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3,5-Diiodo-L-thyronine induces SREBP-1 proteolytic cleavage block and apoptosis in human hepatoma (Hepg2) cells

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ABSTRACT

Thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) is known to affect cell metabolism through both the genomic and non-genomic actions. Recently, we demonstrated in HepG2 cells that T3 controls the expression of SREBP-1, a transcription factor involved in the regulation of lipogenic genes. This occurs by activation of a cap-independent translation mechanism of its mRNA. Such a process is dependent on non-genomic activation of both MAPK/ERK and PI3K/Akt pathways. The physiological role of 3,5-diiodo-L-thyronine (T2), previously considered only as a T3 catabolite, is of growing interest. Evidences have been reported that T2 rapidly affects some metabolic pathways through non-genomic mechanisms. Here, we show that T2, unlike T3, determines the block of proteolytic cleavage of SREBP-1 in HepG2 cells, without affecting its expression at the transcriptional or translational level. Consequently, Fatty Acid Synthase expression is reduced. T2 effects depend on the concurrent activation of MAPKs ERK and p38, of Akt and PKC-δ pathways. Upon the activation of these signals, apoptosis of HepG2 cells seems to occur, starting at 12 h of T2 treatment. PKC-δ appears to act as a switch between p38 activation and Akt suppression, suggesting that this PKC may function as a controller in the balance of pro-apoptotic (p38) and anti-apoptotic (Akt) signals in HepG2 cells.

1. Introduction

Thyroid hormones (THs) play fundamental roles in the regulation of cell functions through two distinct mechanisms: genomic and nongenomic actions. In the genomic action, 3,5,3′-triiodo-L-thyronine (T3) interacts with the nuclear receptors TR-α and TR-β regulating the expression of target genes. Over the past decade growing evidence demonstrated that this is not the only mechanism. THs can generate rapid non-genomic biological responses independent of the interaction with TRs [1,2].

Lipid metabolism is largely recognised as a main target of TH action. Beside the THs, Sterol regulatory element binding protein-1a, -1c and -2 (SREBP-1a), (SREBP-1c), and (SREBP-2) are transcription factors involved in the control of lipid homeostasis through the regulation of several lipogenic genes [3].

SREBPs are synthesized as inactive precursors bound to the endoplasmic reticulum (ER), where their regulatory domain interacts with SCAP (SREBP-cleavage-activating protein), which functions as a sensor of membrane cholesterol levels. When cells become depleted in cholesterol, the SREBP–SCAP complex translocates from the ER to the Golgi, where a two-step proteolytic cleavage releases the N-terminal half of SREBP, allowing its entry into the nucleus. Conversely, in sterol-repleted cells the SREBP–SCAP complex remains in the ER owing to the interaction of this complex with the ER-embedded INSIG (insulin-induced gene) [4].

Evidences are accumulating that SREBPs are not only involved in sterol-regulated events but are also targets of intracellular signalling pathways such as the MAPK cascades (ERK 1/2 and p38) [5,6], and the PI3K/Akt pathway [7,8]. In a recent study we found that in liver cells T3 is able to increase SREBP-1 protein expression enhancing the translation efficiency of its mRNA through an Internal Ribosome Entry Site (IRES)-mediated mechanism [9]. This effect depends on the activation of both the MAPK/ERK and PI3K/Akt pathways. The latter seems to be regulated in duration through a negative feedback loop exerted by S6K-P70 and PCK-α [9].

Several studies indicated that, in addition to T3, also 3,5-diiodo-L-thyronine (T2), which is a product of peripheral deiodination of T3, exhibits important biological effects in the liver as well as in other tissues [10]. Evidences have been presented indicating that T2, previously considered only as a T3 catabolite, is able to mimic some of T3 effects on

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FASN, fatty acid synthase; FL, firefly luciferase; IRES, internal ribosome entry site; mTOR-C1, mammalian target of rapamycin complex 1; MAPK, mitogen activated protein kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; RL, *Renilla* luciferase; SRB, sulforhodamine B; SREBP, sterol regulatory element binding protein; TH, thyroid hormone; T2, 3,5-diiodo-L-thyronine; T3, 3,5,3′-triiodo-L-thyronine

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energy metabolism. T2 metabolic effects seem to be independent of protein synthesis [11–13]. Therefore, T2 action appeared to be more rapid than T3 effect [10,14]. It has been shown that T2 acts as a molecule capable of increasing the metabolic rate of hypothyroid rats [10]. T2 powerfully reduces adiposity and dyslipidemia, and, when administrated to high-fat fed rats prevents excessive body weight gain and the development of liver steatosis. This occurs without unfavourable side effects (i.e. thyrotoxicosis) usually observed when T3 or T4 is administrated [15].

As far as T2-triggered signal transduction is concerned, it has been reported that T2 activates the MAPK and PI3K/Akt pathways in chick embryo hepatocytes during development [16], and exerts non-genomic effects on intracellular calcium and nitric oxide signalling in a pituitary cell model [17].

Despite the great interest around this hormone, the literature data about T2 effects at cellular level are yet incomplete. Furthermore, considering the important role of SREBP-1 on the regulation of lipid metabolism, studies on a putative T2-dependent mechanism of SREBP-1 induction are lacking.

In this work, we provide the first evidence that T2 determines the block of the proteolytic cleavage of SREBP-1 through the activation of ERK, p38, Akt and PKC-δ in HepG2 cells. In turn, the inhibition of SREBP-1 due to T2 decreased the expression of its target fatty acid synthase (FASN).

It is worth to observe that whilst the PI3K/Akt pathway has a well known antiapoptotic potential [22,23], p38 and PKC-δ often act as positive regulators of apoptosis [18–21]. Another important proapoptotic stimulus is represented by the inhibition of FASN, an enzyme generally over expressed in cancer cells [24]. The inhibition of SREBP-1 also promotes cell death [26–28], whilst its upregulation in cancer promotes cell survival [25]. For these reasons, here we also investigated whether the inhibition of SREBP-1 and FASN provoked by T2 caused apoptosis in HepG2 cells. Comparison between the present data and the previously published results on T3 effects [9] has been routinely carried out. The differences between the mechanism of action of T2 and T3 have been highlighted.

2. Materials and methods

2.1. Reagents and antibodies

The MAPK/ERK kinase (MEK) inhibitor PD098059 (PD), the MAPK/p38 inhibitor SB203580, the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, the PKC inhibitor GF109203X, the mTOR-C1 inhibitor rapamycin, and tetrac were purchased from Sigma Chemical Co. (Milan, Italy). Total and phospho-specific ERK1 and ERK2, total and phospho-specific p38 MAPK, SREBP-1, PKCs, goat anti-mouse IgG conjugated with peroxidase, and β-actin antibodies were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Total and phospho-specific Akt and S6K-P70 Kinase antibodies, were purchased from Millipore Corporation (Milan, Italy). Goat anti-rabbit IgG conjugated with peroxidase was from Calbiochem (Darmstadt, Germany).

2.2. Cell culture and transient transfection assay

The human hepatic carcinoma cell line (HepG2) was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum (FBS), 100 μg/ml streptomycin, 60 μg/ml penicillin, and 4 mM glutamine. Cells were incubated at 37 °C under 5% CO_2 atmosphere. All experiments were performed with HepG2 cells kept in a serum-starved medium (0.5% FBS) for 24 h and then incubated with T2 and/or various inhibitors. In dose-dependent experiments HepG2 were treated with the indicated T2 concentrations (0.01–10 μM) for 2 h. For all other experiments 0.1 μM T2 was used.

For transient transfections, 3.5×10^4 cells were seeded into 12-well plates 48 h before transfection. Plasmid pRS1aF was constructed by inserting the cDNA of the SREBP-1a 5′-UTR into the intercistronic site of the pRF vector [9]. Cells (2×10^5) were plated on to 6-well cell culture plates. After 48 h, cells were co-transfected with the dicistronic pRS1aF construct (1.5 μg/well) and pcDNA3.1/HisB/lacZ plasmid (100 ng/well) used as control for transfection efficiency, by using FuGENE6 (Roche Diagnostics). After an 8-h transfection period, the medium was changed to fresh DMEM supplemented with 10% (v/v) FBS and cells were incubated for 24 h. Cells were incubated for a further 24 h in a serum-starved medium (0.5% FBS) and then treated with 0.1 μM T2 for 3 h. After cell lysis, RL (*Renilla* luciferase) and FL (firefly luciferase) activities were measured using the Dual Luciferase Reporter Assay System (Promega). The β-galactosidase activity was determined using a β-galactosidase assay.

2.3. Preparation of nuclear and cytosolic protein extracts

All procedures were carried out at 4 °C. To prevent proteolysis, a mixture of protease inhibitors from Sigma Chemical Co. (Milan, Italy) was included in all the buffers. HepG2 cells from 25 cm^2 flasks were pooled and centrifuged at 900 g for 5 min at 4 °C. The resulting cell pellet was resuspended in Buffer 1 (20 mM Tris–HCl [pH 8.0], 420 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, 0.2% [v/v] Nonidet P-40, 10% [v/v] glycerol). After 10 min incubation on ice, cells were passed several times through a 20 gauge syringe needle and then sonicated until no cells remained intact. The homogenate was centrifuged at 1100 g for 10 min, and the supernatant was collected as cytosolic fraction. The nuclear pellet was washed once in Buffer 1 and then resuspended in high-salt Buffer 2 (20 mM Tris–HCl [pH 7.9], 420 mM NaCl, 10 mM KCl, 0.1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 20% [v/v] glycerol). This suspension was rotated for 30 min and then centrifuged at 15,000 g for 30 min. The resulting supernatant is designated as the nuclear extract fraction. The purity of fraction was tested by immunoblotting with anti-histone-3/4 polyclonal antibody. Protein concentration was determined using the Bio-Rad protein assay kit (Milan, Italy). Lyophilized bovine serum albumin (BSA) was used as a standard.

2.4. Western-blot analysis

To obtain whole protein cell extracts for Western-blot analysis, cells were scraped in the following buffer: 20 mM Tris–HCl (pH 8.0), containing 420 mM NaCl, 2 mM EDTA, 2 mM Na_3VO_4, and 1% (v/v) Nonidet P-40, supplemented with a cocktail of protease inhibitors. Cells were then passed several times through a 20 gauge syringe and centrifuged at 16,000 g for 20 min at 4 °C. Proteins in homogenate and cellular fractions were determined using the Bio-Rad protein assay kit. Lyophilized bovine serum albumin (BSA) was used as a standard. Total cell proteins or proteins of the distinct subcellular fractions were dissolved in sodium dodecyl sulphate (SDS) sample buffer and separated on 10% (w/v) SDS gels. Separated proteins were transferred electrophoretically onto a nitrocellulose membrane (Pall, East Hills, NY, USA). Equal protein loading was confirmed by Ponceau S staining. The filter was blocked with 5% (w/v) non-fat dried milk in buffered saline. Blots were incubated with specific primary antibodies and the immune complexes were detected using appropriate peroxidase-conjugated secondary antibodies and enhanced chemiluminescent detection reagent (Amersham International). Densitometric analysis was carried out on the Western-blots using the NIH Image 1.62 software (National Institutes of Health, Bethesda, MD, USA), normalizing to β-actin used as a control.

2.5. SREBP-1 half-life analysis

HepG2 cells were plated at a density of 1×10^6 cells into 25 cm^2 flasks and incubated in DMEM supplemented with 10% (v/v) FBS for 48 h. Cells were incubated for a further 24 h in a serum-starved medium (0.5% FBS). After starvation cells were treated with 0.1 μM T2 for 3 h. Then, 100 μg/ml cycloheximide, an inhibitor of protein synthesis,