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

Figure 1 Proposed metabolic pathway of T₄ to T₁AM.

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 T₁AM (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 µg/ml), and a six-hormone mixture containing insulin (1 µg/ml), hydrocortison (10⁻⁸ M), transferrin (5 µg/ml), somatostatin (10 ng/ml), glycyl-L-histidyl-L-lysine (10 ng/ml), and bTSH (1 mU/ml) as described previously (Ambesi-Impimbatto *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 µg/ml), and a three-hormone mixture containing insulin (1 µg/ml), transferrin (5 µg/ml), and bTSH (1 mU/ml).

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

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'-CGC-AGGCAGAAGACCTGATT-3'; hTAAR1FW 5'-ATGGT-GAGATCTGCTGAGCA-3'; hTAAR1REV 5'-TCCTCTG-CAGTGAACATGTT-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 (Invitrogen Life Technologies). After the amplification, 5 µl reaction mix were separated on 1% agarose gel and the resulting bands were visualized in u.v. light on a transilluminator, following ethidium bromide staining. Molecular weights were compared with a 100 bp ladder.

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 $\Delta\Delta C_T$ 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 mU/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 mU/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 pmoles 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

five-hormone medium before performing the Na¹²⁵I uptake assay as described previously (Tonacchera *et al.* 2001). Briefly, cells were incubated at 37 °C for 45 min with about 1 00 000 c.p.m. of carrier-free Na¹²⁵I (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 T₁AM. 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 × 10⁵ cells/well in three-hormone medium and were then grown for 7 days in one-hormone medium (transferrin 5 µg/ml) before performing ³H-deoxy-glucose (³H-DG) experiments.

Cells were quickly rinsed in 0.9% NaCl and incubated for 2 h at 37 °C with about 1 µCi 2-deoxy-D-[1-³H]-glucose (Amersham Biosciences) in 500 µl of uptake buffer (HBSS, 0.5% BSA, 10 mM Hepes, 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 ³H-DG/mg protein.

³H-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 T₁AM 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.

T₁AM and T₄ 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 NaHCO₃, 4.5 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.5 mM CaCl₂, and 11 mM glucose) and cells were preincubated in a humidified atmosphere of 5% CO₂ at 37 °C for 30 min. Incubation started by adding 0.5 ml Krebs-Ringer buffer containing 1 µM T₁AM or 1 µM T₄ and returning the plate to a humidified atmosphere of 5% CO₂ at 37 °C for times ranging from 5 min to 24 h. At the end of incubation, the medium was removed

Figure 3 Rat and human TAAR1 expression. RT-PCR expression in FRTL5 cells and rat thyroid tissue (A) and in human thyroid tissue (B); protein expression determined by western blot (C) in FRTL5 cells and in rat control tissues.

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 T₄ uptake.

HPLC–MS/MS

To evaluate the metabolism of T₁AM and T₄, 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 T₃, T₄, T₁AM, and its putative metabolites, namely T₀AM, TA₀, and TA₁. The assay technique has been described in detail elsewhere (Saba *et al.* 2010).

Statistical analysis

Results were expressed as mean $\pm$ 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 (T₁AM, 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 T₃ pathway could be altered by T₁AM, *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 μ M T₁AM for a period of time ranging from 0 to 72 h (data not shown).

cAMP production after bTSH or T₁AM treatment

Time course of cAMP production in FRTL5 cells after stimulation with increasing concentrations of bTSH

Figure 4 Time course of cAMP production in FRTL5 cells after stimulation with increasing concentrations of bTSH (A) and after stimulation with increasing concentrations of T₁AM (B). When error bars are not visible, they are so small that they fall within the symbols. Data are expressed as mean $\pm$ s.d. of three different experiments.

Figure 5 Effects of the monoamine oxidase inhibitor pargyline on cAMP production after bTSH (10 mU/ml) or T₁AM (0.001, 0.01, 0.1, 1, 10, and 100 µM) treatment. When error bars are not visible, they are so small that they fall within the symbols. Data are expressed as mean $\pm$ s.d. of three different experiments.

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).

Figure 6 Iodide uptake in FRTL5 cells after stimulation with 1 mU/ml bTSH for different times (A), after 24 h treatment with increasing doses of bTSH (B), and after treatment with increasing concentrations of T₁AM for different times (C). When error bars are not visible, they are so small that they fall within the symbols. Data are expressed as mean $\pm$ s.d. of three different experiments.

Figure 7 Glucose uptake in FRTL5 cells after treatment with 10 mU/ml bTSH (A) and after treatment with 100 μ M 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 $\pm$ s.d. of three different experiments.

T₁AM and T₄ uptake and catabolism in FRTL5 cells

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 μ M T₁AM are shown in Fig. 8. In incubation medium, T₁AM concentration decreased exponentially to zero, with a rate constant of $0.156 \pm 0.26/\text{min}$ ($r = 0.975$), 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 $T_{0.5}$ of 12.25 ± 3.15 min ($r = 0.958$), peaked at 90 min (1.6 μ M), and then progressively decreased reaching about 0.9 μ M at 360 min. In pilot experiments, lysate T₁AM concentration further decreased to 0.06 μ M 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%.

Figure 9A shows the results obtained in FRTL5 cells exposed to 1 μ M 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 $T_{0.5}$ of 12.87 ± 6.66 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 μ M 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 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.

Using quantitative RT-PCR, the same authors found that *TAARI* gene was expressed at moderate levels in human stomach and at low levels in human amygdala, kidney, lung, and small intestine. Trace amounts of *TAARI* 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 *TAARI*, *TAAR6*, *TAAR8*, and *TAAR9* was demonstrated by RT-PCR in human leukocytes (D'Andrea *et al.* 2003), and *TAAR9* was also

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 not 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 over 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. Theoretical 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 amino 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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