Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

TRβ is the critical thyroid hormone receptor isoform in T3-induced proliferation of hepatocytes and pancreatic acinar cells

Marta A. Kowalik[1], Andrea Perra[1], Monica Pibiri[1], Maria T. Cocco[1], Jacques Samarut[2], Michelina Plateroti[2], Giovanna M. Ledda-Columbano[1], Amedeo Columbano[1,*]

[1]*Department of Toxicology, Oncology and Molecular Pathology Unit, University of Cagliari, Italy;* [2]*Institut de Génomique Fonctionnelle de Lyon, Université Lyon 1, CNRS, INRA, Ecole Normale Supérieure de Lyon, France*

Background & Aims: Thyroid hormones elicit many cellular and metabolic effects in various organs. Most of these actions, including mitogenesis, are mediated by the thyroid hormone 3,5,3′-triiodo-L-thyronine (T3) nuclear receptors (TRs). They are transcription factors, expressed as different isoforms encoded by the TRα and TRβ genes. Here, experiments were performed to determine whether (i) T3-induces hepatocyte proliferation in mouse liver and pancreas, and, (ii) which TR isoform, is responsible for its mitogenic effect.

Methods: Cell proliferation was measured by bromodeoxyuridine (BrdU) incorporation after T3 or the TRβ agonist GC-1 in liver and pancreas of CD-1, C57BL, or TRα$^{0/0}$ mice. Cell cycle-associated proteins were measured by Western blot.

Results: T3 added to the diet at a concentration of 4 mg/kg caused a striking increase in BrdU incorporation in mouse hepatocytes. Increased BrdU incorporation was associated with enhanced protein levels of cyclin D1 and PCNA and decreased levels of p27. Treatment with GC-1, a selective agonist of the TRβ isoform, also induced a strong mitogenic response of mouse hepatocytes and pancreatic acinar cells which was similar to that elicited by T3. Finally, treatment with T3 of mice TRα$^{0/0}$ induced a proliferative response in the liver and pancreas, similar to that of their wild type counterpart.

Conclusions: These results demonstrate that T3 is a powerful inducer of cell proliferation in mouse liver and suggest that the β-isoform is responsible for the hepatomitogenic activity of T3. The same isoform seems to also mediate the proliferation of mouse pancreatic acinar cells.

Keywords: Thyroid hormone; Liver; Pancreas; Thyromimetics; GC-1.

Received 24 December 2009; received in revised form 29 March 2010; accepted 19 April 2010; available online 1 July 2010

*Corresponding author. Address: Dipartimento di Tossicologia, Sezione di Oncologia e Patologia Molecolare, Via Porcell 4, 09124 Cagliari, Italy. Tel.: +39 070 6758345; fax: +39 070 666062.

E-mail address: columbano@unica.it (A. Columbano).

Abbreviations: T3, triiodothyronine; T4, thyroxine; TRs, thyroid hormone receptors; TRα$^{0/0}$, thyroid hormone receptor alpha 0/0; PPAR, peroxisome proliferators-activated receptor; RAR, retinoic acid receptor; RXR, retinoid-X receptor; VDR, vitamin D receptor; CAR, constitutive androstane receptor; BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; wt, wild type.

Introduction

The thyroid hormones 3,5,3′-triiodo-L-thyronine (T3) and thyroxine (T4), influence a variety of physiological processes, including cell growth and metabolism in mammals, metamorphosis in amphibians, and development of the vertebrate nervous system [1–3]. Most of the effects of T3 are mediated by thyroid hormone nuclear receptors, the TRs, which act as transcription factors [4,5]. TRs are members of the steroid/thyroid receptor superfamily of nuclear hormone receptors, which includes the two retinoid acid receptors (RAR and RXR), the vitamin D receptor (VDR), the peroxisome proliferators-activated receptor (PPAR), the constitutive androstane receptor (CAR), and some orphan receptors [6]. Two different TR subtypes, TRα and TRβ, have been identified which are the products of distinct genes [7]. The TRα1 and the TRβ1 isoforms bind thyroid hormone with near-equal affinity, and are ubiquitously expressed, although TRα1 predominates in the heart (50–70% of TRs) while TRβ1 predominates in the liver (80% of TRs) [8]. Data collected from various TR knockout mice suggest that TRα1 mediates the effects of thyroid hormones on heart rate, whereas TRβ1 is important in mediating the cholesterol-lowering and TSH suppressant effects of T3 [7]. However, which isoform is involved in the mitogenic effect exerted by T3 in several organs [9–14] is unknown. Discriminating the different effects of thyroid hormones has both theoretical and practical importance. Indeed, thyroid hormones are widely used in therapy for the treatment of a wide range of thyroid diseases, and their administration is associated with serious side effects, particularly cardiac dysfunction, i.e. tachycardia, arrhythmias, and precipitation of ischemic episodes or heart failure [15]. The availability of isoform selective thyromimetics might significantly reduce the occurrence of side effects while retaining the desired actions, such as TSH inhibition or reduction of cholesterol synthesis. It might also expand the therapeutic indications of thyromimetics, to include conditions for which the therapeutic ratio is presently unacceptable, such as the treatment of obesity [16] and dyslipidemia [17,18]. In addition, since T3 was shown to induce regression of preneoplastic lesions after their initial proliferation, possibly inducing their reversion to a normal phenotype [19], it is important to know which isoform is critical in this process, if one wants to design new drugs to be used as anti-tumoral agents.

Recently, a new class of halogen-free thyroid hormone agonists, which are both highly selective for binding and activation

functions of TRβ1 over the TRα1 receptors was developed. The first molecule synthesized in this group, GC-1 [20], contains several structural changes with respect to the natural hormone T3, including replacement of the three iodines with methyl and isopropyl groups, replacement of the biaryl ether linkage with methylene linkage, and replacement of the amino-acid side chain with an oxyacetic-acid side chain.

Notably, animal studies [21] revealed that treatment with GC-1 induces a reduction of cholesterol levels similar to that obtained with equimolar doses of T3 and even higher than that achieved with the most common drugs currently available on the market for the treatment of hypercholesterolemia, such as the inhibitors of hydroxymethyl glutaryl coenzyme A reductase. In the same studies, GC-1 caused an even higher reduction of triglyceride levels than that produced by equimolar doses of T3 without any significant side effects on heart rate, muscle loss, or increase in the overall catabolic state [21]. Because of the "selective hyperthyroidism" generated by GC-1, this compound has the potential to be developed as a new therapeutic agent for the treatment of a variety of thyroid hormone-related metabolic disorders, like lipid disorders and obesity [22]. Interestingly, GC-1 was shown to induce proliferation in rat liver and pancreas, suggesting that in these two organs, TRβ is the critical isoform in mediating thyroid hormone-induced proliferation [23]. However, from those studies, the contribution of TRα in T3 or GC-1-induced hepatocyte proliferation cannot be completely excluded. Thus, the aim of the present study was to identify the receptor isoform responsible for T3-induced proliferation, taking advantage of the currently available TRs knockout mice [24].

Materials and methods

Animals

Female CD-1 and C57BL mice (6–8 weeks old) purchased from Charles River (Milano, Italy) were maintained on a standard laboratory diet (Ditta Mucedola, Settimo Milanese, Italy). The animals were given food and water *ad libitum* with a 12 h light/dark daily cycle and were acclimated for 1 week before the start of the experiment. Additional experiments were performed with TRα$^{0/0}$ mice, lacking all known products of thyroid hormone receptor alpha gene and created as described previously [24]. Guidelines for the Care and Use of Laboratory Animals were followed during the investigation. T3 (Sigma Chem. Co., St. Louis, MO), was added to a basal diet at a final concentration of 4 mg/kg and mice were sacrificed at 4 and 7 days after the beginning of the diet exposure. GC-1, synthesized in our laboratory as described by Chiellini et al. [20], was added to a basal diet at a final concentration of 5 mg/kg of diet, and the mice were sacrificed 7 days thereafter. Immediately after sacrifice, sections of the liver were fixed in 10% buffered formalin and processed for staining with hematoxylin–eosin or immunohistochemistry. The remaining liver was snap-frozen in liquid nitrogen and kept at −80 °C until use.

Western blot analysis

Total cell extracts were prepared from frozen livers as described previously [23]. For immunoblot analysis equal amounts (from 100 to 150 μg/lane) of proteins were electrophoresed on SDS–12% or –8% polyacrylamide gels. Polyacrylamide was purchased from Sigma Chemicals. After the gels were electrotransferred onto nitrocellulose membranes (Osmonics, Westborough, MA, USA), to ensure equivalent protein loading and transfer in all lanes, the membranes and the gels were stained with 0.5% (wt/vol) Ponceau S red (ICN Biomedicals, Irvine, CA) in 1% acetic acid for 5 min, and with Coomassie blue (ICN Biomedicals, Irvine, CA) in 10% acetic acid for 30 min, respectively. Before staining, gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid (Sigma Ch.). After blocking in TBS containing 0.05% Tween 20 (Sigma Ch.) and 5% non-fat dry milk, membranes were washed in TBS-T and then incubated with appropriate primary antibodies diluted in blocking buffer. Whenever possible, the same membrane was used for detection of the expression of different proteins. Immunoreactive bands were identified with a chemiluminescence detection system, as described by the manufacturer (Supersignal Substrate, Pierce).

Antibodies

For immunoblotting experiments, we used mouse monoclonal antibodies directed against cyclin D1 (72-13 G), PCNA (PC-10) (Santa Cruz Biotechnology, CA), actin (Sigma Ch.), and p27^{kip} (Pharmingen, Erembodeyem, Belgium). Anti-mouse horseradish peroxidase conjugated IgG was from Santa Cruz Biotechnology.

Immunohistochemistry

Mice, fed a T3 or GC-1-supplemented diet, received BrdU, dissolved in drinking water (1 mg/ml), throughout the experimental period. Mouse monoclonal anti-BrdU antibody was obtained from Becton Dickinson (Becton Dickinson, San Jose, CA) and the peroxidase method was used to stain BrdU-positive hepatocytes. Peroxidase goat anti-mouse immunoglobulin was obtained from Dako (Dako EnVision^{+}™ Peroxidase Mouse, Dako Corporation, Carpinteria, CA). Four micron thick sections were deparaffinized, treated with HCl 2 N for 20 min, then with 0.1% trypsin type II (crude from porcine pancreas, Sigma Ch.) for 20 min and treated sequentially with normal goat serum 1:10 (Dako), mouse anti-BrdU 1:200 for 1 h and 30′ and Dako EnVision^{+}™ Peroxidase Mouse ready-to-use. The sites of peroxidase binding were detected by 3,3′-diaminobenzidine. Labelling index (LI) was expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Mitotic activity was determined as the number of mitoses/1000 hepatocytes. Results are expressed as means ± SE of 4–5 mice per group. At least 5000 hepatocyte nuclei per liver were scored. LI of pancreatic acinar cells was expressed as number of BrdU-positive nuclei/100 nuclei. Results are expressed as means ± SE of 4–5 animals per group. At least 2000 acinar cell nuclei per pancreas were scored.

Statistical analysis

Comparison between treated and control group was performed by Student's *t* test.

Results

While T3 is a well known inducer of hepatocyte proliferation in rats [9–11], no evidence has been provided about its mitogenic effect on mouse liver. Initial experiments aimed at determining the effect of a single dose of T3 indicated that, unlike rat liver, only a negligible hepatocyte proliferation (LI was less than 2%) could be obtained in mice treated with a single dose of 100, 200, or 400 mg/kg of T3 (data not shown). Notably, these results are similar to what was observed with ligands of another nuclear receptor, namely PPARα, which are, at a single dose, strongly mitogenic for rat but not for mouse hepatocytes [25].

Next, we aimed to determine the effect of continuous administration of T3 in the diet of mice. Previous studies have shown that a dose of 4 mg/kg is the strongest mitogenic dose in rat liver [26]; therefore, experiments were performed wherein CD-1 mice were fed a basal diet supplemented with 2, 4, or 8 mg/kg of T3 for 7 days. To determine the LI, T3-fed mice and controls were given BrdU in drinking water (1 mg/ml), throughout the experimental period. The results (Fig. 1) showed that a final concentration of 4 mg/kg induced the highest proliferative response. (LI was 10.8 ± 3.3% vs. 5.6 ± 2.5% and 5.8 ± 2.3% of 2 and 8 mg/kg, respectively. LI of controls was 1.5 ± 1.3%.) Histological examination of the livers from mice administered T3 did not reveal signs of necrosis nor any significant increase in the number of apoptotic bodies compared to controls (not shown).

Based on these results a final concentration of 4 mg/kg of T3 was chosen for future experiments. To determine whether the mitogenic effect induced by T3 could be exerted also in other