Elevated Serum Tetrac in Graves Disease: Potential Pathogenic Role in Thyroid-Associated Ophthalmopathy

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Context: The sources and biological impact of 3,3′,5,5′ tetraiodothyroacetic acid (TA4) are uncertain. $CD34^+$ fibrocytes express several proteins involved in the production of thyroid hormones. They infiltrate the orbit in Graves disease (GD), an autoimmune process known as thyroid-associated ophthalmopathy. It appears that the thyrotropin receptor plays an important role in the pathogenesis of thyroid-associated ophthalmopathy.

Objective: To quantify levels of TA4 in healthy participants and those with GD, determine whether fibrocytes generate this thyroid hormone analogue, and determine whether TA4 influences the actions of thyroid-stimulating hormone and thyroid-stimulating immunoglobulins in orbital fibroblasts.

Design/Setting/Participants: Patients with GD and healthy donors in an academic medical center clinical practice were recruited.

Main Outcome Measures: Liquid chromatography–tandem mass spectrometry, autoradiography, real-time polymerase chain reaction, hyaluronan immunoassay.

Results: Serum levels of TA4 are elevated in GD. TA4 levels are positively correlated with those of thyroxine and negatively correlated with serum levels of triiodothyronine. Several cell types in culture generate TA4 from ambient thyroxine, including fibrocytes, HELA cells, human Müller stem cells, and retinal pigmented epithelial cells. Propylthiouracil inhibits TA4 generation. TA4 enhances the induction by thyrotropin and thyroid-stimulating immunoglobulins of several participants in the pathogenesis of thyroid-associated ophthalmopathy, including interleukin 6, hyaluronan synthase 1, prostaglandin endoperoxide H synthase 2, and haluronan production.

Conclusion: TA4 may be ubiquitously generated in many tissues and enhances the biological impact of thyrotropin and thyroid-stimulating immunoglobulins in orbital connective tissue. These findings may identify a physiologically important determinant of extrathyroidal thyroid-stimulating hormone action. **(*J Clin Endocrinol Metab* 102: 776–785, 2017)**

Recognition that thyroid hormones (THs) are metabolized in tissues peripheral to the thyroid gland provided important insights into their regulation of target cell function (1–4). When untreated, the autoimmune condition, Graves disease (GD) (5), is frequently associated with elevated serum thyroxine (T4) and triiodothyronine (T3)

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Abbreviations: bTSH, bovine TSH; DMEM, Dulbecco's modified Eagle medium; FT3I, free triiodothyronine index; FT4I, free thyroxine index; GD, Graves disease; HA, hyaluronan; HAS1, hyaluronan synthase 1; IL-6, interleukin 6; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MeOH, methanol; PGHS-2, prostaglandin endoperoxide H synthase 2; PTU, propylthiouracil; TAO, thyroid-associated ophthalmopathy; TA4, 3,3′,5,5′ tetraiodothyroacetic acid; TH, thyroid hormone; TSH, thyroid-stimulating hormone; TSHR, thyrotropin receptor; TSI, thyroid-stimulating immunoglobulin.

concentrations. Besides T4 and T3, less abundant TH metabolites can also be detected and quantified in the circulation. Among these is 3,5,3′,5′ tetraiodothyroacetic acid (tetrac, TA4), the product of T4 decarboxylation and oxidative deamination (6). The enzyme(s) required for these reactions has never been identified. Furthermore, the cellular origins and function of TA4 have remained uncertain. Whether this TH analogue might play a role in disease pathogenesis has not been explored in detail.

In GD, activating antibodies against the thyrotropin receptor (TSHR), known as thyroid-stimulating immunoglobulins (TSIs), drive thyroid gland overactivity (7). In addition, TSIs are also thought to participate in the pathogenesis of thyroid-associated ophthalmopathy (TAO) (8, 9). Human $CD34^+$ fibrocytes, derived from monocytes, express relatively high levels of TSHR (10). They participate in tissue repair and remodeling such as those occurring in experimental models of lung fibrosis (11). These cells appear to infiltrate the thyroid (12) and orbit (10) in GD. They participate in tissue repair and fibrosis (12). Fibrocytes express several other "thyroid-specific" proteins, including thyroglobulin and thyroperoxidase (10, 13, 14). Thus, we postulated that fibrocytes might produce TH.

We now report that serum TA4 levels in GD are considerably higher than in healthy individuals and positively correlate with serum free thyroxine index (FT4I) but correlate inversely with free triiodothyronine index (FT3I). Several candidate cell types, including fibrocytes, were found to release [^{13}C6]TA4. Because of their putative involvement in TAO, orbital fibroblasts were investigated for responses to exogenous TA4. TA4 enhances the induction by thyroid-stimulating hormone (TSH) and TSI of interleukin 6 (IL-6), prostaglandin endoperoxide H synthase 2 (PGHS-2), hyaluronan synthase 1 (HAS1), and hyaluronan (HA) accumulation, all considered integral to the pathogenesis of TAO (15–20). The current findings suggest widespread generation of TA4, an enhancing factor of extrathyroidal actions of TSH and TSI.

Methods

Materials

TA4 (cat. T3787), charcoal-stripped fetal bovine serum (cat. F6765; T4, 6.3 mcg/dL; T3, 62 ng/dL), 2-mercapto-1 methylimidazole (cat. 301507), 6-propyl-2-thiouracil (cat. P3757), and sodium perchlorate monohydrate (cat. 71853) were from Sigma (St Louis, MO). [^{13}C6]T4 (cat. 41654) was from Cerilliant Corporation (Round Rock, TX). Dulbecco's modified Eagle medium (DMEM; cat. 11965) and fetal calf serum (cat. 16000-044; T4, 14.6 mcg/dL; T3, 184 ng/dL) were from Life Technologies (Grand Island, NY). Bovine TSH (bTSH) (cat. 609385) was obtained from Calbiochem (La Jolla, CA), and M22 activating anti-TSH monoclonal antibody (cat. M22-5c/00-690) came from Kronus (Star, ID). Anti-TSHR phycoerythrin ab (Sc-53542; Santa Cruz) and phycoerythrin-mouse immunoglobulin G isotype control (cat. 555749) came from BD Pharmingen (San Diego, CA).

Participant recruitment

Procurement of human blood was conducted after obtaining informed consent as approved by the Institutional Review Board of the University of Michigan Health System. Participants derived from the outpatient clinic population at the Kellogg Eye Center. Those with GD were diagnosed using widely accepted clinical criteria (5). Healthy participants were recruited from patients receiving routine eye care.

Individuals with any autoimmune condition were excluded from the control cohort. Two separate participant cohorts were recruited because many serum samples from cohort 1 were exhausted during initial studies. Thus, additional samples comprising cohort 2 were obtained and used for the subsequent comparisons of TA4, FT4I, FT3I, and TSH.

Fibrocyte and orbital fibroblast cultivation

Fibrocytes were isolated from blood obtained from the American Red Cross, patients with GD, or healthy donors from the clinic population. They were isolated and cultured as described previously (11). Culture purity and viability were verified to be >90% fibrocytes by fluorescence-activated cell sorting analysis. Orbital fibroblasts were cultivated from fat removed during surgical orbital decompression as described (21). Thyroid fibroblasts were cultivated as reported previously (12). HELA and human Müller stem cell line cells were supplied by American Type Culture Collection (Manassas, VA). Retinal pigmented epithelial cells were kindly supplied by Dr Steve Abcouwer, University of Michigan.

Isolation of [^{125}I]TA4 from fibrocytes

Fibrocyte monolayers were incubated in the presence of 1 mCi Na[^{125}I] for 24 hours at 37°C. Media and cells were applied to preequilibrated (with 1 mL water) C18 cartridges, washed with 5 mL water, and eluted with 5 mL acetonitrile. Samples were dried down with air and resuspended in 200 μL 4 M NH_4OH in methanol (MeOH). They were then divided in half and were spotted onto 2 normal phase thin-layer chromatography plates. The mobile phase consisted of 2.5% MeOH/dichloromethane + 1% acetic acid. For autoradiography, thin-layer chromatography plates were exposed to film for 12 days at −80°C.

Identification of endogenous and [^{13}C6]-labeled TA4 generation in several cultured cell types, including fibrocytes

Fibrocytes (10^6–10^7 cells) from healthy individuals and those with GD were cultured for 14 days in DMEM without or with 100 nM [^{13}C6]T4. In those experiments, fresh [^{13}C6]T4 was added to cultures every 72 hours. DMEM (4 mL) was spiked with 4 μL (0.5 ng/μL) d4 3-iodothyroacetic acid internal standard, and protein was precipitated with acetonitrile (8 mL). Samples were vortexed for 1 minute and subjected to centrifugation at 10,000 × *g* at 4°C for 10 minutes. Resulting supernatants were dried down with a SpeedVac at 45°C (ThermoScientific, Waltham, MA). Bond Elut Plexa PAX cartridges (60 mg/3 mL) (Agilent, Santa Clara, CA) were preconditioned with MeOH (2 mL) and deionized H_2O (2 mL) using gravity flow. Dried samples were resuspended in 2% NH_4OH (aqueous) solution (2 mL), applied to cartridges, and washed with

3 mL H_2O followed by 3 mL MeOH. TA4 and [^{13}C6]TA4 were eluted with 20% formic acid in MeOH (800 μL, then 450 μL). Organic solvent was removed with a SpeedVac at 45°C, and dried samples were resuspended in MeOH (200 μL), briefly centrifuged at 14,000 × *g*, filtered through 0.22-μm filters, and subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Detection of TA4 in human serum

Serum (1 mL) was spiked with 4 μL 0.5 ng/μL d4 3-iodothyroacetic acid internal standard and protein removed with 2 mL acetonitrile. Samples were subjected to centrifugation at 10,000 × *g* at 4°C for 10 minutes. Resulting supernatants were dried down under a vacuum at 45°C. Bond Elut Plexa PAX cartridges (60 mg/3 mL) were preconditioned with MeOH (2 mL) and deionized H_2O (2 mL) using gravity flow. Dried samples were resuspended in 2 mL 2% NH_4OH (aqueous) solution, applied to cartridges, and washed with 3 mL H_2O followed by 3 mL MeOH. TA4 or [^{13}C6]TA4 was eluted with 20% formic acid in MeOH (800 μL, then 450 μL). Organic solvent was removed with a SpeedVac at 45°C. Samples were then resuspended in 200 μL MeOH, briefly centrifuged at 14,000 × *g*, filtered through 0.22-μm filters, and subjected to LC-MS/MS analysis. Serum FT4I, FT3I, and TSH were kindly determined by Dr. Neal Scherberg and Professor Samuel Refetoff (University of Chicago).

LC-MS/MS conditions

Instrumentation was located in the Bioanalytical Shared Resource/Pharmacokinetics Core at Oregon Health & Science University. TA4 was monitored by LC-MS/MS in the multiple-reaction monitoring mode at 746.6/126.9 and 746.6/702.7 transitions, and [^{13}C6]TA4 was monitored at 752.6/126.9 and 752.6/708.7 transitions. A Poroshell 120 SB-C18 (100 × 2.1 mm) 2.7 μm high-performance liquid chromatography column (Agilent) and Thermo Betabasic 18 (10 × 2.1 mm) 5-μm guard column were used for chromatography (Thomas Scientific, Waltham, MA). Mobile phases consisted of (A) H_2O with 0.05% acetic acid start at 90% and (B) acetonitrile with 0.05% acetic acid with a flow rate of 0.3 mL/min. Gradient conditions were as follows: 10% B to 90% B from 0 to 18 minutes, 90% B from 18 to 21 min, 90% B to 10% B from 21 to 23 minutes, and 10% B from 21 to 28 minutes. The autosampler and oven temperatures were 4°C and 35°C, respectively. Electrospray in negative ionization mode was used with curtain gas, 20; collision gas, high; ionization source, −4500 V; temperature, 550°C; ion gas source 1, 40; ion gas source 2, 40; dwell time, 75 seconds; declustering potential, −95 V for both transitions; entrance potential, −10 eV for both transitions; collision energy, −130 eV for 746.6/126.9 and -16 eV for 746.6/702.7 transitions; and collision cell exit potential, −15 V for 746.6/126.9 and −11 eV for 746.6/702.7 transitions.

RNA preparation and real-time polymerase chain reaction

Orbital fibroblast strains were inoculated into 6-well plates in 10% charcoal-stripped fetal bovine serum–supplemented DMEM. Confluent cultures were shifted to DMEM with 1% serum for 16 hours and treated with TA4 (100 nM) for 48 hours and bTSH (5 mIU/mL) or M22 (100 ng/mL) for the final 6 hours. Cellular RNA was extracted with an Aurum TM Total RNA Mini Kit (cat. 732-6820; Bio-Rad, Hercules, CA). RNA (2 μg) was reverse-transcribed and real-time polymerase chain reaction was performed using an Applied Biosystems (Rockford, IL) instrument and QuantiTect SybrGreen polymerase chain reaction kits (cat. 204143; Qiagen, Frederick, MD). Primer sequences synthesized by Life Technologies (Waltham, MA) were as follows: GAPDH, 5′-TTGCCATCAATGACCCCTTCA-3′ (forward) and 5′-GCCCCACTTGATTTTGGA-3′ (reverse); IL-6, 5′-TGAGAAAGGAGACATGTAACAAGAGT-3′ (forward) and 5′-TTGTTCCTCACTACTCTCAAATCTGT-3′ (reverse); PGHS-2, 5′-GAGCAGGCAGATGAAATG-3′ (forward) and 5′-TACCAGAAGGGCAGGTAC-3′ (reverse); and HAS1, 5′-CGATACTGGGTAGCCTTCAATG-3′ (forward) and 5′-GGAGGTGTACTTGGTAGCATAACC-3′ (reverse).

Quantitect primer assays were from Qiagen. Sample values were generated against a standard curve and normalized to GAPDH signals.

HA enzyme-linked immunosorbent assay

Washed monolayers were homogenized in 0.1 M NaCl and supernatants were assayed for HA content using an enzyme-linked immunosorbent assay kit (K-1200) from Echelon Biosciences, Inc (Salt Lake City, UT).

Flow cytometric analysis

Surface TSHR was quantified by flow cytometry as described previously (10) using an LSR II instrument (Becton-Dickinson, San Jose, CA).

Statistics

Sample characteristics were summarized with means, medians, and standard deviations for continuous variables and frequencies and percentages for categorical variables. Patients with GD were compared with healthy control participants on measures of serum TA4, FT3I, FT4I, and TSH with 2-sample *t* tests for measures with normal distributions or 2-sample Wilcoxon tests for measures with skewed distributions. *P* values were adjusted for multiple comparisons by the Holms method. Statistical significance for the *in vitro* studies was determined by a 2-tailed Student *t* test. Analyses were performed with SAS version 9.4 (SAS Institute, Cary, NC).

Results

Description of patient cohorts examining serum TH levels

Cohort 1 comprised 146 participants, including 58 healthy controls and 88 with GD diagnosed on established criteria (5, 22). Of those with GD, 97% manifested TAO (85/88). Control participants and those with GD were of similar age, but 84.1% with GD were female, whereas 55.2% in the control group were female ($P < 0.0001$ by χ^2), indicating a significant sex imbalance. Cohort 2 included 56 participants (23 healthy controls and 33 with GD). Of those with GD, 97% exhibited TAO (32/33). Neither age nor sex imbalances were identified in this cohort. Data from 1 healthy individual and 6 participants with GD are represented in both cohorts. Demographic details of these cohorts are presented in Table 1 and Supplemental Table 2, respectively. All participants in

Table 1. Demographics and Baseline Characteristics of Cohort 1: Healthy Controls and Graves Disease

Characteristic	Value
Healthy controls (n = 58)	
Age, y	
Mean (SD)	55.9 (15.0)
Median (range)	59 (21–89)
Sex, No. (%)	
Male	26 (45)
Female	32 (55)
Graves disease (n = 88)	
Age, y	
Mean (SD)	56.8 (13.1)
Median (range)	58 (25–87)
Sex, No. (%)	
Male	14 (16)
Female	74 (84)
Time from diagnosis, y	
Mean (SD)	7.4 (8.4)
Median (range)	4 (0.1–43)
Treatment at time of study participation, No. (%)	
Methimazole	10 (11)
PTU	1 (1)
Levothyroxine	62 (70)
RAI	40 (45)
Thyroidectomy	15 (17)

Abbreviation: RAI, radioactive iodine.

the studies were clinically euthyroid at the time of participation.

Serum TA4 levels in GD exceed those in healthy individuals

TA4 levels were quantified in serum samples from individuals with GD and healthy controls by LC-MS/MS (Fig. 1). The mean ± SD serum TA4 concentration in healthy individuals in cohort 1 was 104.5 ± 64.7 pM (median, 98.3 pM). In contrast, the mean ± SD level in patients with GD was 232.2 ± 226.3 pM (median, 197.8 pM; $P < 0.0001$). When analyzed separately, female participants with GD had significantly higher average TA4 levels than did their healthy counterparts (247.7 ± 238.7 pM vs 150.1 ± 117.5 pM, $P < 0.0001$) (Supplemental Table 1). In contrast, there was no difference between healthy males and those with GD (107.2 ± 53.5 pM vs 150.1 ±117.5 pM, $P = 0.5052$). All participants with GD in this cohort were clinically euthyroid at the time of participation.

In cohort 2, the mean ± SD TA4 concentration was 249.1 ± 121.2 pM in participants with GD (median, 242.6 pM) and 140.1 ± 159.8 pM in healthy controls (median, 76 pM) ($P < 0.00012$) (Supplemental Table 3). In those with GD, the mean FT4I was 9.42 ± 2.22 vs 7.96 ± 0.99 ($P = 0.0016$), and mean FT3I was 98.08 ± 0.22.02 in GD vs 119.56 ± 22.94 in controls ($P = 0.0009$). Serum TSH levels were similar in healthy controls and those with GD. Examination of potential relationships between serum TA4 levels and those of FT4I, FT3I, and TSH were assessed. Analysis revealed a significant positive correlation exists between serum TA4 levels and FT4I ($r = 0.52$; $P < 0.0001$) [Fig. 2(A)], congruent with an earlier report from Ramsden and Crossley (23). In contrast, a negative correlation was detected between TA4 and FT3 ($r = -0.28$; $P = 0.0245$) [Fig. 2(B)]. No significant correlation could be identified between TA4 concentrations and serum TSH [Fig. 2(C)]. These results suggest that the ambient FT4 serum concentration might represent a determinant of circulating TA4 levels.

Several cell types can generate TA4 from ambient T4

To our knowledge, the source of TA4 has never been established, although kidney homogenate appears to possess the capacity for its generation (6). Our previous observation that human fibrocytes express several proteins involved in thyroid hormone biosynthesis (13, 14) prompted further examination of TH and TA4 generation in these cells. Fibrocyte cultures were incubated with sodium [^{125}I] iodide, and labeled cell extracts and conditioned media were subjected to TLC and autoradiography [Fig. 3(A)]. As indicated in Fig. 3(A), the second highest migrating radioactive compound was identified as TA4, based on comparison of retention time with a TA4 standard; the highest migrating and major radioactive compound was not unequivocally characterized but is believed to be an intermediate in the conversion of T4 to TA4. Although radioactive iodide incorporation

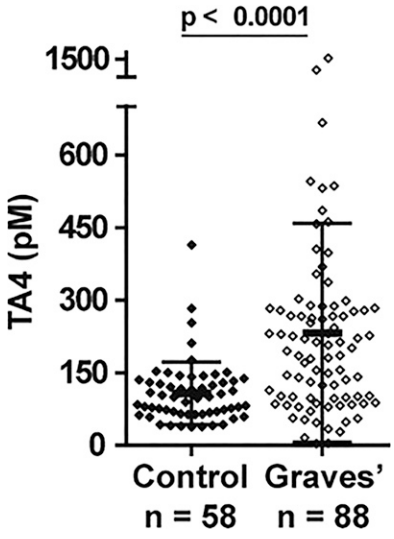

Figure 1. Serum 3,3′,5,5′ tetraiodothyroacetic acid (TA4) concentrations in healthy donors and those with Graves disease. Serum samples from a cohort of individuals were analyzed by liquid chromatography–mass spectrometry for TA4 concentrations.

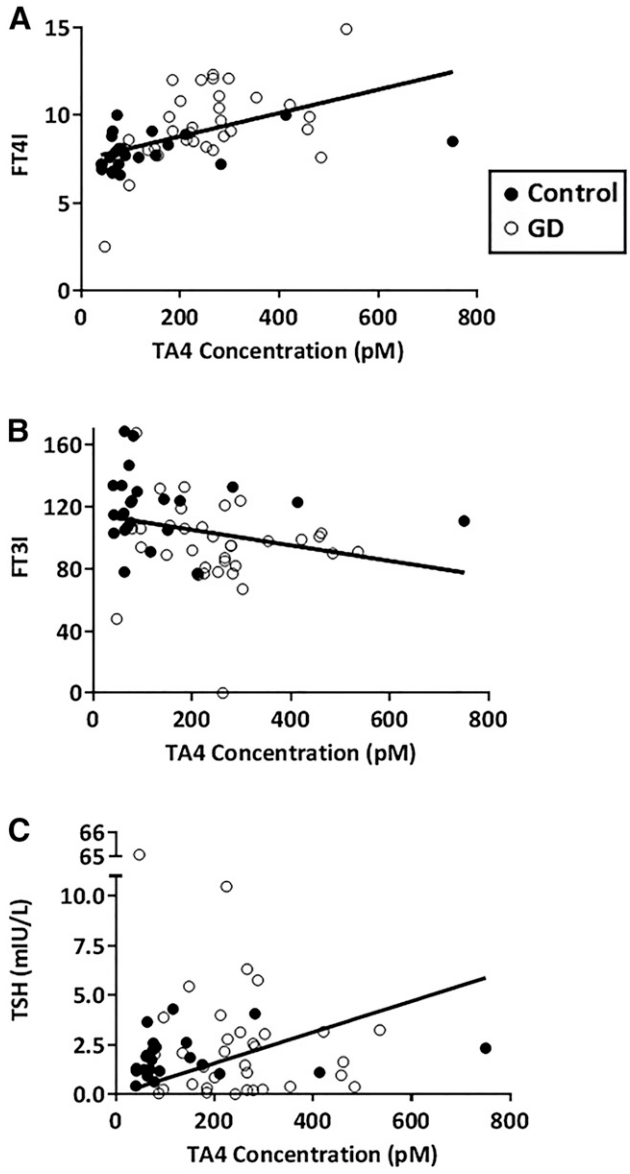

Figure 2. Relationship between serum 3,3′,5,5′ tetraiodothyroacetic acid (TA4) levels and those of free thyroxine index (FT4I), free triiodothyronine index (FT3I), and thyroid-stimulating hormone (TSH). (A) Scatterplot for the relationship between FT4I and TA4. A significant, positive correlation was observed ($r = 0.52$; $P < 0.0001$) between FT4I and TA4. The scatterplot includes the best-fit linear regression line. (B) Scatterplot for the relationship between FT3I and TA4. A significant, negative correlation was observed ($r = -0.28$; $P = 0.0245$) between FT3I and TA4. The scatterplot includes the best-fit linear regression line. (C) Scatterplot for the relationship between TSH and TA4. No significant correlation was found between TSH and TA4 ($r = -0.09$; $P = 0.4610$). The scatterplot includes the best-fit linear regression line. As a sensitivity analysis, the outlying TSH value of 65.08 mIU/L was removed and the correlation recalculated. The magnitude and direction of the correlation changed but was still not significantly different from zero ($r = 0.16$, $P = 0.2210$). Serum samples were simultaneously assessed for FT4I, FT3I, TSH, and TA4 concentrations and were collected over a 4-month period. Fifty-six serum samples were used for analysis, including samples both with and without Graves disease (GD). The points differentiate between samples from donors with GD and healthy controls.

into these T4 metabolites was initially thought to result from fibrocyte-specific thyroid hormone biosynthesis involving the expressed thyroid hormone biosynthetic machinery discussed previously, the mechanism of [^{125}I] incorporation into TA4 was determined to be spontaneous iodide exchange between radioactive iodide and unlabeled TA4 produced in the fibrocyte culture based on comparable labeling of TA4 with sodium [^{125}I] iodide in cell-free buffer (data not shown). Further confirmation of TA4 production in fibrocytes was obtained by LC-MS/MS analysis of human fibrocyte-conditioned media [Fig. 3(B)]. The TA4 analyte was detected in negative ion mode at 2 transitions corresponding to decarboxylation (746/703) and deiodination with detection of iodide (746/127). Next, cultured fibrocytes were incubated for 10 days without or with [^{13}C6]T4 (100 nM), and cell extracts and conditioned media were subjected to LC-MS/MS analysis. TA4 containing the expected 6 m/e shift in mass at both of the aforementioned transitions was detected in conditioned fibrocyte media [Fig. 3(C)] but absent in media unexposed to fibrocytes (data not shown), indicating that fibrocytes contain the biochemical machinery necessary for conversion of T4 to TA4. Most of detected TA4 was found in the medium and not in the cell layer-associated fractions. Generation of TA4 in fibrocytes from donors with GD was compared with that in cells from healthy individuals. The GD fibrocytes generated significantly higher levels of TA4 on a per-cell basis compared with fibrocytes from healthy controls [Fig. 3(D)]. In an effort to provide insight on TA4 biosynthesis, fibrocytes were incubated with the thyroperoxidase inhibitor, propylthiouracil (PTU). PTU inhibited the generation of TA4 in fibrocytes [Fig. 3(E)], suggesting that thyroperoxidase or a similar PTU-sensitive enzyme plays a role in TA4 production in these cells.

To determine whether TA4 generation was unique to fibrocytes, several different cell types were examined. As the data in Supplemental Fig. 1 demonstrate, HELA cells, human Müller stem cells, thyroid fibroblasts, and human retinal pigmented epithelial cells incubated with [^{13}C6]T4 (100 nM) for 14 days could also generate TA4 and [^{13}C6]TA4, indicating that a wide array of cultured cell types is capable of converting ambient T4 into TA4. Thus, TA4 generation appears to be widespread and is not unique to a single cell type such as fibrocytes.

TA4 enhances the actions of TSH and TSI on orbital fibroblasts

TA4 enhances the actions of TSH and TSIs in TAO orbital fibroblasts. Confluent cultures were incubated with TA4 (10 nM) alone or in combination with bTSH or

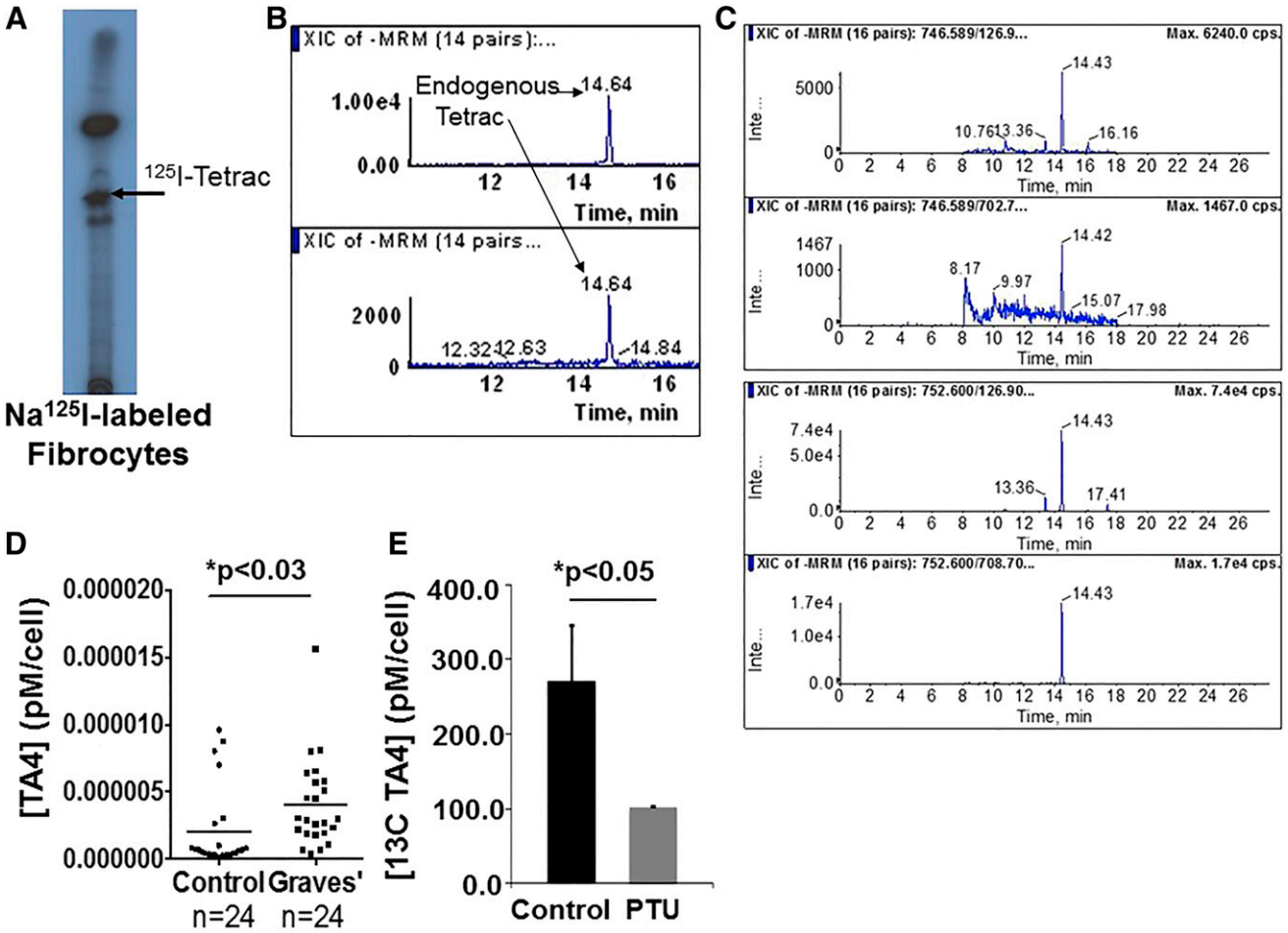

Figure 3. Generation of 3,3′,5,5′ tetraiodothyroacetic acid (TA4) in fibrocytes. (A) Fibrocytes were metabolically labeled with 1 mCi Na[^{125}I]. Cells and media were combined and homogenized, and labeled compounds were identified by thin-layer chromatography followed by autoradiography. (B) The identity of labeled tetrac was confirmed by retention time against authentic material. (C) A liquid chromatography–mass spectrometry (LC-MS) chromatogram example of TA4 produced by fibrocytes from healthy patients. TA4 was extracted using acetonitrile crash and analyzed on the Applied Biosystems 5500 triple quadrupole mass spectrometer, in negative mode. The *m/z* transitions for TA4 are 746/127 (upper panel) and 746/703 (lower panel). (D) Data generated from LC-MS analysis of unlabeled TA4 produced by fibrocytes in culture. Fibrocyte monolayers were incubated for 24 hours in medium that was then collected and analyzed. Each datum point represents the concentration of TA4 in conditioned medium, which was incubated for 24 hours with fibrocytes. Each point represents results from fibrocytes from a unique donor (healthy controls, n = 24; Graves disease, n = 24). *$P < 0.05$, unpaired *t* test. (E). PTU inhibits [^{13}C6]TA4 production in fibrocytes. Confluent fibrocytes were incubated with [^{13}C6]T4 (100 nM) for 14 days without or with PTU (75 μm). Medium was then analyzed by LC-MS and the concentration of [^{13}C6]TA4 determined from these measurements was corrected for the differences in fibrocyte cell numbers between the PTU-treated and control cultures. *$P < 0.05$, unpaired *t* test.

M22 (24). As a single agent, TA4 failed to influence steady-state messenger RNA levels for IL-6 [Fig. 4(A)], PGHS-2 [Fig. 4(B)], and HAS1 [Fig. 4(C)]. However, the TH analogue synergistically enhanced the induction by bTSH and M22 of all 3 transcripts. Induction of IL-6 by bTSH and M22 was enhanced by 2-fold ($P < 0.05$) and 5-fold ($P < 0.005$), respectively, by cotreatment with TA4, that of PGHS-2 was 2-fold ($P < 0.05$) and 5-fold ($P < 0.01$), while HAS1 was enhanced by 10-fold ($P < 0.01$) and 3-fold ($P < 0.05$). bTSH modestly increases the HA accumulation in TAO orbital fibroblasts as a single agent [Fig. 4(D)]; however, concomitant treatment with bTSH and TA4 substantially enhances the levels above those resulting from bTSH alone [mean ± SD; control, 1781 ± 375 ng/μg protein; bTSH, 5657 ± 923 ng/μg; TA4, 1781 ± 230 ng/μg; bTSH plus TA4, 18,218 ± 1970 ng/μg ($P < 0.01$ vs bTSH alone)]. The enhancing effects of TA4 on bTSH and TSI actions do not appear to be mediated through increased levels of cell-surface TSHR since the extremely low level of receptor density is unaffected by TA4 [Fig. 4(E)]; however, levels of phosphorylated Akt, a component of a critical signaling pathway downstream from TSHR in orbital fibroblasts, were enhanced with TA4 [>2-fold, Fig. 4(F)]. It would appear from these data that TA4 generation and accumulation within tissues such as those in the orbit might enhance local TSH/TSI actions and could therefore drive localized tissue remodeling in TAO. The effects of TA4 on

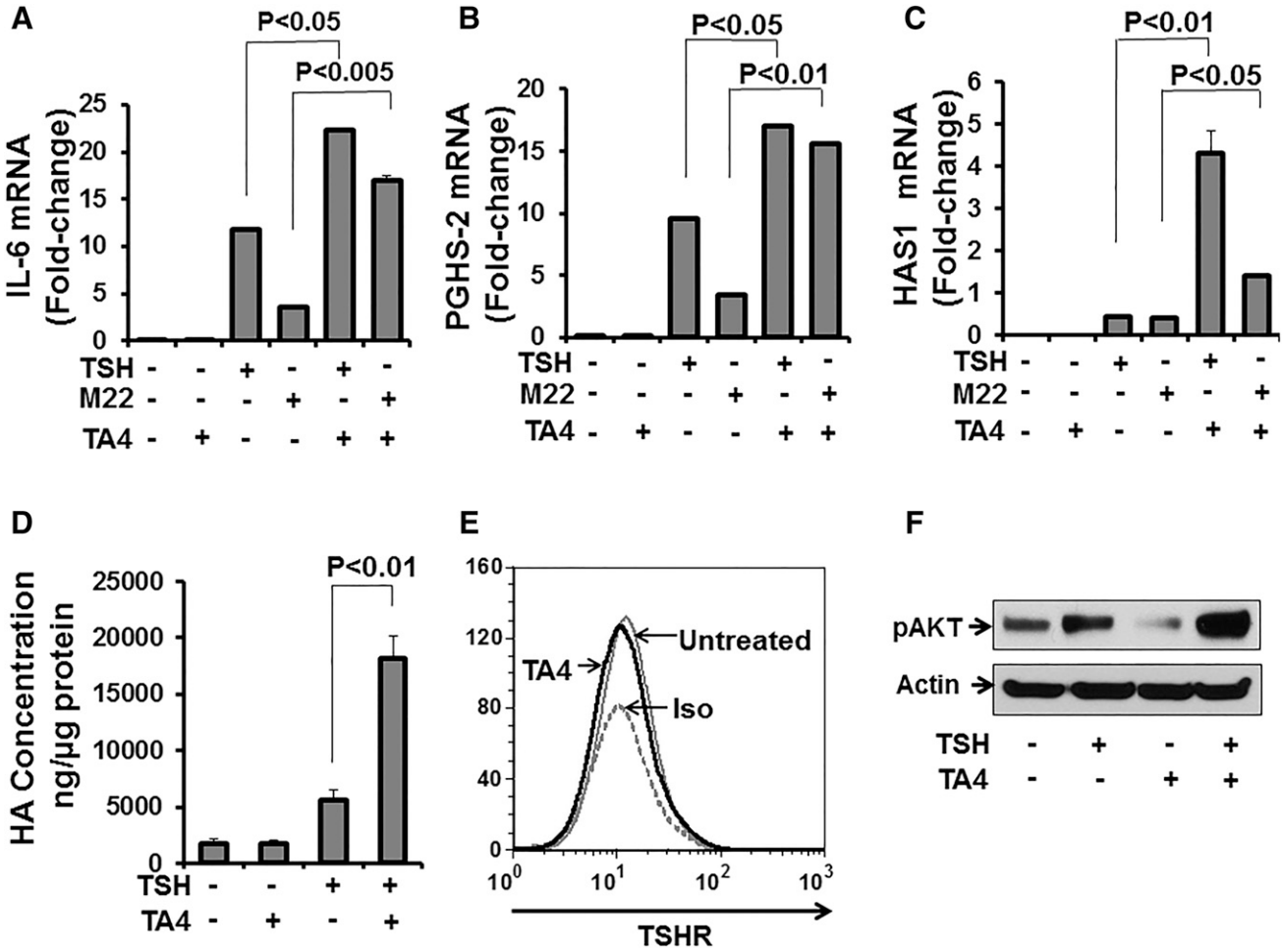

Figure 4. Effects of 3,3′,5,5′ tetraiodothyroacetic acid (TA4) on orbital fibroblasts from individuals with thyroid-associated ophthalmopathy. Shown are results from representative studies where confluent orbital fibroblasts were incubated in medium supplemented with 1% fetal bovine serum in the absence or presence of bTSH (5 mU/L), M22 (2 μg/mL), or TA4 (10 nM) alone or in combination. Effects of TA4, bTSH, and M22 added as single agents or in the combinations indicated along the abscissas on (A) interleukin 6 (IL-6) mRNA, (B) prostaglandin endoperoxide H synthase 2 (PGHS-2) messenger RNA (mRNA), and (C) hyaluronan synthase 1 (HAS1) mRNA expression in orbital fibroblasts. (D) Effects of TA4 and bTSH added alone or in combination on hyaluronan (HA) accumulation. (E) Flow cytometric gating strategy assessing effects of TA4 on surface-displayed thyrotropin receptor (TSHR) by orbital fibroblasts. The plot illustrates an absence of change. (F) TA4 (10 nM) enhances bTSH-dependent phosphorylation of Akt in orbital fibroblasts. Cultures were treated with TA4 for 16 hours followed by no treatment or bTSH for 30 minutes. Cell layers were analyzed by Western blot for phosphorylated Akt (pAkt). (Densitometry of signals corrected for respective β-actin: control, 0.45; TSH, 0.63; TA4, 0.2; TSH + TA4, 1.36 AU).

TSH actions appear to be both time and dose dependent [Fig. 5(A) and 5(B)]. The induction of HA was enhanced near-maximally at the first time point examined (8 hours) where the effects of TSH were increased 3-fold. Even at the lowest concentration examined (10 pM), TA4 significantly enhanced the induction by TSH on HAS1 messenger RNA. At 100 nM, the highest concentration tested, the effects of TSH were enhanced by more than 4-fold [Fig. 5(B)].

Discussion

Despite its initial detection decades ago (6), little is currently known about either the site(s) of TA4 generation or the biological roles it might play in health and disease. TA4 and its deiodinated congener, TA3, are components of the endogenous thyroid hormone metabolome (6, 25–27). The far better characterized pathway of this complex involves the enzymatic deiodination of T4 to iodothyronines such as T3 and reverse T3. This occurs not only within the thyroid gland but also in peripheral target tissues. Investigation of the transformation of T4 to TA4 and T3 to TA3 was reported initially by Tomita and colleagues (6) almost 60 years ago. They found enzymatic activity in T4-treated rat kidney homogenates that resulted in the formation of TA4. TA3 could also be detected in extracts of T3-treated kidneys and was posited as representing a degradation product of T3 (28), but this concept lacks empirical support (29). Even less is known about the biosynthetic origins of TA4. Conversion of T4 to TA4 was once considered essential for its thyromimetic actions (27). Braverman and colleagues (30) detected conversion of

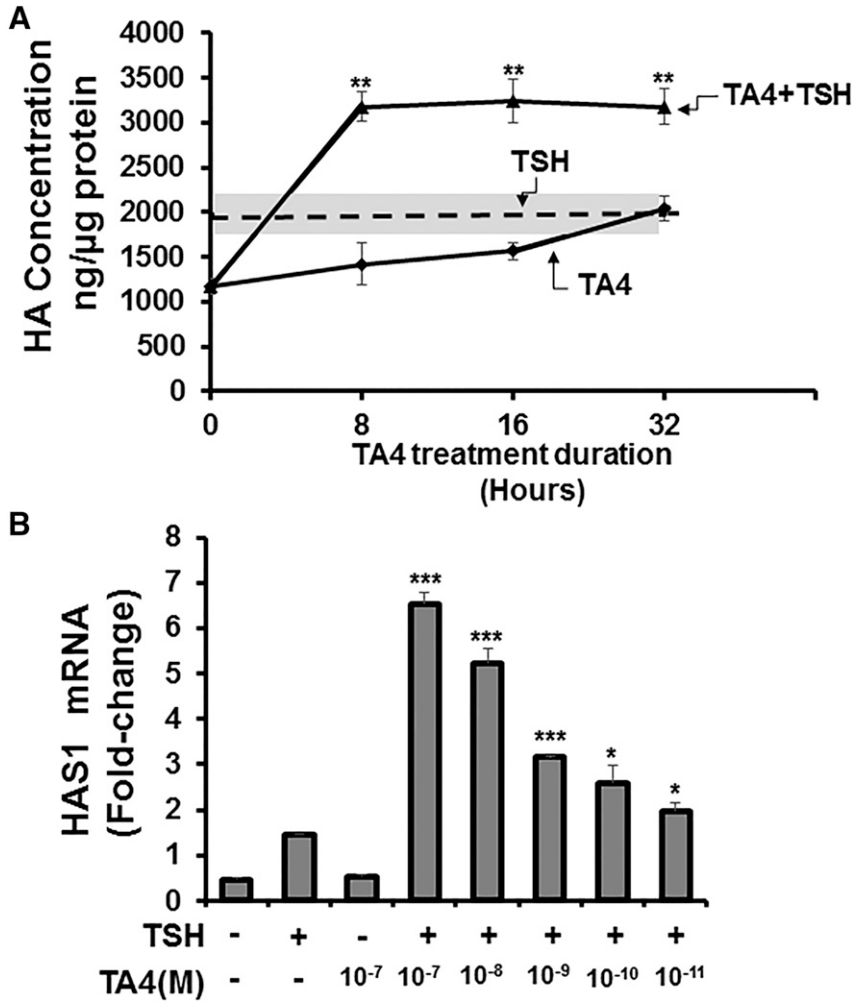

Figure 5. (A) Time course of 3,3′,5,5′ tetraiodothyroacetic acid (TA4) effects on bTSH–dependent hyaluronan (HA) accumulation production. Confluent fibroblast cultures were treated with TA4 (100 nM) for duration of time indicated along the abscissa. They were then treated with bTSH (5 IU/mL) for the final 6 hours of incubation. Cell layers were subjected to the hyaluronan (HA) enzyme-linked immunosorbent assay as described in the "Methods." Data were normalized to the protein content of the samples. Dashed line represents HA levels in wells treated with TSH alone for 6 hours ± SD shown as shaded area. (B) Concentration dependence of TA4 effects on TSH-dependent hyaluronan synthase 1 (HAS1) messenger RNA expression. Cultures were incubated with graded concentrations of TA4 indicated along the abscissa for 48 hours without or with bTSH (5 mIU/mL) for the final 6 hours. Cell layers were lysed for RNA extraction and reverse transcribed, and the complementary DNAs were subjected to real-time polymerase chain reaction. Data were normalized to corresponding GAPDH levels. Data are expressed as the mean ± SD of 3 independent determinations. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with bTSH alone.

[^{125}I]T4 to [^{125}I]TA4 in athyreotic human subjects. Fasting appears to shunt T4 metabolism away from T3 production toward TA4 formation (31). Despite detecting both TA4 and TA3 in biological fluids, including human serum samples (23, 32), no tissue or cellular source besides kidney for either analogue has yet been identified. The generation of TA4 by fibrocytes and several other cell types demonstrated here unmasks additional putative sources of TA4 and suggests that its generation might occur in many tissues.

Earlier estimates of serum TA4 concentrations in healthy donors varied between 300 and 1000 pM (25, 33), where the unbound fraction was found to be 0.001%. More recent estimates of TA4 levels in a cohort of euthyroid participants analyzed using gas fragmentography are in better agreement with those we report here (114.5 ± 26.4 pmol/L) (26). Refinements in assay precision and specificity may account for the lower values reported in more recent studies. Although T4 binds to several serum proteins, including thyroxine-binding protein, transthyretin, and albumin, only transthyretin has been identified as a TA4-binding serum protein (28). Concentrations of this binding protein do not appear to determine those of TA4 in serum (26).

The current results suggest strongly that a major determinant of serum TA4 concentration is that of T4. In both healthy individuals and those with GD, a significant positive correlation was detected between concentrations of the 2 molecules (Figure 2). Moreover, an inverse correlation was observed between T3 and TA4, suggesting that either TA4 is produced from T4 at the expense of T3 or that negative feedback regulation exists between T3 and TA4. The direct conversion of T4 to TA4 was demonstrated unequivocally in a variety of cell types by incubating cells with isotopically labeled T4 and detecting labeled TA4 in the media using LC-MS/MS (Figure 3C and Supplemental Figure 1). Thus, TA4 appears to be a metabolite of T4, and therefore T4 concentrations represent the dominant reservoir of substrate destined for conversion to TA4. Whether endogenous generation of TA4 occurs in thyroid epithelial cells and fibrocytes cannot be ruled out without further studies.

Most biological actions of TH are attributed to T3 due to its relatively high affinity and potency at nuclear receptors. The biological functions of endogenously generated TA4 are as yet unidentified. TA4 and TA3 appear to be more potent than T4 and T3, respectively, in inhibiting TSH release from the pituitary (33, 34). TA4 has been found effective at reducing TSH response to thyrotropin-releasing hormone in rat anterior pituitary

cells at a free concentration of 5 pM (34, 35). In contrast, TA4 and TA3 exhibit substantially weaker nuclear actions in a recently developed reporter assay than do T4 and T3 (36). TA4 can substitute for T4 in neocortex embryonic development (37) and in developing mouse brain where monocarboxylate transporter 8 is knocked out (38). In hypothyroid myxedema, TA4 was shown to normalize basal metabolic rate and serum cholesterol (39). Thus, some actions of T4 are shared with TA4, whereas others appear to diverge. Directly relevant to the current findings, TA4 can oppose the actions of T4 and T3 at the cell membrane receptor, $\alpha V\beta 3$, and in so doing modulate angiogenesis (40). Given the evidence we now present where TA4 generation appears to be widespread (Supplemental Fig. 1), it is possible that this TH analogue imposes a modulatory influence on T4 and T3 action in many if not all target tissues.

A completely unanticipated finding is the enhancement by TA4 of TSH and TSI actions (Figure 4). Induction of key factors involved in TAO is amplified substantially, including IL-6 (15), PGHS-2 (16–18), HAS1 (41), and HA accumulation (8, 9, 19). Although the TA4 concentration achieved within the TAO orbit is uncertain, it is possible that tissue TA4 levels might exceed those in serum. Although the current results unambiguously demonstrate that several different cell types can convert T4 to TA4, additional studies will be necessary to identify the mechanisms involved in TA4 generation, action, and whether fibrocytes can generate T4 or TA4 *de novo* from the thyroglobulin backbone that they endogenously express (13, 14).

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