T$_0$		14
T$_1$		24
3,5-T$_2$	738	56
3,3′-T$_2$	776	54
T$_3$	479	53
rT$_3$	818	34
T$_4$	1518	21
Tyramine		3
Serotonin		3

[a] Percent inhibition at 1 μM concentration of inhibitors was determined in a separate experiment.

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 (10 μg/ml) compared with that in LDL-free media (Fig. 7*A*). 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. 7*B*). 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. 7*C*). About 20% of this was surface-associated and could be released with heparin treatment, whereas about 80% was intracellular and heparin-resistant (Fig. 7*D*). 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. 7*E*). 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. 7*F*).

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
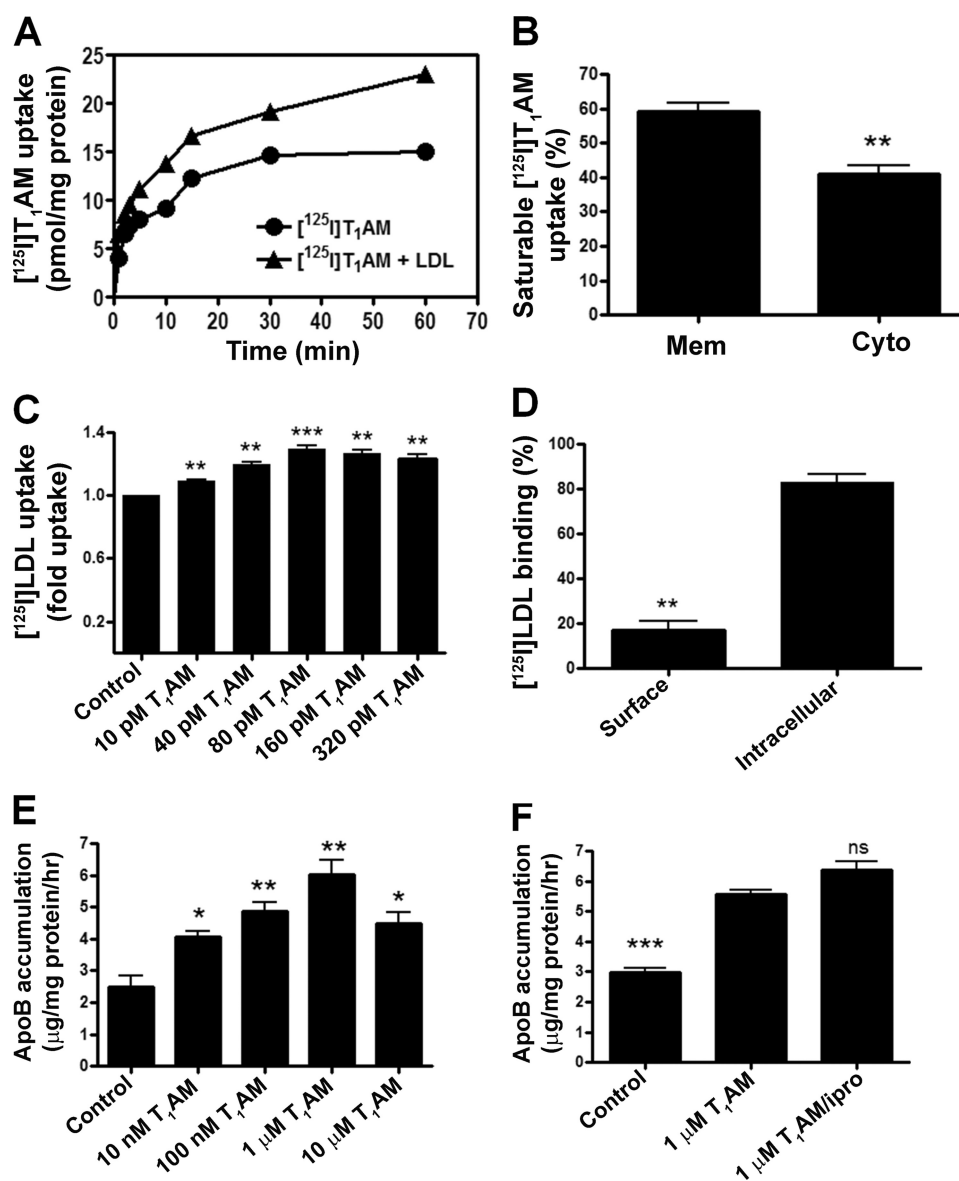
FIGURE 7. *In vitro* functional effects of T_1AM. *A,* saturable uptake of $[^{125}I]T_1AM$ by LDL receptor-competent HepG2 cells in the absence or presence of LDL (10 μg/ml). Plotted data are the mean $\pm$ S.D. of three experiments, each performed in triplicate. *B,* subcellular localization (membrane and cytoplasm) of $[^{125}I]T_1AM$ taken up by HepG2 cells in an LDL-dependent manner after 60 min of incubation. *C,* saturable uptake of ^{125}I-labeled LDL (10 μg/ml) by LDLR-competent fibroblasts cells at different concentrations of T_1AM (0, 10, 40, 80, 160, and 320 pM). The data are plotted as fold uptake of ^{125}I-labeled LDL relative to the control (T_1AM untreated). *D,* subcellular localization (surface and intracellular) of ^{125}I-labeled LDL in the presence of T_1AM. Human fibroblasts were incubated with ^{125}I-labeled LDL at 37 °C and preincubated with 80 pM T_1AM, 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). *E,* effect of T_1AM on apoB production by HepG2 cells. HepG2 cells were treated with T_1AM or vehicle for 2 days in DMEM supplemented with 10% human LDS (28). *F,* effect of iproniazid (100 μM) on the production of apoB stimulated by T_1AM in HepG2 cells. *, $p \le 0.05$; **, $p \le 0.01$; ***, $p \le 0.001$; *ns,* not significant.

higher than $\sim$0.5 mg/kg induce anorexia, which would interfere with the circulating lipid levels.[3] In addition, these T_1AM doses do not induce hypothermia, hyperglycemia, or any of the other previously reported actions of T_1AM. 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. S3, *A–E*). As expected, the human apoB-100 transgenic mice had elevated levels of apoB-100-containing LDL and VLDL (supplemental Fig. S3, *A and B*) but reduced levels of HDL (supplemental Fig. S3*C*) com-

pared with WT/high cholesterol-fed mice. This also corresponded to elevated triglycerides (supplemental Fig. S3*D*) and unchanged total cholesterol (supplemental Fig. S3*E*). T_1AM 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_1AM, like the thyroid hormones T_4 and T_3, is largely protein bound in circulation. Using affinity chromatography, we isolated the predominant T_1AM-binding protein from human and rodent serum, which turned out to be

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[3] B. Hettinger and T. S. Scanlan, unpublished data.

apoB-100, the unique protein component of low density lipoprotein particles. More than 95% of specifically bound T_1AM was found to be associated with the lipoprotein fraction in human serum, and more than 90% of the lipoprotein-bound T_1AM was distributed approximately equally between apoB-100-containing LDL and VLDL particles. T_1AM binds reversibly to apoB-100 with a K_D of 17 nM and a T_1AM/apoB-100 stoichiometry of 1:1. This binding site is highly selective for T_1AM as none of the iodothyronamines or iodothyronines competed effectively with T_1AM in ligand competition binding assays and had IC_{50} values 50–1000 times higher than that of T_1AM.

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 T_4 and T_3 (3% of T_4 and 6% of T_3) 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 T_4 with a K_D of 1 μM, and apoB-100-bound T_4 may facilitate entry of T_4 into cells via LDL/LDL receptor-mediated endocytosis. We found a higher affinity interaction between T_1AM and apoB-100, and T_4 was unable to compete against T_1AM for binding to apoB-100. We conclude from this that the T_1AM-binding site on apoB-100 is distinct from the previously reported T_4-binding site and that the T_4-binding site does not allosterically influence the T_1AM-binding site.

The finding that circulating T_1AM is largely bound to apoB-100 as part of a lipoprotein particle may explain the discrepancy in the reported serum levels of T_1AM using different bioanalytical methods (43). Using an LC/MS/MS approach, Saba *et al.* (9) reported high tissue concentrations of T_1AM but very low serum concentrations, on the order of 0.3 nM. In contrast, Hoefig *et al.* (10) measured substantially higher serum T_1AM concentrations averaging ~66 nM using an immunoassay based on a monoclonal antibody that selectively binds T_1AM. 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 T_1AM from its unique binding site residing within a lipoprotein particle and that extraction-based approaches quantify free and not total circulating T_1AM. Additionally, if total T_1AM 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 T_1AM serum-binding sites are normally in excess compared with the total T_1AM 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 T_1AM to apoB-100-containing lipoprotein particles. In experiments aimed to address this, we observed modest effects by T_1AM *in vitro* on LDL uptake and apoB-100 secretion; however, none of these effects were apparent *in vivo*. For this we used multiple dose T_1AM 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 T_1AM 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 T_1AM 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, T_1AM 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 T_1AM resulted in a 50% enhancement in the cellular uptake of T_1AM, suggesting that the physiological role of T_1AM association with apoB-100 may be to provide a mechanism for transportation and entry of T_1AM 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 T_1AM is also most abundant in the liver (9).

The robust, specific uptake of T_1AM 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 T_1AM transport. It has been further demonstrated that T_1AM is not a substrate for monocarboxylate transporter (MCT)-8 or MCT-10, both established specific transporters of T_4 and T_3 (45). A T_1AM uptake mechanism based on receptor-mediated endocytosis could not be ruled out, and the prospect of this as the uptake route of T_1AM 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 T_1AM, then it suggests that a target of the biological action of T_1AM 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 T_1AM.

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