3-Iodothyronamine metabolism and functional effects in FRTL5 thyroid cells

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Abstract

3-Iodothyronamine (T1AM), produced from thyroid hormones (TH) through decarboxylation and deiodination, is a potent agonist of trace amine-associated receptor 1 (TAAR1), a G protein-coupled receptor belonging to the family of TAARs. In vivo T1AM induces functional effects opposite to those produced on a longer time scale by TH and might represent a novel branch of TH signaling. In this study, we investigated the action of T1AM on thyroid and determined its uptake and catabolism using FRTL5 cells. The expression of TAAR1 was determined by PCR and western blot in FRTL5 cells, and cAMP, iodide uptake, and glucose uptake were measured after incubation with increasing concentrations of T1AM for different times. T1AM and its catabolites thyronamine (T0AM), 3-iodothyroacetic acid (TA1), and thyroacetic acid (TA0) were analyzed in FRTL5 cells by HPLC coupled to tandem mass spectrometry. The product of amplification of TAAR1 gene and TAAR1 protein was demonstrated in FRTL5 cells. No persistent and dose-dependent response to T1AM was observed after treatment with increasing doses of this substance for different times in terms of cAMP production and iodide uptake. A slight inhibition of glucose uptake was observed in the presence of 100 μM T1AM after 60 and 120 min (28 and 32% respectively), but the effect disappeared after 18 h. T1AM was taken up by FRTL5 cells and catabolized to T0AM, TA1, and TA0 confirming the presence of deiodinase and amine oxidase activity in thyroid. In conclusion, T1AM determined a slight inhibition of glucose uptake in FRTL5 cells, but it was taken up and catabolized by these cells.

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Introduction

Iodothyronines (including thyroid hormones (TH)) might be substrates for aromatic amino acid decarboxylase with production of iodothyronamines having structural similarities with several biogenic amines (Borowsky et al. 2001, Bunzow et al. 2001) present in trace levels in mammalian nervous system. Endogenously produced 3-iodothyronamine (T1AM) has been detected in human, rat, mouse, and guinea pig blood as well as other tissues (Scanlan et al. 2004, Saba et al. 2010). A specific group of mammalian G protein-coupled receptors, now called trace amine-associated receptors (TAARs), was recently discovered and classified (Borowsky et al. 2001, Bunzow et al. 2001). In humans, nine TAAR genes, including three pseudogenes, have been identified (Gloriam et al. 2005, Lewin 2006). The entire family of TAAR genes maps to a narrow region of a single chromosome spanning about 109 kb of human chromosome 6q23.1, 192 kb of mouse chromosome 10A4, and 216 kb of rat chromosome 1p12 (Lindemann et al. 2005). Structural similarities among trace amines and iodothyronamines suggest that the latter are able to activate TAARs; in fact, T1AM has been shown to be the most potent agonist stimulating cAMP production via activation of heterologously expressed mouse and rat TAAR1 (Scanlan et al. 2004).

In target tissues, thyroxine (T4), the predominant form of TH, is enzymatically deiodinated to 3,5,3′-triiodothyronine (T3), a high-affinity ligand for nuclear TH receptors controlling normal vertebrate development, growth, and metabolism (Yen 2001). T3-modulated transcription of target genes via activation of TH nuclear receptors is a slow process that requires hours or days. In addition to the classical nuclear mode of action of TH, a number of rapid effects at cytosol and plasma membrane levels have been identified (Davis & Davis 1996, Falkenstein et al. 2000). These nongenomic actions of TH are extranuclear, independent of TH receptors, and occur at posttranscriptional level. It has been proposed that T1AM is a novel signaling molecule
that can rapidly influence several physiological manifestations of TH action, including body temperature, heart rate, and cardiac output (Scanlan et al. 2004, Chiellini et al. 2007, Ghelardoni et al. 2009). In vivo, T1AM induces profound hypothermia and bradycardia within minutes, consequences that are opposite to those associated with TH excess, and it has been hypothesized that T1AM exerts its influence via nongenomic effectors including TAAR1 (Scanlan et al. 2004).

T1AM is usually assumed to be produced from TH through decarboxylation and deiodination (Zucchi et al. 2006), but there is no direct demonstration of T4 conversion to T1AM (Fig. 1). T1AM is a substrate of deiodinases, particularly D3 deiodinase (Piehl et al. 2008), and it is a potential substrate of amine oxidases, such as monoamine oxidase and semicarbazide-sensitive amine oxidase. Oxidative deamination followed by aldehyde oxidation by the ubiquitous enzyme aldehyde dehydrogenase would produce 3-iodothyroacetic acid (TA1; Scanlan et al. 2004, Gereben et al. 2008, Klieverik et al. 2009). Although TA1 production has been observed in hepatic tissue and cardiac tissue after administration of exogenous T1-AM (Wood et al. 2009, Saba et al. 2010), a quantitative and comprehensive analysis of T1AM catabolism (Fig. 2) in thyrocytes has not been performed.

In this study, we investigated the possible action of T1AM on thyroid by using a differentiated thyroid follicular cell line derived from normal rat thyroid (FRTL5 cells). In particular, we investigated the effects of T1AM on basal and bovine TSH (bTSH)-stimulated cAMP production and iodide and glucose uptake. Moreover, we evaluated whether exogenous T1AM can be taken up and/or catabolized by FRTL5 cells measuring the levels of T1AM and its catabolites thyronamine (T0AM), TA1, and thyroacetic acid (TA0) in cell medium and lysate by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). Uptake and catabolism of exogenous T4 in FRTL5 cells was also investigated.

**Materials and methods**

**Cell culture**

FRTL5 cells (Fisher rat thyroid cells) were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum, gentamicin (50 mg/ml), and a six-hormone mixture containing insulin (1 mg/ml), hydrocortison (10^-8 M), transferrin (5 mg/ml), somatostatin (10 ng/ml), glycy1- L-histidyl- L-lysine (10 ng/ml), and bTSH (1 mU/ml) as described previously (Ambesi-Impiombato et al. 1980). All the reagents were purchased from Sigma Chemical Co. FRTL5 cells were seeded in 96-well plates for the determination of cAMP production, in 24-well plates for the measurement of iodide uptake, and in 60 mm diameter dishes for total RNA extraction. For glucose uptake experiments, FRTL5 cells were grown in 24-well plates in Coon’s modified Ham’s F-12 medium supplemented with 5% calf serum, gentamicin (50 mg/ml), and a three-hormone mixture containing insulin (1 mg/ml), transferrin (5 mg/ml), and bTSH (1 mU/ml).

Rat thyroid primary cells were obtained from a tissue explant as described previously (Chiovato et al. 1989).

**Figure 1 Proposed metabolic pathway of T4 to T1AM.**

**Figure 2 Chemical structure of 3-iodothyronamine (T1AM), thyronamine (T0AM), 3-iodothyroacetic acid (TA1), and thyroacetic acid (TA0).**
Total RNA extraction, DNase treatment, and reverse transcription

Total RNA was isolated directly from cultured FRTL5 cells and from rat and human thyroid tissues using the AquaPure RNA Isolation Kit (Bio-Rad Laboratories) according to the manufacturer’s instructions. Contaminating DNA was removed by incubating the RNA for 15 min at 37 °C with 0.5 µg DNase I (Invitrogen Life Technologies) in the presence of a ribonuclease inhibitor. The quality of RNA samples was analyzed by microfluidic electrophoretic separation on chip using the Agilent 2100 BioAnalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). For each sample, 1 µg of total RNA was reverse transcribed for 1 h at 42 °C in a 20 µl reaction volume using 200 units of Superscript II RNase H− reverse transcriptase (Invitrogen Life Technologies) in the presence of 1·5 µM random examers (Pharmacia Biotech), 0·01 M dithiothreitol, and 1 mM dNTP mix.

PCR amplification

The expression of rat TAAR1 mRNA was determined in FRTL5 cells and in rat and human thyroid tissues by PCR amplification. Reverse-transcribed cDNA (500 ng) was amplified using specific primers for a 300 bp portion of rat TAAR1 gene or human TAAR1 gene. The sequences of primers are as follows: rTAAR1FW 5'-GTGAGAACAGTTGAGCA-3'; rTAAR1REV 5'-ATGGTGAGTCTGGGAGCA-3'; hTAAR1FW 5'-ATGGTGGATCTGCTGAGCA-3'; hTAAR1REV 5'-TCCCTCTGCAGTGAACATGTT-3'.

PCR amplification was carried out in 50 µl reaction mixture containing 20 pM specific primers and 1·5 mM MgCl2 in the presence of 1 U Taq DNA Polymerase (Biotech), 0·01 M dithiothreitol, and 1 mM dNTP mix.

Protein extraction and western blot analysis

Proteins were extracted from FRTL5 cells and rat tissues (brain, stomach and lung) and western blot analysis was performed as described previously (Peri et al. 2002). To detect TAAR1 in rat FRTL5 cells and in rat tissues used as positive controls, a polyclonal antibody raised against a peptide mapping within an extracellular domain of the protein was used (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Trypan blue dye exclusion

Cells (100,000) were grown in 24-well plates in six-hormone medium for 4–5 days and maintained for 6–7 days in five-hormone medium (in the absence of bTSH). Then, cells were incubated at 37 °C for 1, 6, 24, 48, and 72 h with increasing concentrations of T1AM (0·1, 1, 10, and 100 µM), with increasing concentrations of monoamine oxidase inhibitor pargyline (1, 10, and 100 µM), or with increasing concentrations of the tyrosine kinase inhibitor genistein (1, 10, and 100 µM). Cellular viability was determined with 0·2% trypan blue dye, and cell counts were performed using a hemocytometer. Results were expressed as percentage of alive cells with respect to the number of cells counted.

Quantitative real-time PCR

The expression level of type I iodothyronine deiodinase (Dio1) gene was determined in FRTL5 cells treated with 100 µM T1AM for 0–72 h by real-time PCR as reported previously (Agretti et al. 2002). After isolation of total RNA and reverse transcription, levels of Dio1 were measured using TaqMan gene expression assay (Rn00572183_m1; Applied Biosystems, Foster City, CA, USA). Analysis of relative gene expression data was performed using the ΔΔCT method with the house-keeping gene β-actin (Actb) as an endogenous control/reference assay. Results were expressed as the amount of target gene normalized to the endogenous reference and relative to a calibrator (untreated FRTL5 cells).

cAMP assay

FRTL5 cells seeding in 96-well plates were grown for 4–5 days in six-hormone medium and 6–7 days in five-hormone medium before performing the cAMP assay. Cells were washed once with Hanks’ balanced salt solution (HBSS) and incubated for 5, 15, 30, 60, 90, and 120 min at 37 °C in hypotonic medium containing 0·5 mM 3-isobutyl-1-methylxanthine (IBMX) as a cAMP phosphodiesterase inhibitor alone (basal value) or in the presence of increasing concentrations of bTSH (from 0·001 to 10 µM/ml) or T1AM (from 0·1 to 100 µM). FRTL5 cells were also subjected to treatment with increasing concentrations of bTSH (0·01, 0·1, 1, 10, and 100 µM/ml) together with 0·1, 1, and 10 µM T1AM. Additional experiments were performed in the presence of the monoamine oxidase inhibitor pargyline at the final concentration of 10 µM for 60 min. Extracellular cAMP was measured in the medium collected at the end of the incubation using an in-house RIA assay with a commercial polyclonal anti-cAMP antibody as described previously (Vitti et al. 1993) and expressed as pmol per well.

Iodide uptake

Cells (100,000) were plated in 24-well plates and grown for 4–5 days in six-hormone medium and 6–7 days in six-hormone medium for 4–5 days and maintained for
five-hormone medium before performing the Na$^{125}$I uptake assay as described previously (Tonacchera et al. 2001). Briefly, cells were incubated at 37°C for 45 min with about 1 000 000 c.p.m. of carrier-free Na$^{125}$I (Amersham Biosciences) in the presence of 10 μM NaI, washed twice and solubilized with 0.1 M NaOH. The radioactivity from each well was counted using a γ-counter, and the data of iodide uptake were expressed as pmol/well. Iodide uptake was measured in FRTL5 cells after 6, 12, 24, 36, 48, 60, and 72 h of stimulation with 1 mU/ml bTSH and after 24 h of treatment with increasing concentrations of bTSH. Time course of iodide uptake was also determined after treatment with increasing doses of T$_1$AM. Experiments were also performed in the presence of the monoamine oxidase inhibitor pargyline at the final concentration of 10 μM or the tyrosine kinase inhibitor genistein at the final concentration of 37 μM.

Glucose uptake

FRTL5 cells were grown for 3 days in 24-well plates at a density of about 2×10$^5$ cells/well in three-hormone medium and were then grown for 7 days in one-hormone medium (transferrin 5 μg/ml) before performing $^3$H-deoxy-glucose ($^3$H-DG) experiments. Cells were quickly rinsed in 0.9% NaCl and incubated for 2 h at 37°C with about 1 μCi 2-deoxy-[$^3$H]-glucose (Amersham Biosciences) in 500 μl of uptake buffer (HBSS, 0.5% BSA, 10 mM Hapes, pH 7.4). Finally, cells were rinsed three times in ice-cold uptake buffer and solubilized in 500 μl RIPA buffer (50 mM Tris, pH 8-0, 150 mM NaCl, 1% NP-40, 0-5% sodium deoxycholate, and 0-1% SDS). In order to measure incorporated radioactivity, 450 μl of each sample were removed and counted in a liquid scintillation β-counter (LKB Wallac, Turku, Finland). For protein quantity determination, 20 μl of each sample were used (Bradford 1976). All experiments were conducted in triplicate, and in order to study the specificity of the glucose transporter-mediated uptake, we incubated cells from two wells in the same conditions as described above, but with the addition of 50 μM cytochalasin B (Sigma–Aldrich). The values of radioactivity expressed in counts per minute were normalized with respect to the value of protein concentration and results were expressed as fmol $^3$H-DG/mg protein.

$^3$H-DG uptake was measured in FRTL5 cells after 0, 5, 30, 60, and 120 min and 18, 24, and 42 h of incubation with 10 mU/ml bTSH and after 0, 5, 30, 60, and 120 min and 18 h of incubation with T$_1$AM 100 μM. Experiments were also performed in the presence of the monoamine oxidase inhibitor pargyline at the final concentration of 10 μM or the tyrosine kinase inhibitor genistein at the final concentration of 37 μM.

T$_1$AM and T$_4$ uptake and catabolism

FRTL5 cells were seeded into 24-well plates and grown in six-hormone medium. At 80–90% confluence, the culture medium was replaced with 0.5 ml of Krebs-Ringer medium (118 mM NaCl, 25 mM NaHCO$_3$, 4.5 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 1.5 mM CaCl$_2$, and 11 mM glucose) and cells were preincubated in a humidified atmosphere of 5% CO$_2$ at 37°C for 30 min. Incubation started by adding 0.5 ml Krebs-Ringer buffer containing 1 μM T$_1$AM or 1 μM T$_4$ and returning the plate to a humidified atmosphere of 5% CO$_2$ at 37°C for times ranging from 5 min to 24 h. At the end of incubation, the medium was removed.
from each well and transferred to a tube for HPLC–MS/MS analysis. Cells adherent to plate were dissolved in 0.1 ml NaOH 0.1 M and 0.01 ml 1.0 M HCl were added for pH neutralization. Cell lysates were then diluted with 0.390 ml of Krebs-Ringer medium, transferred to a tube for LC/MS/MS analysis, and centrifuged at 14 000 g for 2–3 min before performing the analysis. Rat thyroid primary cells were grown and treated as above to investigate $T_4$ uptake.

**HPLC–MS/MS**

To evaluate the metabolism of $T_1AM$ and $T_4$, we modified previously published methods (Scanlan et al. 2004, Tai et al. 2004, Chiellini et al. 2007, Piehl et al. 2008) to develop an assay based on MS/MS coupled to HPLC, which allowed the contemporary detection of $T_3$, $T_4$, $T_1AM$, and its putative metabolites, namely $T_0AM$, $TA_0$, and $TA_1$. The assay technique has been described in detail elsewhere (Saba et al. 2010).

**Statistical analysis**

Results were expressed as mean ± s.d. Differences between groups were evaluated by either one-way or two-way ANOVA as appropriate. If ANOVA showed significant differences between groups, individual groups were compared with the control group by Bonferroni post hoc test. GraphPad Prism version 4.1 for Windows (GraphPad Software, San Diego, CA, USA) was used for data analysis.

**Results**

**PCR amplification of $TAAR1$ gene**

Reverse transcription and PCR using specific primers for rat $TAAR1$ gene demonstrated the amplification product of about 300 bp in FRTL5 cells and in rat thyroid tissue (Fig. 3A). RT-PCR analysis also showed the expression of nine different TAAR subtypes ($TAAR2$, $TAAR3$, $TAAR4$, $TAAR5$, $TAAR6$, $TAAR7a$, $TAAR8a$, and $TAAR9$) in FRTL5 cells (data not shown). The amplification product of human $TAAR1$ gene was also demonstrated in human thyroid tissue (Fig. 3B).

**$TAAR1$ protein expression determined by western blotting**

$TAAR1$ protein was detected in FRTL5 cells using a polyclonal antibody. $TAAR1$ protein corresponding to a 39-1 kDa band was identified in FRTL5 cells extract and in rat control tissues extracts (Fig. 3C).

**Trypan blue dye exclusion**

In this assay, no substance ($T_1AM$, pargyline, and genistein) showed a cytotoxic effect at the tested doses (data not shown) on FRTL5 cells, and concentrations used for further experiments did not interfere with cell functions.

**Quantitative real-time PCR**

In order to assess whether intracellular $T_3$ pathway could be altered by $T_1AM$, $Dio1$ gene expression was analyzed by quantitative PCR. $Dio1$ was expressed in FRTL5 cells and no significant change in expression level was observed after incubation with 100 µM $T_1AM$ for a period of time ranging from 0 to 72 h (data not shown).

**cAMP production after bTSH or $T_1AM$ treatment**

Time course of cAMP production in FRTL5 cells after stimulation with increasing concentrations of bTSH
showed that the maximum response to bTSH was obtained after 60 min of incubation (Fig. 4A). No significant response to T1AM stimulation, in terms of cAMP production, was observed after treatment of FRTL5 cells with increasing doses of this substance for times ranging from 5 to 120 min as shown in Fig. 4B (P=NS for the effect of T1AM by two-way ANOVA). Concentrations of T1AM ranging from 0-1 to 10 µM were not able to modify the response of FRTL5 cells to stimulation with 0·01–100 mU/ml bTSH (data not shown). The monoamine oxidase inhibitor pargyline was not able to modify the response to bTSH or T1AM stimulation in FRTL5 cells (Fig. 5).

**Iodide uptake after bTSH or T1AM treatment**

Time course of iodide uptake in FRTL5 cells after stimulation with a maximal dose (1 mU/ml) of bTSH showed that a significant increase of iodide uptake was reached just after 24 h of treatment (Fig. 6A). The treatment of FRTL5 cells with increasing doses of bTSH for 24 h determined an increase of iodide uptake with a plateau reached at the concentration of 0·1 mU/ml bTSH (Fig. 6B). No persistent and clearly dose-dependent response to T1AM stimulation was observed after treatment of FRTL5 cells with increasing doses of this substance for times ranging from 1 to 72 h (Fig. 6C). However, a slight reduction of iodide uptake occurred in the presence of 10 or 100 µM T1AM at 1 and 24 h (P<0·01). The effect of T1AM on iodide uptake of FRTL5 cells was not increased in the presence of the monoamine oxidase inhibitor pargyline or the tyrosine kinase inhibitor genistein (data not shown).

**Glucose uptake after bTSH or T1AM treatment**

Treatment of FRTL5 cells with 10 mU/ml bTSH for 5, 30, 60, and 120 min and 18, 24, 42, and 60 h determined an increase in 3H-DG uptake starting from 120 min and reaching the maximum plateau level after 42 h (Fig. 7A). No change in 3H-DG uptake was observed after treatment of FRTL5 cells with 100 µM T1AM for times ranging from 5 to 30 min. Between 60 and 120 min, a slight reduction of glucose uptake was observed (P<0·05 at 60 min and P<0·01 at 120 min) but the effect was not persistent after 18 h (Fig. 7B). The effect of T1AM on 3H-DG uptake of FRTL5 cells was not increased in the presence of the monoamine oxidase inhibitor pargyline or the tyrosine kinase inhibitor genistein (data not shown).
T1AM are shown in Fig. 8. In incubation medium, T1AM concentration decreased exponentially to magnitude lower than T1AM, and after 360 min, it was still one order of magnitude lower. After 360 min, the overall recovery, i.e. the ratio of (T1AM + T0AM + TA1 + TA0) to initial T1AM, was close to 100%.

Figure 9A shows the results obtained in FRTL5 cells exposed to 1 μM T4. After 360 min, the amount of T4 still present in the incubation medium was 71% of the initial dose. About 11% of the initial dose was detected in cell lysate, and T4 accumulation was roughly interpolated by a first-order (hyperbolic) kinetics with T0.5 of 12.87 ± 0.66 min (r=0.671). About 21% of the administered T4 was converted into T3, giving a complete recovery, while other catabolites were not detected. T3 was detected both in the incubation medium and in the cellular lysate, and after 360 min, the ratio of lysate T3 to medium T3 was close to 4. In both compartments, the increase in T3 concentration was appropriately interpolated by polynomial or exponential fitting, with r>0.980.

Primary rat thyroid cells were also able to accumulate T4 and to produce a minimal amount of T3 when exposed to 1 μM T4 for times ranging from 0 to 360 min (Fig. 9B). Although T4 uptake was similar or even slightly higher in primary culture cells than in FRTL5 cells, T3 production was remarkably lower and corresponded to <1% of the administered T4.

**Discussion**

It has recently been proposed that T0AM, decarboxylated derivatives of TH, may constitute a class of signaling molecules similar to several biogenic amines present in trace levels in mammalian nervous system, able to activate the members of a recently identified family of G protein coupled receptors (GPCRs) called TAARs. In particular, T1AM has been identified as the most potent agonist of the rat TAAR1 with an EC50 of 14 nM (Scanlan et al. 2004). When heterologously expressed, rat or mouse TAAR1 rapidly couple to the stimulation of cAMP production when exposed to T1AM. The types of G protein coupled to each TAAR and the immediate downstream effectors have yet to be established, although there is widespread evidence that activated recombinant TAAR1 heterologously expressed in HEK293 cells or *Xenopus* oocytes couple to Gs and stimulate cAMP production (Scanlan et al. 2004). In *vivo* T1AM has been found to produce physiological effects in a matter of minutes through a nontranscriptional mechanism, which are opposite to those induced over time by TH (Scanlan et al. 2004, Chiellini et al. 2007, Ghelardoni et al. 2009). Several authors reported TAAR transcripts to have a broad tissue distribution. The presence of TAAR1 mRNA was detected using in situ hybridization histochemistry in many areas of the mouse brain (Borowsky et al. 2001).

![Figure 7](https://www.endocrinology-journals.org/content/47/1/29/F7.large.jpg)  
Figure 7 Glucose uptake in FRTL5 cells after treatment with 10 μM BTSH (A) and after treatment with 100 μM T1AM (B) for different times. When error bars are not visible, they are so small that they fall within the symbols. Data are expressed as mean±s.d. of three different experiments.

FRTL5 is a rat thyroid cell line, which does not express active thyroid peroxidase but has a high deiodinase activity (Borges et al. 1990, Derwahl et al. 1990). The results obtained in FRTL5 cells exposed to 1 μM T1AM are shown in Fig. 8. In incubation medium, T1AM concentration decreased exponentially to zero, with a rate constant of 0.156 ± 0.005 min⁻¹, corresponding to a half-life of 4 min. In cellular lysate, T1AM concentration showed complex changes: at first, it increased according to a first-order (hyperbolic) kinetics with T0.5 of 12.25 ± 3.15 min (r=0.958), peaked at 90 min (1.6±0.9 μM), and then progressively decreased reaching about 0.9 μM at 360 min. In pilot experiments, lysate T1AM concentration further decreased to 0.06 μM after 1140 min of incubation. The assay of T1AM catabolites showed production of T0AM, TA1, and TA0. Significant amounts of T0AM were detected only in cell lysate: a plateau was reached after 20–30 min, and then further increase occurred after 180 min. TA1 and TA0 were detected mainly in the incubation medium, where their concentration increased over time and could be interpolated by linear regression. Over the first 180 min of the experiment lysate, T0AM concentration was about two orders of magnitude lower than T1AM, and after 360 min, it was still one order of magnitude lower. After 360 min, the overall recovery, i.e. the ratio of (T1AM + T0AM + TA1 + TA0) to initial T1AM, was close to 100%.

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Using quantitative RT-PCR, the same authors found that TAAR1 gene was expressed at moderate levels in human stomach and at low levels in human amygdala, kidney, lung, and small intestine. Trace amounts of TAAR1 mRNA were detected in human liver, pancreas, prostate, skeletal muscle, spleen, and in many areas of central nervous system (Borowsky et al. 2001). Recently, the expression of mRNA for TAAR1, TAAR6, TAAR8, and TAAR9 was demonstrated by RT-PCR in human leukocytes (D’Andrea et al. 2003), and TAAR9 was also

Figure 8 Representative experiments showing T1AM uptake and catabolism in FRTL5 cells. T1AM, T0AM, TA1, and TA0 concentrations are measured in lysate (1) and medium (2) from FRTL5 cells. Please note that as cellular lysate was diluted by about 25-fold (Saba et al. 2010), actual cellular concentrations are about 25-fold higher than lysate concentrations.

Figure 9 Representative results showing T4 uptake and catabolism in FRTL5 cells (A) and in cultured rat thyroid primary cells (B). T4 and T3 concentrations were measured in lysate (1) and medium (2) from cells. Please note that as cellular lysate was diluted by about 25-fold (Saba et al. 2010), actual cellular concentrations are about 25-fold higher than lysate concentrations.
found in pituitary and skeletal muscle (Vanti et al. 2003). Transcripts for TAAR1, TAAR2, TAAR3, TAAR4, and TAAR8a have been amplified from rat heart mRNA (Chiellini et al. 2007).

As we observed that TAAR1 gene is expressed in human thyroid tissue, we planned this study to investigate the possible action of T1AM, the main agonist of TAAR1, on thyroid. We performed in vitro experiments on FRTL5 cells, a differentiated thyroid follicular cell line derived from normal rat thyroid, to investigate the effects of T1AM on basal and bTSH-stimulated cAMP production and iodide and glucose uptake. The product of amplification of rat TAAR1 gene was demonstrated in FRTL5 cells by RT-PCR. The protein was also demonstrated in these cells by western blotting. Treatment of FRTL5 cells with increasing doses of T1AM, for times ranging from few minutes to several hours, was able to produce major changes in cAMP production and iodide or glucose uptake. However, a transient decrease in glucose and possibly in iodide uptake was observed after a period of 1–2 h in the presence of 10–100 μM T1AM. The monoamine oxidase inhibitor pargyline or the tyrosine kinase inhibitor genistein did not modify the effects of T1AM on FRTL5 cells. The noncytotoxic effect of T1AM, pargyline, or genistein at the used concentrations, was determined with trypan blue dye.

The second issue, which we investigated, is T1AM accumulation and catabolism in FRTL5 cells. T1AM uptake has been reported in different neoplastic cell lines (Ianculescu et al. 2009) but has never been demonstrated in thyroid. The molecular mechanism responsible for T1AM uptake has not been defined, but it does not appear to involve known transporters for monoamines or TH. On the basis of transport inhibition by siRNA technique, eight candidate transporters belonging to the SLC family have recently been identified (Ianculescu et al. 2009). We obtained evidence that T1AM is accumulated by FRTL5 cells, since a few minutes after adding T1AM to the incubation medium, it could be detected in cellular lysate. The noncytotoxic effect of T1AM, pargyline, or genistein at the used concentrations, was determined with trypan blue dye. Theoretic considerations suggest that T1AM may be a substrate for D1 and D3 deiodinases and for amine oxidases, so that expected catabolites include T0AM, TA0, and TA1 (Zucchi et al. 2006, Gereben et al. 2008, Wood et al. 2009). To establish whether T1AM can undergo deiodination, experiments were performed with FRTL5 cells. This thyroid cell line cannot produce TH, because thyroid peroxidase is not functional (Derwahl et al. 1990), but it shows higher deiodinase activity (either on the tyrosyl or on the phenolic ring) than normal thyroid tissue (Borges et al. 1990, Derwahl et al. 1990), as confirmed in the present investigation by determining Dio1 expression through quantitative PCR. In FRTL5, cellular uptake of T1AM was confirmed and deiodinated metabolites were detected. T0AM accumulated in the lysate, whereas TA1 and TA0 were released in the incubation medium. If the dilution of intracellular content in the lysate (over 25-fold) is taken into account, it seems likely that intracellular concentration exceeded extracellular concentration also for TA1 and TA0. These results are consistent with the known subcellular location of deiodinases and amine oxidases, because intracellular monoamine oxidase A (MAO-A) appears to be the prevailing amine oxidase in thyroid cells (Andres et al. 2001), and deiodination also occurs intracellularly (Friesema et al. 2006), although D3 is a plasma membrane enzyme (Gereben et al. 2008). Notably, the progressive decrease in T1AM concentration may account for the transient effects observed in functional experiments. Moreover, it was demonstrated that T4 enters in FRTL5 cells and is converted to a small extent to T3, whereas other catabolites of T4 are not detectable over this time scale. The intracellular T3 pathway was not altered by T1AM, because the level of Dio1 expression was unchanged after prolonged exposure to T1AM. As expected, T4 was also trapped and converted to T3 by normal rat thyroid primary cells. Although the extent of T4 uptake was similar in FRTL5 cells and in adult thyrocytes, T3 production was higher in the former model, consistent with a greater deiodinase activity.

In summary, we demonstrated that (i) TAAR1 is expressed in FRTL5 cells and in human thyroid tissue; (ii) its most potent agonist T1AM is not able to exert any effect on cAMP accumulation, while it may transiently decrease glucose uptake in FRTL5 cells; (iii) FRTL5 cells are able to accumulate, deiodinate, and oxidatively deaminate exogenous T1AM; and (iv) FRTL5 are able to accumulate exogenous T4 and to convert it into T3.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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