Steroids 77 (2012) 589–595

Contents lists available at SciVerse ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

3,5,3′-Triiodothyronine (T_3) stimulates cell proliferation through the activation of the PI3K/Akt pathway and reactive oxygen species (ROS) production in chick embryo hepatocytes

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ARTICLE INFO

Article history:
Received 18 November 2011
Received in revised form 30 January 2012
Accepted 31 January 2012
Available online 11 February 2012

Keywords:
3,5,3′-Triiodothyronine (T_3)
Nongenomic effects
Integrin αvβ3
PI3K/Akt pathway
Reactive oxygen species (ROS)
Chick embryo hepatocytes

ABSTRACT

Thyroid hormones (THs) have a wide variety of essential roles in vertebrates, ranging from the regulation of key metabolic processes to cell proliferation and apoptosis. The classical mechanism of action of THs is genomic; 3,5,3′-triiodothyronine (T_3) binds to specific nuclear receptors (TRs) and modifies the expression of specific genes.

Recently, a new category of mechanisms, termed nongenomic, has been discovered for T_3. These mechanisms include, among others, the rapid activation of signal transduction pathways, such as PI3K/Akt and MAPK, which eventually lead to cell proliferation. These effects are mediated in some cell types by a plasma membrane receptor, identified as integrin αvβ3, and in other cell types by cytoplasmic TRβ1.

The aim of this work was to analyze the effect of T_3 on the cell growth of chick embryo hepatocytes at two different stages of development, 14 and 19 days, and to determine the activation of the signal transduction pathways, focusing on the potential involvement of a plasma membrane receptor and the possible participation of PI3K/Akt and reactive oxygen species (ROS).

Our results clearly show that T_3 stimulates cell proliferation at both stages of development through the activation of the PI3K/Akt pathway and the production of small amounts of ROS, which operate as effective second messengers. Moreover, we prove that these effects are not initiated at the plasma membrane receptor for T_3.

1. Introduction

Thyroid hormones (THs) perform fundamental roles in the correct growth, development and metabolism of almost all cell types in vertebrates [1].

Until a few decades ago, most scientists believed that the principal mechanism of action of THs was through its interaction with nuclear thyroid hormone receptors (TRs) for 3,5,3′-triiodothyronine (T_3) (genomic actions). The actions of thyroid hormones that are independent of the nuclear ligand binding of hormone receptors are called nongenomic [2]. The possible existence of thyroid hormone receptors in the plasma membrane was noted several decades ago [3] and has now been clearly demonstrated in integrin αvβ3 [4,5]. A large number of short-term or nongenomic actions involving the rapid activation of protein kinase-dependent signaling pathways are being discovered for T_3 in almost all cell types. The activation of important plasma membrane proteins, such as Ca^{2+}-ATPase in human erythrocytes [6] and K^+ channels in guinea pig ventricular myocytes [7], by T_3 is well documented, and recently, the regulation of these channels in cardiomyocytes has been proposed, which involves PKA, PKC, PI3K and MAPK [8]. The Na^+/H^+ antiporter is also stimulated in rat myoblasts and chick embryo hepatocytes through the involvement of PKCα and ERK 1/2 [9,10]. The activation of Na^+/K^+-ATPase in rat alveolar epithelial cells through a PI3K-mediated mechanism has been reported [11]; however, PKA- and PKCα-mediated inhibition in chick embryo hepatocytes has been described [12]. Moreover, in chick embryo hepatocytes, T_3 increases the intracellular levels of DAG, IP_3 and Ca^{2+} through a PLC-mediated mechanism, leading to the activation of PKCα [13].

The effects on cell proliferation are remarkable, as shown in CAM (chick chorioallantoic membrane angiogenesis model) and human osteoblast-like cells, in which T_3 increases cell growth through a MAPK-dependent mechanism initiated at integrin

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0039-128X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.steroids.2012.01.022

αvβ3 [14–16]. In rat hepatocytes, T_3 induces proliferation through enhancing the synthesis of cyclin D1 [17]. In addition, during hepatic regeneration, T_3 regulates the expression of cyclins A, D1, and E, their inhibitors (p16, p21, and p27), p53 and p73 and stimulates ERK 1/2 and p38 MAPK activation [18]. In chick embryo hepatocytes, a role in enhancing cell growth has been found for T_3, and in this system, the process is characterized by the increased expression of cyclin D1 and mediated through the activation of ERK 1/2 and PKCα [19].

In several cell types, including hepatocytes, a novel mechanism for the T_3-mediated induction of proliferation has recently been reported, which starts with the direct interaction of the hormone with cytoplasmic TRβ1 followed by the activation of PI3K through binding to the p85 regulatory subunit in the cytoplasm. This binding increases the kinase activity of the enzyme and also leads to Akt activation [20–23].

In this context, therefore, this work was aimed at analyzing the association of signaling pathways induced by T_3 with the cellular proliferation of chick embryo hepatocytes at two different developmental stages (14 and 19 days). More specifically, we evaluated the possible involvement of integrin αvβ3 in the induction of cell growth with the inhibitory tripeptide Arg-Gly-Asp (RGD) and the deaminated derivative tetraiodothyroacetic acid (Tetrac), both of which block the binding site on integrin αvβ3 [15,24]. We also used non-permeant T_3-agarose (T_3-Ag) to determine the presence of another plasma membrane receptor different from integrin αvβ3. We also studied the contribution of reactive oxygen species (ROS) to the signaling pathways activated by T_3. At the cellular level, oxidative stress leads to a wide spectrum of responses depending on the cell type, the level of ROS and the length of exposure. Furthermore, recent studies have noted the importance of these molecules in transducing a wide variety of signals in many cell types [25–28]. Interestingly, a direct role has been reported for NADPH oxidase (NOX)-derived ROS as promoters of cell proliferation because they can activate some key kinases of the MAPK system, such as p38 and ERK 1/2 [24,25,28]. In this regard, it is also important to emphasize that a subunit of NOX2, $p47^{phox}$, binds to 3′-phosphorylated phosphatidylinositols, the end products of PI3K, and that this subunit is highly expressed in hepatocytes; the functional meaning of this interaction is still unknown, but it may have a role in enzyme activation [24]. Therefore, we also examined the effect of T_3 on the activation of the PI3K/Akt pathway and ROS production and determined whether ROS generation itself may be NADPH oxidase- and PI3K/Akt-dependent.

2. Experimental

2.1. Materials

Collagenase (type D), 3,5,3′-triiodo-L-thyronine (T_3), wortmannin, LY-294,002, tetraiodothyroacetic acid (Tetrac), the tripeptide Arg-Gly-Asp (RGD), diphenyleneiodonium chloride (DPI) and 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc) were supplied by Sigma Aldrich (St. Louis, MO). All of the other chemicals were supplied by Merck (Darmstadt, Germany). Primary and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA): anti-phospho-ERK 1/2 (Cat. No. 4695), anti-p85 (Cat. No. 4257), anti-phospho-p85 (Cat. No. 4228), anti-Akt (Cat. No. 4685), anti-phospho-Akt (Cat. No. 4058), and anti-rabbit (Cat. No. 7054).

2.2. Hepatocyte preparation and culture

Chick embryo hepatocytes were isolated using a previously described non-perfusive method [29]. Briefly, livers from White Leghorn (*Gallus gallus*) chick embryos (14 and 19 days of embryonic age) were rapidly removed, pooled, cut into small pieces, washed in ice-cold Hanks' buffer, and incubated three times for 10 min at 37 °C with 0.3 mg/mL collagenase in Hanks' buffer (7 mL/g liver). During the incubation, the buffer was continuously gassed under an atmosphere of 5% CO_2. At the end of each incubation, the media were aspirated, pooled and centrifuged twice for 5 min at 80*g* at 4 °C. The isolated hepatocytes (4.0×10^6/60 mm collagen-coated dish) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 200 mM L-glutamine, 5% (v/v) fetal calf serum, 2.5 ng/mL amphotericin, 0.1 mg/mL gentamicin, and 0.25 μM dexamethasone in a humidified atmosphere of 5% CO_2 at 37 °C and used after 48 h. For ROS determination experiments, the cells were used in suspension.

2.3. The preparation of T_3-agarose

T_3-agarose was prepared as previously reported [9]. Activated CH Sepharose 4B (Sigma) was washed in HCl at low speed to discard contaminants and impurities. T_3 was then added and mixed gently with Sepharose at a 2:1 ratio at room temperature for 2 h. Next, the suspension was washed three times at 500*g* for 5 min with a solution of 0.1 M Tris·HCl and 0.5 M NaCl, pH 8, and three times at 500*g* for 5 min with a solution of 0.1 M sodium acetate and 0.5 M NaCl, pH 4. T_3-agarose prepared following this protocol had a final concentration of ∼10 μmol/mL and was stored at 4–8 °C for a couple of months. The contamination with free T_3 was less than 0.070 pmol/mL, as assessed with a radioimmunoassay.

2.4. In vitro DNA synthesis

DNA synthesis was examined by measuring the incorporation of methyl-^{3}H-thymidine into whole-cell DNA 24 h after stimuli. The cells were pulse-labeled for 2 h with 1 μCi/mL of methyl-^{3}H-thymidine in medium containing 5% FCS as previously described [30].

2.5. Gel electrophoresis and Western blot

To prepare cell lysates, the cells were washed twice with ice-cold PBS, collected and centrifuged for 8 min at 900*g* at 4 °C. The supernatant was discarded, and the pellet was resuspended and sonicated in sample buffer solution (10% SDS, 0.125 M Tris·HCl, pH 6.8, 1% phenylmethylsulfonylfluoride (PMSF), 1% sodium orthovanadate ($NaVO_3$), and 1% leupeptin), and the protein concentration was measured with Lowry's procedure [31]. A total of 100 μg of sample proteins was subjected to 7.5% SDS–PAGE and transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA, USA). The membranes were incubated in Tris-buffered saline (TBS) containing 5% non-fat dried milk powder and 0.1% Tween20 for 60 min at room temperature and then probed with a specific primary antibody overnight. The antibody reaction was revealed after incubation for 3 h with an alkaline phosphatase-conjugated secondary antibody, and the immunoelectrophoretic profile was visualized using the NBT/BCIP reagent.

The densitometry determinations were performed using ImageJ software, which is available online at www.nih.gov/ij/download.html, and the values were normalized to GAPDH.

2.6. ROS determination

The cell suspension was centrifuged at 100*g* for 5 min at 24 °C. The supernatant was discarded, and the pellet was resuspended in 5 mL PBS with 5.0 mM glucose at 37 °C (PBSG). The cells were then incubated with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) from a stock solution (10 mM in DMSO) to a final concentration of 10 μM for 30 min in the dark at 37 °C,