3-Iodothyronamine and 3,5,3′-triiodo-L-thyronine reduce SIRT1 protein expression in the HepG2 cell line

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Abstract:
Background: 3-Iodothyronamine (T1 AM) is an endogenous messenger chemically related to thyroid hormone. Recent results indicate significant transcriptional effects of chronic T1 AM administration involving the protein family of sirtuins, which regulate important metabolic pathways and tumor progression. Therefore, the aim of this work was to compare the effect of exogenous T1 AM and 3,5,3′-triiodo-L-thyronine (T3) chronic treatment on mammalian sirtuin expression in hepatocellular carcinoma cells (HepG2) and in primary rat hepatocytes at micromolar concentrations.

Materials and methods: Sirtuin (SIRT) activity and expression were determined using a colorimetric assay and Western blot analysis, respectively, in cells treated for 24 h with 1–20 μM T1 AM or T3. In addition, cell viability was evaluated by the MTT test upon 24 h of treatment with 0.1–20 μM T1 AM or T3.

Results: In HepG2, T1 AM significantly reduced SIRT1 (20 μM) and SIRT4 (10–20 μM) protein expression, while T3 strongly decreased the expression of SIRT1 (20 μM) and SIRT2 (any tested concentration). In primary rat hepatocytes, T3 decreased SIRT2 expression and cellular nicotinamide adenine dinucleotide (NAD) concentration, while on sirtuin activity it showed opposite effects, depending on the evaluated cell fraction. The extent of MTT staining was moderately but significantly reduced by T1 AM, particularly in HepG2 cells, whereas T3 reduced cell viability only in the tumor cell line.

Conclusions: T1 AM and T3 downregulated the expression of sirtuins, mainly SIRT1, in hepatocytes, albeit in different ways. Differences in mechanisms are only observational, and further investigations are required to highlight the potential role of T1 AM and T3 in modulating sirtuin expression and, therefore, in regulating cell cycle or tumorigenesis.

Keywords: rat hepatocyte, sirtuin, thyroid hormone, thyronamine

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Introduction

3-Iodothyronamine (T1 AM) is an endogenous compound chemically related to thyroid hormone. At nanomolar concentrations, it can activate G protein-coupled receptors [trace amine-associated receptors (TAARs)], in particular TAAR1 [1], but it may also interact with other targets, such as transporters on plasma membrane, vesicular biogenic amine transporters and mitochondrial proteins [2], [3]. Endogenous T1 AM has been detected in human and rodent blood and tissue samples [4], and circulating T1 AM is largely bound to apolipoprotein B100 (apoB100) [5]. Although the exact T1 AM biosynthetic pathway is still unknown, it has been hypothesized to derive from decarboxylation and deiodination of thyroxine (T4), and a tentative of biosynthetic pathway has been suggested [6].

T4, the predominant form of thyroid hormones, in target tissues, is enzymatically deiodinated to 3,5,3′-triiodo-L-thyronine (T3), which modulates the transcription of target genes via activation of thyroid hormone receptor α (TRα) and TRβ. Non-genomic effects have also been described, concerning glucose and calcium uptake, oxygen consumption, ion channel activation and cardiac function [7]. Moreover, evidences highlight the
influence of T3 in the regulation of tumor development [8]. General consensus exists regarding the oncosuppressor role of TRβ1 in hepatocarcinoma; however, controversial data have been reported about its role upon T3 activation [9]. Furthermore, hypothyroidism has been reported to represent a risk factor for hepatocarcinoma [10].

Several T1AM functional effects have been described, namely severe hypothermia [1], decrease in oxygen consumption and in respiratory quotient, the latter resulting in a shift from carbohydrate to lipid as metabolic energetic source [11]. Moreover, T1AM stimulates both gluconeogenesis and ketogenesis [12]. While hypothermia is observed only after administration of high T1AM dosages, metabolic effects occur at doses close to the physiological range [13].

The molecular mechanisms underlying T1AM metabolic effects are still unknown. In liver of obese mice (CD-1) evidence of changes in sirtuin expression has been reported [14]. The mammalian genome encodes for seven sirtuins (SIRT1–7) whose activities are controlled directly by the cellular levels of nicotinamide adenine dinucleotide (NAD+) and inhibited by nicotinamide [15]. Sirtuins regulate important pathways involved not only in stress resistance, energy efficiency and metabolism during caloric restriction, but also in aging, regulation of transcription, apoptosis and tumorigenesis [15].

So far, the effects of thyronamines and thyronines on sirtuins have not been fully unraveled. SIRT1 is a coregulator of TRβ, enhancing T3 activity [16]. Transgenic mice, bringing a dominant-negative mutation in TRβ, have higher hepatic SIRT1 activity, similar to hypothyroid wild-type mice [17], [18]. Conversely, in hypothyroid mice, T4 supplementation reduces liver SIRT1 protein [18]. In liver of CD-1 mice, T1AM is able to increase SIRT6 and reduce SIRT4 expression [14].

The aim of the present study was to get a better understanding of the activity of exogenous thyroid hormone and T1AM in liver, using two different cell lines: a cancer line, HepG2, hepatocellular carcinoma (HCC) cells, and a primary line of rat hepatocytes. We analyzed their potential cytotoxic activity and then their action on sirtuin activity and protein expression at pharmacological doses.

Materials and methods

Chemicals

Human hepatocellular carcinoma cells (HepG2) were obtained from American Type Culture Collection (Manassas, VA, USA). Unless otherwise specified, all reagents were obtained from Sigma Aldrich (St Louis, MO, USA).

Cell culture and treatment

Primary rat hepatocytes were prepared as previously described [19] with minor modifications. Experimental procedures were approved by the Ethical Committee of the University of Pisa (protocol no. 51814/2016). Briefly, adult Wistar rats (200–250 g; EnvigoRms, Udine, Italy) were sacrificed by exsanguination under diethyl ether anesthesia. Livers were quickly perfused without recirculation through the vena cava at 20 mL/min for 5 min with calcium and magnesium-free Dulbecco’s phosphate-buffered saline, followed by 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing collagenase 0.5% (w/v) and CaCl2 5 mM until the tissue was softened. The partially isolated hepatocytes were then collected and incubated for 5 min in the same collagenase-containing buffer. Hepatocytes were then washed 3 times by centrifugation in Dulbecco’s modified Eagle medium/nutrient mixture F-12 (DMEM/F12) supplemented with 100 IU penicillin/mL, 100 µg streptomycin/mL, 10% (v/v) fetal bovine serum (FBS), and suspended in fresh supplemented medium. The cell yield and viability were about \(10^7\) cells/mL and 85–90%, respectively, the latter being assessed by the trypan blue exclusion test. The cells were then plated, and treated after 4 h of seeding.

HepG2 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1 mM pyruvate, 100 U/mL penicillin/mL, 100 µg streptomycin/mL with 10% (v/v) fetal bovine serum (FBS), and suspended in fresh supplemented medium. The cell yield and viability were almost above \(10^5\) cells/mL. HepG2 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1 mM pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO2 and subcultured before confluence. Unless otherwise specified, all reagents were obtained from Sigma Aldrich (St Louis, MO, USA).

To start treatment, in any experimental procedure, medium was replaced with fresh medium supplemented with exogenous T1AM, or T3, in the range from 1 to 20 µM, unless otherwise indicated; control cells were incubated with supplemented DMEM containing equal volume of vehicle [dimethyl sulfoxide (DMSO)] for T1AM and NaOH 0.1 mM for T3.

To assess sirtuin expression, cells were seeded in six-well plates (3×10^5 cells/well). Upon treatment with T1AM or T3, HepG2 cells or primary hepatocytes were lysed in ice-cold buffer, containing 20 mM Tris pH 7.5,
150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 2.5 mM sodium pyrophosphate, 1% Igepal CA-630, 1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM phenylmethanesulfonyl fluoride (PMSF) and protease inhibitor cocktail. After sonication, cell lysates were centrifuged at 10,000×g for 10 min at 4 °C to pellet cellular debris, and supernatants were collected and frozen at −80 °C. The protein concentration in supernatant fraction was determined using the Bradford method [20].

**NAD concentration assay**

HepG2 cells or primary rat hepatocytes were seeded in 96-well plates (10,000 cells/well) and treated with T3 or T1 AM for 24 h; then, NAD concentration was assessed using a NAD/NADH cell-based colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer’s instructions. The absorbance was measured at 450 nm using a microplate reader (BioRad) and NAD concentration in samples was calculated according to standard curve. NAD concentration was measured as nM and plotted as % of control.

**Sirtuin activity assay**

Sirtuin activity was measured using a colorimetric assay kit (Universal SIRT Activity Assay Kit, Abcam, Cambridge, UK), according to the manufacturer’s instructions. Cells were seeded in six-well plates and treated with T1 AM or T3 for 24 h. At the end of treatment, the cells were solubilized in the appropriate buffer, and nuclear and cytoplasmic extracts were prepared according to the manufacturer’s protocol (Nuclear Extraction Kit, Abcam, Cambridge, UK). Sirtuin activity was evaluated in 10 μg of nuclear or cytoplasmic extract. The absorbance was read at 450 nm with a reference wavelength of 655 nm. The activity was calculated as optical density (OD)/min/mg of protein and plotted as % of control.

**Western blotting**

Western blotting was performed according to the manufacturer’s instructions (BioRad Laboratories, Hercules, CA, USA). In brief, 40 μg of proteins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–20% acrylamide separating gel, Criterion TGX Stain-Free precast gel, BioRad). The separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA). Membranes were then incubated using the appropriate primary and secondary antibodies. Immunoblots were visualized by means of a chemiluminescence reaction (Millipore) using Image Lab™ Software (BioRad) under a luminescent image analyzer (Chemidoc XSR+, BioRad). Chemiluminescence was expressed in terms of OD of specific immunoreactive bands, and the protein level was normalized to the OD of total proteins in each lane, previously acquired. The trihalo compounds included in the TGX stain-free gel (BioRad) react with tryptophan residues in an ultraviolet (UV)-induced reaction, allowing total protein detection by fluorescence. Only bands below the saturation limit were analyzed.

**MTT staining**

Cells were seeded in 96-well plates at a density of 5000–10,000 cells/well. After 24 h, T1 AM and T3 were added at different concentrations (0.1–20 μM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining was determined 24 h after incubation as described by Mossman [21]. Briefly, MTT (0.5 mg/mL) was added to the medium, and after an additional 4 h of incubation, SDS-HCl (0.05 g/mL) was added to solubilize formazan salt. After 18 h, the absorbance of the solution was read at 570 nm in a microplate reader (BioRad Instruments). In this test, cellular staining requires MTT reduction by endogenous oxidoreductases, particularly but not exclusively, mitochondrial dehydrogenases, using NADH or reduced nicotinamide adenine dinucleotide phosphate (NADPH) as reducing substrates. Therefore, MTT staining reflects the integrity and the rate of oxidative metabolism.

**Statistical analysis**

Results are expressed as the mean ± standard error of the mean (SEM). Differences between groups were analyzed by one-way analysis of variance (ANOVA). In experiments aimed at determining differences versus a
single control group, Dunnett’s post-hoc test was used. The threshold of statistical significance was set at $p < 0.05$. GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA) was used for data processing and statistical analysis.

Results

MTT staining

In order to evaluate the role of T1AM and T3 in liver cell damage, we treated HepG2 cells and primary hepatocytes with exogenous T1AM or T3 for 24 h.

As shown in Figure 1A, the results indicated that T1AM was slightly but significantly cytotoxic at any tested concentration in HepG2 cells, decreasing MTT staining from 100 nM to 20 $\mu$M by 10–20% (100 nM, $p < 0.01$; 500 nM, $p < 0.05$; 1 $\mu$M, $p < 0.0001$; 10 $\mu$M, $p < 0.001$; 20 $\mu$M, $p < 0.001$).

In primary rat hepatocytes, T1AM was cytotoxic starting from 500 nM ($p < 0.05$) to 20 $\mu$M ($p < 0.05$, Figure 1B) reducing MTT staining by about 30%. T3 exhibited a significant reduction of about 10–15% ($p < 0.05$) in HepG2 cells starting from 1 $\mu$M (1 and 10 $\mu$M, $p < 0.05$; 20 $\mu$M, $p < 0.001$), but no effect was observed in primary hepatocytes (Figure 1C and D). These results showed a different sensitivity of cell lines to the exposure of T1AM or T3.

NAD concentration and sirtuin activity assays

As NAD+$^+$ functions as a cofactor in sirtuin enzyme activity, we measured first the total cellular NAD concentration, and then we assessed sirtuin activity in the nuclear and cytoplasmic extracts, upon 24 h of treatment with T1AM or T3, in both liver cell lines.

Values of NAD concentration in control cells averaged in each cell line as follows: in primary hepatocytes 0.221 ± 0.008 nM, and in the HepG2 cell line 0.785 ± 0.022 nM.

As shown in Figure 2A, in HepG2 cells, NAD concentration was reduced only by T3 by about 15–20% (1 $\mu$M and 10 $\mu$M both $p < 0.001$ vs. vehicle, Con), while T1AM was unable to affect NAD concentration. Similar
results were obtained in primary rat hepatocytes (Figure 2B) where only T3 at 10 μM (p < 0.01 vs. vehicle, Con) was able to reduce NAD concentration by about 25%.

![Figure 2: NAD concentration assay results in HepG2 cells and primary rat hepatocytes (primary hep.) for T1AM (A) and for T3 (B). HepG2 cells or primary rat hepatocytes were seeded in 96 well plates (10.000 cells/well) and treated with T3 or T1AM for 24 h; then, NAD concentration was assessed by a NAD/NADH cell-based colorimetric assay kit in cell lysates, according to manufacturer's instructions. All treatments received the same amount of vehicle (DMSO for T1AM and NaOH 0.1 mM for T3). Data are represented as mean ± SEM. (one-way ANOVA, p < 0.01, Dunnett's post-hoc test for multiple comparison, **p < 0.01; ***p < 0.001 vs. control (Con), n = 5 per group).](image)

Sirtuin activity was measured in the nuclear extracts which contained, according to the handbook of nuclear extraction kit, described in the Materials and methods, SIRT1, SIRT6 and SIRT7, and in the cytoplasmic extracts containing SIRT2, SIRT4 (present also in mitochondria) and SIRT5. Baseline values of sirtuin activity in control cells averaged in the nuclear and cytoplasmic extracts of each cell line, respectively, as follows: in primary rat hepatocytes, 0.159 ± 0.011 OD/min/mg and 0.042 ± 0.006 OD/min/mg; in the HepG2 cell line, 0.086 ± 0.010 OD/min/mg and 0.0253 ± 0.004 OD/min/mg.

As shown in Figure 3A, T1AM significantly reduced sirtuin activity only in the nuclear extracts of primary rat hepatocytes by about 40% (T1 AM 10 μM, p < 0.05), without inducing any change in the HepG2 cell line. Conversely, in the nuclear extract of primary rat hepatocytes, T3 exhibited opposite effects on sirtuin activity (Figure 3B), being decreased at 20 μM (~60%, p < 0.01 vs. Con) and enhanced at 1 μM by about 50% (p < 0.01 vs. Con). In HepG2 cells, the nuclear extract showed a remarkable increase in activity at any T3 tested concentration (+150% with T3 1–20 μM, p < 0.05 vs. vehicle, Figure 3B). Lastly, cytoplasmic activity was never affected by T3.
Figure 3: Sirtuin activity assay results in primary rat hepatocytes (prim. hep.) and HepG2 cells. Enzymatic activity was measured in nuclear (n.e.) and cytoplasmic (c.e.) extracts for T\(_1\)AM (A) and for T3 (B). Cells were seeded in 6-well plates and treated with T\(_1\)AM or T3 for 24 h. At the end of treatment, cells were solubilized in the assay buffer, and nuclear and cytoplasmic extractions were performed according to manufacturer’s protocol. Sirtuin activity was evaluated in 10 μg of each extract. Control groups received only vehicles in the same amount (DMSO for T\(_1\)AM and NaOH 0.1 mM for T3). Data are represented as mean ± SEM (one-way ANOVA, p < 0.05, Dunnett’s post-hoc test for multiple comparison, *p < 0.05; **p < 0.01 vs. control (Con), n = 3–4 per group).

Western blot analysis

To investigate and compare the effect of T\(_1\)AM and T3 on mammalian sirtuin protein expression, we exposed HepG2 cells and primary hepatocytes to T\(_1\)AM or T3.

In HepG2 cells (Figure 4A), 24 h of infusion of T\(_1\)AM significantly reduced SIRT1 expression (20 μM, p < 0.05) by 47% and SIRT4 expression (10 μM, p < 0.05; 20 μM, p < 0.05) by 45%, whereas T3 strongly decreased SIRT1 (20 μM, p < 0.05) by 70%, and SIRT2 expression by 55%, 65% and 45%, respectively, at 1 μM (p < 0.05), 10 μM (p < 0.001) and 20 μM (p < 0.05). In this tumor cell line, other sirtuins were unaffected.
Western blot analysis of T₁AM and T3 effects on sirtuin expression in (A) HepG2 cells and (B) primary rat hepatocytes. Cells were seeded in six-well plates (3×10⁵ cells/well). Upon treatment with T₁AM or T3, HepG2 cells or primary hepatocytes were lysed in ice-cold buffer. Proteins were then subjected to SDS-PAGE, transferred to a PVDF membrane and incubated using the appropriate primary and secondary antibodies. Representative blots of sirtuins, which reached significance, were showed. Histograms represent mean ± SEM. All results are normalized against total protein densitometric values in each lane, previously acquired by fluorescence. (one-way ANOVA, p < 0.05, Dunnett’s post-hoc test for multiple comparison *p < 0.05 vs. control group (Con), DMSO for T₁AM and NaOH 0.1 mM for T3, n = 3–4 per group).

As shown in Figure 4B, in primary rat hepatocytes, sirtuin expression was unchanged by T₁AM while T3 decreased SIRT2 levels by 62% (10 μM, p < 0.05).

Discussion

In this study, we compared the effect of T₁AM on sirtuin activity and protein expression and cell viability in HepG2 cells and primary rat hepatocytes. We observed a reduction in SIRT1 and SIRT4 levels in HepG2 cells and in nuclear sirtuin activity in primary rat hepatocytes. As for MTT-estimated cell viability, the HepG2 cell line resulted more sensible to T₁AM than primary hepatocytes. Given the structural similarity between T₁AM and thyronines, investigations were extended to T3 by using the same in vitro model. We observed a reduction in the expression of SIRT1 and SIRT2 in HepG2 cells, and SIRT2 in primary hepatocytes, while cell viability was affected by T3 only in the HepG2 cell line. Furthermore, T3 was able to reduce cellular NAD concentration and enhance nuclear sirtuin activity.

Sirtuins (SIRT1–7) are a class of enzymes which catalyze NAD-dependent substrate deactivation, mainly removing an acetyl group from a variety of substrates which are involved in critical processes such as stress response, cellular metabolism, DNA repair, cancer and aging. All the members of the sirtuin family are expressed in liver and, among them, SIRT1–4 and SIRT6 are sirtuins whose functions have been extensively investigated and reviewed, especially their implication in cancer (i.e. [22], [23]).

In our investigation, both tested compounds downregulated SIRT1 only in the tumor cell line, and they slightly reduced MTT staining. The role of SIRT1 in tumorigenesis is still controversial. Several reviews debate about SIRT1 as an oncogene or tumor suppressor and it has been reported to be overexpressed in HCC, when compared to the normal cell line [24]. SIRT1 modifies the activity of many molecules which play pivotal roles in cell proliferation, senescence, apoptosis and angiogenesis [25]. On one hand, SIRT1 acts as a tumor suppressor, inhibiting inflammation, proliferation, oxidative stress and multistage carcinogenesis [26]. On the other hand, SIRT1 accelerates tumorigenesis via multiple mechanisms, which promote genomic instability, and develop microenvironments suitable to growth and survival of cancer cells [24]. These controversial roles of SIRT1 in tumorigenesis have been attributed to different circumstances, such as distribution of up- and downstream inhibiting factors, subcellular localization and different experimental models [27].
The concept of inhibiting SIRT1 as an antitumor strategy has been explored by several groups, which have developed various SIRT1 inhibitors as alternative approaches to cancer therapy [28], [29]. In HCC, SIRT1 co-operates with the oncogene c-Myc in the tumorigenesis process, promoting cancer cell survival and reducing tumor prognosis [30]. Considering the above, in our experimental model, T1AM and T3, if infused at pharmacological doses, may function as regulators of SIRT1, decreasing its protein expression when upregulated. On the other hand, cell viability was reduced by T1AM or T3 also at lower concentrations, which did not affect SIRT1 expression, indicating that other mechanisms might have been involved. In sharp contrast with protein expression results, nuclear sirtuin activity was increased: this could be explained considering the presence of various sirtuins, namely SIRT1, SIRT6 and SIRT7 [22] (even though SIRT1 is well known to shuttle from nucleus to cytoplasm under specific circumstances [31]), which might have differently contributed to the total enzymatic activity in the nuclear fraction.

In HepG2 cells, T1AM downregulated mitochondrial SIRT4. These results are consistent with previous observations of SIRT4 downregulation in livers, obtained from mice or rats treated with T1AM [32], [14]. SIRT4 functions mainly as a negative regulator of fatty acid oxidative metabolism, facilitating glycolytic metabolism [33], albeit SIRT4 may also act as a potential tumor suppressor, by inhibiting mitochondrial glutamine metabolism [34]. This may indicate a cancer-promoting role for T1AM, selectively in tumor cells, because primary hepatocytes were unaffected.

Different from T1AM, T3 was able to reduce also SIRT2 protein expression and intracellular NAD concentration in both cell lines, without affecting, in cytoplasm, total enzyme activity. Other sirtuins, which included SIRT4 as indicated by the enzymatic assay kit and usually located in mitochondria, might have contributed to the total cytoplasmic activity, counteracting a negative effect. Literature regarding SIRT2 expression in HCC is quite contradictory. Several evidences suggest a dual role for SIRT2 in carcinogenesis, similar to SIRT1: it turns out to be an oncogene when it promotes epithelial-mesenchymal transition and motility of cancer cells [28], whereas it acts as a tumor suppressor by maintaining genomic stability [35]. For this reason, the concept of inhibiting SIRT1 and SIRT2 using a dual SIRT1/SIRT2 inhibitor has been proposed [28], and T3 could be one of the potential molecules to test as a novel strategy for targeted therapy of tumor overexpressing these sirtuins.

In conclusion, we compared the effects of exogenous T1AM and T3 in primary rat hepatocytes and HepG2 cells, focusing our attention on expression of sirtuins, regulators of several cell processes. In the experimental models taken into account in this investigation, a higher susceptibility of tumor cell lines was highlighted. Moreover, the decrease of SIRT1 expression, shared by both compounds, and the downregulation of SIRT2 or SIRT4, exclusive of T3 or T1AM, respectively, could allow to consider these molecules as potential sirtuin modulators. However, summing up the functions of sirtuins in cancer and the overall effects of T1AM on sirtuin expression, the role of this thyronamine is still ambiguous and compensatory: on one hand, it may blunt the antitumoral effects of sirtuin and, on the other hand, it may enhance their action as cancer suppressors, accounting for its marginal effects on cancer cell viability. These differences are simply observational. Therefore, further investigations (e.g. specific activity assays for each sirtuin) are required to reveal step by step the biological mechanisms by which sirtuins are affected by thyroid hormone and its putative derivatives.

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