Differentiated thyroid carcinoma : diagnostic and therapeutic studies

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Chapter 4

The In Vitro Effects of Triiodothyronine on Iodide Uptake in FRTL-5 Cells

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Abstract

Background: Thyrotropin (TSH) stimulated radioiodide scintigraphy and therapy are important in the clinical care of patients with differentiated thyroid carcinoma (DTC). The introduction of recombinant human TSH (rhTSH) is an attractive alternative for thyroid hormone withdrawal (THW). Some reports suggest however that radioiodide uptake after rhTSH is inferior to THW. One of the explanations is that there is a direct effect of triiodothyronine (T3) on iodide uptake.

Aim: To study the effects of triiodothyronine (T3) on iodine uptake and expression of the sodium iodide symporter (NIS).

Methods: Iodide uptake (both steady state and initial rate) were studied in the rat thyroid cell line FRTL-5. FRLT-5 cells were cultured in medium with stripped serum in the absence or presence of 1pM, 2nM or 50nM T3 and all in presence of 1mU/ml TSH for 72 hours. NIS and TSH receptor mRNA and NIS protein expression were studied by quantitative PCR and Western-Blot.

Results: T3 inhibited iodine uptake both at initial rate and during steady state in a concentration dependent manner at steady state. NIS and TSHR expression at mRNA level were both reduced. Western blot of NIS protein showed a significant reduction of NIS protein after 2 nM.

Conclusion: T3 reduces radioiodine uptake and NIS and TSHR expression in FRTL-5 cells. We speculate that this is not caused by iodide being released from T3, as this amount is negligible, but that these are direct genetic effects, of which the mechanism needs further investigation.

Introduction

The concepts of therapy and diagnostic procedures during follow-up in differentiated thyroid carcinoma (DTC) are based on the responsiveness of thyroid carcinoma cells to thyrotropin (TSH)(1). TSH stimulated radioiodine uptake is important for both the ablation of thyroid hormone remnants during initial therapy and treatment of residual or metastatic DTC. In addition, TSH stimulated serum thyroglobulin (Tg) measurements have superior diagnostic value to detect recurrent DTC (2).

High serum TSH levels can be realized by conventional thyroxin withdrawal or more recently by recombinant human TSH (rhTSH), which has advantages with respect to quality of life (3). rhTSH has initially been used for diagnostic radioiodine scintigraphy and Tg measurements (4-13). In addition, rhTSH has also been used for radioiodine therapy in active DTC (14-18) and for the ablation of thyroid remnants (19-21).

The assumption for rhTSH treatment is that the pharmacodynamic properties of rhTSH and thyroxin withdrawal are comparable and that continuation of thyroxin therapy does not influence iodide uptake and Tg synthesis.

It is generally acknowledged that Tg measurements during rhTSH have comparable accuracy as thyroxine withdrawal (2;7). Some authors, however, have observed a lower sensitivity of diagnostic radioiodine scintigraphies performed after rhTSH (22;23). The efficacy of radioiodine therapy after rhTSH may be comparable with withdrawal, but no randomized studies have been performed to allow a direct comparison (14;24). Efficacy of radioiodine ablation after rhTSH was comparable after thyroxin withdrawal in a recent randomized trial (21), although earlier studies with lower activities of radioiodine showed a lower efficacy (25). One of the possible explanations for the supposedly decreased radioiodine uptake during rhTSH may be that triiodothyronin (T3) directly influences iodine uptake in the thyroid. We therefore studied the in vitro effects of T3 on iodide uptake.

Materials and Methods

Cell culture and cell proliferation assay

The rat thyroid FRTL-5 cell-line derived from the ATCC (ATCC, Manassas USA) expresses endogenously NIS which is subjected to TSH regulation (26). FRTL-5 cells were routinely cultured in Coon's F-12 modification medium (Sigma, Missouri USA) supplemented with 5% of stripped bovine calf serum, 1 mM non-essential

amino acids (Life Technologies, Inc.), 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and a six-hormone mixture (6H) containing insulin (1.3 μM), hydrocortisone (1 μM), transferrin (60 pM), L-glycyl-histidyl-lysine (2.5 μM), somatostatin (6.1 nM), and TSH (1 milliunits/ml) as reported previously (27).

For the proliferation assay, 500 cells/well were seeded in 96-well culture plates. T3 was added at concentrations varying from 1 pm to 50 nM. Two nM T3 is the average serum T3 concentration in rats and therefore considered physiological. Cell growth was measured using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay in conjunction with the addition of the electron coupling reagent phenazine methosulfate (PMS) (Promega). Briefly, 1, 3, 6 and 16 days after addition of T3, 180 μl culture medium was replaced by medium containing 10 μl of MTS/PMS mixture for 3 hours and placed at 37°C in a humidified incubator with 5% CO2. The absorbance of each well was measured with a microplate reader (Rainbow reader) at 570 nm wavelengths.

Radioiodide uptake assay

For uptake experiments, FRTL-5 cells were grown in 12-well plates. T3 was added in concentrations ranging from 0, 0.5, 1 and 2 nM for 72 hours prior to the uptake studies. For steady state iodide uptake assessments, cells were also cultured in medium without TSH (5H). The radioiodine uptake was performed as previous described (28). Briefly, the cells were washed 3 times with Hanks Balanced Salt Solution (HBSS) prior to the uptake assay. For the steady state uptake experiments, FRTL-5 cells were incubated with HBSS containing 10 μM $Na^{125}I$ with a specific activity of 50 mCi/mmol for 30 min 37 °C. Thereafter, the radioiodine was washed twice with cold HBSS. Cells were lysed with ice-cold ethanol. Radioactivity was subsequently measured in a gamma emitter counter. The DNA content of each well was subsequently determined after trichloroacetic acid precipitation, by the diphenylamine method (29). Based on the specific activity of the substrates, the efficiency of the γ-counter, and the DNA content of each well, iodide uptake was expressed as picomoles of substrate transported per microgram of DNA or as percentage of control conditions.

In the initial rate experiments, the effect of substrate concentration on uptake was determined by incubating washed FRTL-5 cells for 2 min in HBSS containing NaI from 0.625 to 160 μmol/L. After 2 min, radioiodide uptake was quantified as indicated above.

RNA isolation and real-time quantative PCR

Total RNA of FRTL-5 cells was extracted after 72 hour culturing without or with 1pM, 2 nM or 50 nM T3, using TRIzol LS reagent (Invitrogen Life Technologies, Inc.), followed by RNA cleanup with the RNeasy mini kit (Qiagen, Valencia, CA).

RNA concentrations were determined by measuring the absorbance at 260 nm. RNA was reverse transcribed into cDNA using the SuperScript First-stand Synthesis System for RT-PCR(Gibco BRL).

The following primer sets were used for quantitative PCR (qPCR): TSHR5'-3' TGC TTTCAA TGG AAC AAA GC; 3'-5' GGA AGG AAG AGC AGT AAC GC. NIS 5'-3' GGT TGT GGT AAT GCT CGT TG; 3'-5' GGG TCA AAG TCC ATC AGG TT. beta-actin 5'-3'TCC TTC CTG GGT ATG GAA TC; 3'-5' GCA CTG TGT TGG CAT AGA GG. All PCR amplicons spanned exon-intro boundaries.

The qPCRs were performed in the presence of 5ul Taq Gold buffer, 1.75ul 50mM MgCl2, 1ul 5mM dNTPs, 0.1ul 5U Aplitaq Gold DNA polymerase, 0.25ul 10uM stock solution of sense and antisense primers, 1.5ul sybrgreen and 1ul 5ng/ul cDNA in a final volume of 25ul. Water was used as a negative control. qPCR reactions perform on an iCycler (Biorad, Hercules, CA, USA) using the SybrGreen qPCR core-kit (Eurogentec, Seraing, Belgium). Cycle conditions were: 10 minutes at 94°C followed by 40 cycles of 10 s at 94°C and 1 minute at 60°C. Cycle threshold (Ct) extraction was performed using the iCycler IQ software (version 3, Biorad). The Ct value for NIS and TSHR are subtracted from the Ct values of actin (delta Ct values) (Fig.1). The relative delta Ct was calculated by 2^deltCT. The mean delta Ct value of an individual sample was based on three independent measurements.

Western blot analysis

Western-blot was performed as described previously (30). FRTL-5 cells were grown in the absence or presence of 0.5, 1 and 2 nM T3. Proteins were extracted and quantified using the Lowry method. All samples were diluted 1:2 with loading buffer and heated at 37°C for 30 min prior to electrophoresis.

Western blot analysis was carried out as follows: Twenty-five micrograms of protein per lane were loaded on a 9% SDS polyacrylamide gel and subjected to electophoresis at a constant voltage (150 V). Electroblotting to a nitrocellulose membrane was performed for 1 h. Blocking was done overnight using TTBS/milk (TBS, 1% Tween 20 and 5% milk). The membrane was incubated for 1 hr with a 1:5000 dilution of affinity-purified anti-rNIS antibody (30), which was kindly provided by Dr. Carrasco (Albert Einstein College of Medicine, Bronx, USA) in TTBS/milk. After washing, the membrane was incubated with a 1:5.000 dilution of a horseradish peroxidase-linked donkey anti-rabbit IgG (Amersham) in TTBS/milk. Quantitation of the signal intensity was performed by densitometry (Molecular Dynamics, Inc.). Membranes were also stained with a beta-actin antibody to check the amount of protein loaded.

Results

Cell proliferation assay

The results of the proliferation assay are given in Figure 1. Proliferation was assessed at 1, 3, 6 and 16 days after addition of T3. Addition of different concentrations of T3 (1 pM, 2nM or 50 nM) did not influence the proliferation. It was verified that T3 itself did not directly influence the MTS assay in a separate experiment in which both MTS and DNA concentrations were measured (data not shown).

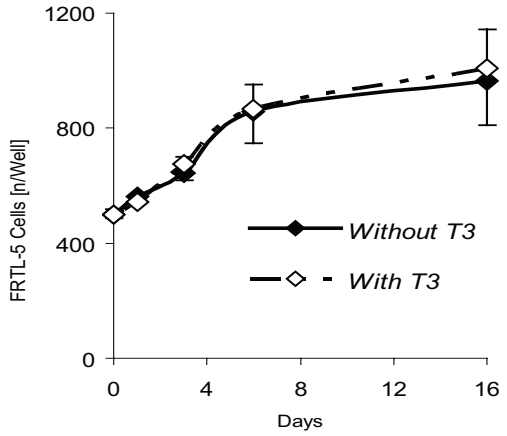

Figure 1. Proliferation of FRTL-5 cells, cultured without or in the presence of 50 nM T3. Cells were cultured in H-6 medium with stripped serum. Proliferation was measured with the MTS assay (see Materials and Methods).

Iodide Uptake

Iodide uptake was measured both in steady state conditions and in an initial rate experiment.

In steady state conditions, as expected, iodide uptake was much higher in the presence of TSH than in FRTL-5 cells without TSH (Figure 2a). Addition of T3 significantly decreased iodide accumulation, in a concentration dependent manner, irrespective whether the cells were cultured in the presence or absence of TSH (Figure 2a and Figure 2b). T3 decreases uptake even with absence of TSH although in a less pronouced level.

In the initial rate experiment, 1 and 2 nM T3 lowered the V_{max} of iodide uptake to about 50% of the control curve, whereas K_m was not influenced (Figure 2b).

NIS mRNA and protein expression

NIS mRNA expression as assessed by quantitative PCR was significantly reduced

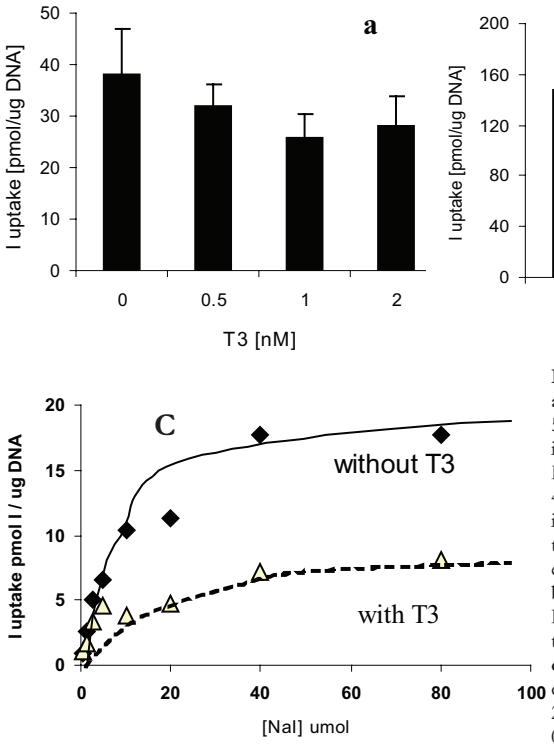

Figure 2.
a. FRTL-5 cells were cultured for 10 days in H-5 medium with stripped serum. T3 was added in indicated concentrations in the presence of H-6 medium. Iodide uptake was measured at 45 min after addition of I-125 (Specific activity 50 mCi/mmol. Activity was extracted from the cells by addition of ethanol. Uptake was expressed as pmol iodide/ug DNA.
b. Same as described in Fig.2a, except that FRTL-5 cells were cultured in H-6 throughout the whole experiment.
c. Initial rate (2 min) iodide uptake by FTRL-5 cells, cultured in H-6 medium with or without 2 nM T3 in a concentration range of NaI of 0.525 – 80 uM, with or without T3.

by the addition of 1 pM, 2 nM and 50 nM T3. The relative concentration of mRNA expression versus control was 0.86 for 2 nM T3 and 0.55 for 50 nM T3.

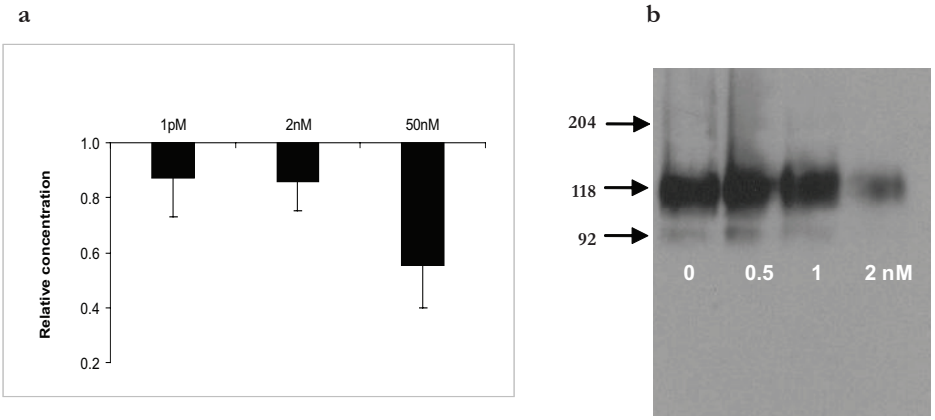

Figure 3.
a. Effects of T3 on NIS mRNA of FRTL-5 cells, cultured in H-6 medium with stripped serum. Proliferation expression as assessed by real time PCR, expressed as relative concentration (2^ delta delta CT)
b. NIS protein expression of FRTL-5 cells cultured in H-6 medium with stripped serum with or without T3.

Western Blot analysis showed that NIS protein expression was significantly reduced in FRTL5 cells cultured in 2 nM T3.

Discussion

The present study was conducted to investigate whether T3 had direct effects on iodide uptake in the thyroid irrespective of presence of TSH. We found indeed a decreased uptake of iodide in the rat cell-line FRTL-5 cultured in the presence of physiological concentrations of T3 even with absence of TSH although in a less pronouced level. Thus we speculate that T3 has TSH idenpendent effects on iodine uptake. This decreased uptake was accompanied by decreased NIS mRNA and protein expression.

The background of this experiment is the advent of rhTSH for the preparation of radioiodide scintigraphy and therapy in DTC (6;21). As patients will continue thyroxin therapy during rhTSH therapy, the question is whether T3 itself may affect iodide uptake as thyroid tissue contains functional T3 receptors (31;32). There have indeed been some suggestions that radioiodide scintigraphies after rhTSH have a lower sensitivity than after thyroxin withdrawal (5;6) and that ablation with 30 mCi radioiodide is less efficient after rhTSH than after thyroxin withdrawal (25). Several explanations for these observations have been proposed.

It has been suggested that the iodide content of levo-thyroxine (T4) therapy during rhTSH may dilute the specific activity of the radioiodide administered. Indeed, 65.4% of the molecular weight of T4 consists of iodide which may result in a net daily supply of 25-60 ug iodide, when taking 100 ug/day. Indeed increased urinary iodide excretion has been observed during rhTSH as compared with thyroxin withdrawal (33;34).

In our study, we found a substantial decrease in iodide uptake of up to 50% after T3. The amount of iodide coming from T3 in our experiment (In case of 2 nM T3: 9 nM of iodide) cannot explain the decrease in iodide uptake, as the steady state experiments were performed in the presence of 10 uM NaI. The resulting dilution of radioactivity may thus only be 0.001, which is negligible.

Another explanation for the diminished quality of radioiodide scintigraphies after rhTSH may be the altered iodide kinetics in euthyroidism as compared with hypothyroidism. Indeed, renal clearance of iodide is higher in euthyroidism, thereby reducing the whole body dose of radioiodine after rhTSH (35;36). In the latter study it was concluded that the effective half life of radioiodide in the thyroid after rhTSH was decreased but that the residence time of radioiodide in the thyroid was longer than after withdrawal. In our study, using an in vitro iodide uptake assay, the influence of whole body iodide kinetics was ruled out.

From our results it seems likely that T3 has effects on NIS gene expression at least in FRTL5 cells, resulting in lower functional NIS protein. It has been debated whether the promoter for NIS contains T3 responsive elements. In one study, it was suggested that T3 in fact stimulates the NIS promoter (37). However, these experiments were not performed with stripped serum. In earlier studies, it has been observed that T3 decreases the mRNA and protein expression of NIS as well as the uptake of iodide (32;38). In several experiments, it has been found that the promoter of the TSHR gene contains T3 responsive elements and that T3 suppresses the expression of the TSHR (39;40) (41). Another explanation for the repression of TSHR gene transcription by T3 has been suggested by Tagami et al (42) who found that unliganded thyroid hormone receptor recruits histone deacetylase (HDAC) from the TSHR promoter, resulting in increased histone acetylation and transcriptional activation of the TSHR. In the presence of T3 HDAC comes available to repress TSHR promoter activity. However, we observed that T3 also decreased iodide uptake in FRTL5 cultured in medium without additional TSH.

In conclusion, we found evidence for a TSH and iodide independent effect of T3 on NIS gene expression. The mechanism remains to be resolved and also the question whether the effect is present and relevant in humans. The clinical relevance of this finding is not clear. Randomized trials with clearly defined endpoints can provide answers to this question. The similar ablation efficacy in rhTSH treated patients and patients undergoing thyroxin withdrawal suggest that the contribution of T3 induced NIS suppression may be limited.

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