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3,5,3'-Triiodo-L-Thyronine Regulates Actin Cytoskeleton Dynamic in The Differentiated PC-12 Cells during Hypoxia through An αvβ3 Integrin

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Abstract

Objective: Thyroid hormones are involved in the pathogenesis of various neurological disorders. Ischemia/hypoxia that induces rigidity of the actin filaments, which initiates neurodegeneration and reduces synaptic plasticity. We hypothesized that thyroid hormones via alpha-v-beta-3 (αvβ3) integrin could regulate the actin filament rearrangement during hypoxia and increase neuronal cell viability.

Materials and Methods: In this experimental study, we analysed the dynamics of actin cytoskeleton according to the G/F actin ratio, cofilin-1/p-cofilin-1 ratio, and p-Fyn/Fyn ratio in differentiated PC-12 cells with/without T3 hormone (3,5,3'-triiodo-L-thyronine) treatment and blocking αvβ3-integrin-antibody under hypoxic conditions using electrophoresis and western blotting methods. We assessed NADPH oxidase activity under the hypoxic condition by the luminometric method and Rac1 activity using the ELISA-based (G-LISA) activation assay kit.

Results: The T3 hormone induces the αvβ3 integrin-dependent dephosphorylation of the Fyn kinase (P=0.0010), modulates the G/F actin ratio (P=0.0010) and activates the Rac1/NADPH oxidase/cofilin-1 (P=0.0069, P=0.0010, P=0.0045) pathway. T3 increases PC-12 cell viability (P=0.0050) during hypoxia via αvβ3 integrin-dependent downstream regulation systems.

Conclusion: The T3 thyroid hormone may modulate the G/F actin ratio via the Rac1 GTPase/NADPH oxidase/cofilin1signaling pathway and αvβ3-integrin-dependent suppression of Fyn kinase phosphorylation.

Keywords: Actin Filament, Hypoxia, Integrin, PC-12, Thyroid Hormone

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Introduction

The thyroid hormones, including 3,5,3´-triiodo-L-thyronine (T3) and L-thyroxine (T4), play a significant role as a regulator of cell growth, development, and metabolism Thyroid hormones involve in the pathogenesis of various neurological diseases. There is evidence linking alterations in thyroid hormone levels to the pathogenesis of Alzheimer disease (1). Also, Thyroid dysfunction was observed in children with autism spectrum disorder (2). Low T3 plasma level after an acute ischemic stroke is associated with a greater stroke severity and worse functional outcomes (3, 4). However, the relationship between thyroid hormones, functional post-stroke outcomes, and recovery time is complex and requires further investigation.

Thyroid hormones primarily target the nuclear receptor; however, recent investigations have suggested a non-nuclear, non-genomic action (5, 6). The non-genomic effects of thyroid hormones occur partly due to the activation of the cell surface-exposed integrin alpha-v-beta-3 (αvβ3) receptor (5). Thyroid hormones binding to the αvβ3 receptor can enhance the adhesion to extracellular matrix substrates (7) and activate the protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) signalling pathways (8, 9). The αvβ3 receptor seems to contain a T3-specific binding site and a region at which both T4 and T3 bind in the hormone-binding domain (6). Binding to the T3-specific site activates the phosphoinositide 3-kinase (PI3K) pathway. In contrast, the T4 does not activate the PI3K pathway, but acts on the ERK-dependent signalling (9). In the differentiation state, PC-12 cells are predominantly regulated by the T3 via the P13K/Akt pathway (8, 9), suggesting that T3 and T4 act differently on the intracellular regulatory systems via the αvβ3 integrin.

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Generally, a cytoskeleton is responsible for various functions in all cell types, such as the organization of subcellular organelles and regulating cell motility and dynamics (10). Dysregulation of the cytoskeletal organization contributes to many pathological disorders, including hypoxia-related diseases (11, 12). Therefore, the cytoskeleton dynamic nature helps its components rapid rearrangement in response to extracellular signals (10). The Actin is the central component of the cytoskeleton that contributes to most cell functions and a tissue organization (13). Thyroid hormones are involved in an actin polymerization in the central nervous system (14). Several actin-binding proteins control an actin polymerization and its dynamic. They maintain a monomeric G-actin pool, promote a F-actin polymerization, restrict an actin filament length, and regulate a filament assembly and disassembly (13). There is an evidence that an actin polymerization is mainly modulated by the rT3 and T4, but not by the T3 in astrocytes, which directly regulates the F-actin elongation in the neurites of neurons (15). However, there are not enough data about the integrin-mediated action of thyroid hormones on the actin polymerization during hypoxia.

The thyroid hormone-αvβ3 integrin interaction results in a small guanosine triphosphatase (GTPase) signalling (16), which controls the cytoskeleton. A Rho family GTPases (Rho, Rac) acts on the actin cytoskeleton by modulating the actin-binding protein activity (17). The reciprocal effects of Rho and Rac (Rac activation and Rho inhibition) on a dendritic motility can be involved in a dendritic dynamic (18). The Rho family members play a critical role in the mediating ischemia/reperfusion injury-induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, reactive oxygen species (ROS) generation, and oxidative stress in the brain, which greatly contributes to neuronal degeneration and cognitive dysfunction after cerebral ischemia (19).

Actin-depolymerizing factor/cofilin can severe filaments increasing their depolymerization and thus regulating the dynamics of actin filaments (20). Cofilins play an important role in normal development, and dysregulation of their functions causes severe complications of tissue homeostasis and health (21, 22). They can be potentially involved in the progression of neurodegenerative disorders forming cofilin-actin rods that affect normal neuronal function (23). Cofilin phospho-regulation is crucial for a wide variety of cellular processes, such as neuronal development, and synaptic plasticity. Through it a variety of extracellular stimuli regulate actin cytoskeletal reorganization (20).

Recent data highlighted the importance of cytoskeletal dynamic in hypoxia (17); however, further research is needed to determine the role of thyroid hormones in the recovery of cellular pathological changes caused by hypoxia-ischemic processes. Despite of the growing interest in the actin cytoskeleton and hypoxia interactions, the molecular mechanisms of these interactions are not completely understood. We hypothesized that thyroid hormones via the αvβ3 integrin signalling and actin dynamic could affect the hypoxia-induced cell damage and increase the cell survival during hypoxia.

Materials and Methods

In this experimental study, differentiated PC-12 cells were used for investigation the role of T3 thyroid hormone in cell survival during hypoxia. This study was approved by the LEPL Ilia State University Committee of Establishing Ethical Norms Adherence in Research Projects (R/135-22).

Cell line culturing

In this experimental study, PC-12 pheochromocytoma cells (CRL-1721™, ATCC, USA) were used. PC-12 cells display a chromaffin cell-like morphology, but undergo rapid changes following treatment with the nerve growth factor (NGF), including neurite outgrowth. These cells are widely employed as model systems to examine the signal transduction process in neurons, predominantly of the dopaminergic origin. PC-12 cells were cultured in the T25 flasks (690 170, Greiner Bio-One GmbH, Austria) in a humidified atmosphere containing 5% CO_2 at 37°C in a high-glucose Dulbecco's modified Eagle's medium (DMEM, 30-2002™, ATCC, USA) supplemented with 10% heat-inactivated horse serum (HS, H1138, Sigma-Aldrich, USA), 5% fetal bovine serum (FBS, F2442, Sigma-Aldrich, USA), and 100 U/mL penicillin/streptomycin (15140148, Gibco™, USA) as well as 50 µg/mL gentamicin sulphate (15750060, Gibco™, USA). To induce differentiation, PC-12 cells were incubated in a low serum-containing DMEM 1% HS and 1% FBS (H1138 and F2442, Sigma-Aldrich, USA) supplemented with 100 ng/mL NGF (N-245 Alomone Labs, Israel) in T25 flasks for five days. The NGF-containing medium was replaced every two days with a fresh complete DMEM medium. Cells were considered differentiated if the length of one or more neurites exceeded the diameter of the cell body. Cell viability was counted using Trypan blue dye assay (145-0013, Bio-Rad, USA) and an Automated Cell Counter (TC20TM, Bio-Rad, USA) (24). Differentiated PC-12 cells (5×10^6 cells per sample) were treated with T3 and αvβ3 integrin blocking antibody 1 µg/mL (23C6; sc-7312, Santa Cruz, USA), and incubated for 1 hour under hypoxic condition. The 10 nM T3 (T6397, Sigma-Aldrich, USA) concentration was used in this experiment. The αvβ3 blocking antibody 1 µg/mL (23C6; sc-7312, Santa Cruz, USA) was used as an αvβ3 integrin inhibitor (25) to evaluate the involvement of the αvβ3 integrin in thyroid hormone-induced effects. Hypoxia conditions (0-1% oxygen) were maintained using nitrogen gas in a BioSpherix C-Chamber placed in a CO_2 incubator and controlled by a controller (ProOx Model P110, BioSpherix, USA). Hypoxia was maintained for an hour. A differentiated PC-12 cells (5×10^6 cells per sample) without any treatment during one-hour hypoxia was used as a control (control, H).

Preparation of cell fractions from the PC-12 cell line for electrophoresis and western blotting

After one hour of exposition to the hypoxic condition (0-1% oxygen), PC-12 cells (5×10^6 cells per sample) were detached from the cell culture flasks using 0.025% trypsin/EDTA (59418C, Sigma-Aldrich, USA) containing phosphate-buffered saline (PBS, 137 mM NaCl (S9888, Sigma-Aldrich, USA), 2.7 mM KCl (P3911, Sigma-Aldrich, USA), 10 mM Na_2HPO_4 (S9763, Sigma-Aldrich, USA), and 1.8 mM KH_2PO_4 (P8709, Sigma-Aldrich, USA pH=7.4) (incubation with trypsin containing buffer for 1 minute at 37°C) and scraped using scrapers (CSL2518, Bioland Scientific LLC, USA). After the detachment and scraping of the PC-12 cells from the flasks, trypsin inactivation was performed using resuspension of detached PC-12 cells in one volume of aprotinin-containing PBS (1 mkg/mL) (78432, Thermo Scientific™, USA) and pelleted by centrifugation at 300 × g. After centrifugation, the pelleted PC-12 cells were washed twice with 5 ml PBS buffer. Washed PC-12 cells (as pellet) were lysed using a lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES, H3375, Sigma-Aldrich, USA), pH=7.4, 10 mM KCl (P3911, Sigma-Aldrich, USA), 10 mM $MgCl_2$ (M8266, Sigma-Aldrich, USA), 1 mM ethylenediaminetetraacetic acid (EDTA, E9884, Sigma-Aldrich, USA), 1 mM ethylene glycol-bis (β-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA, 324628, Millipore, USA), 250 mM sucrose (35580, Serva, Germany), 1 mM dithiothreitol (DTT, 43819, Sigma-Aldrich, USA), and protease inhibitor cocktail (P8340, Sigma-Aldrich, USA)), and passed through a 25 Ga needle ten times using a 1 mL syringe. Lysed cells were centrifuged at 720 × g for 5 minutes, nuclei and intact cells were removed, and the supernatant was used for electrophoresis and western blotting analysis.

F-actin and G-actin fractionation

Fractionation was performed according to the method described by Bhambhvani et al. (26), with some modifications. Incubated PC-12 cells were homogenized in the F-actin stabilization buffer 0.1 M 1,4-piperazinediethanesulfonic acid, pH=6.9 (PIPES, sc-216099, ChemCruz, USA), 30% glycerol (G7893, Sigma-Aldrich, USA), 5% dimethyl sulfoxide (DMSO, D8418, Sigma-Aldrich, USA), 1 mM $MgSO_4$ (39773.01, Serva, Germany), 1 mM EGTA (324628, Millipore, USA), 1% Triton X-100 (X100, Sigma-Aldrich, USA), 1 mM adenosine triphosphate (ATP, A1852, Sigma-Aldrich, USA), and protease inhibitor (P8340, Sigma-Aldrich, USA). The protein concentration of the homogenates was measured using the Pierce Micro BCA™ Protein Assay Kit (23235, Thermo Fisher Scientific, USA), incubated at 37°C for 10 minutes and then, centrifuged at 720 × g for 5 minutes at room temperature. The supernatant was centrifuged at 134,000 × g at 37°C for one hour in an ultracentrifuge (CS150NX, Himac, Japan) to separate G-actin (in the supernatant) from F-actin (in pellet) fractions. Actin proteins were detected in the both fractions by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using an anti-β actin antibody (sc-47778, Santa Cruz, USA).

Western blotting

For the analysis of cofilin1, p-cofilin1 (phosphorylated cofilin), Fyn, and p-Fyn (phosphorylated Fyn) proteins in the supernatant of cell lysates (see "Preparation of cell fractions") and β-actin in the F (pellet) and G (supernatant) fractions (see in the F-actin and G-actin fractionation section), appropriate samples (the same amount of protein) were boiled at 90°C with Laemmli 2× Concentrate Sample Buffer containing 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH approx. 6.8. (S3401, Sigma-Aldrich, USA) for 5 minutes, cooled at room temperature, and centrifuged at 16,000 × g 3 minutes using centrifuge (Sigma 3-16 PK, Germany), followed by separation via sodium dodecyl SDS-PAGE on 7.5-15% gels and transfer to nitrocellulose membranes (88018, Thermo Scientific, USA) (26). After blocking with 5% bovine serum albumin (8076.1, Roth, Germany) dissolved in TBST [Tris-HCl-buffer: 250 mM Tris-HCl (648317, Millipore, USA), 27 mM KCl (P3911, Sigma-Aldrich, USA), 1.37M NaCl (S9888, Sigma-Aldrich, USA), pH=7.4] with 0.05% Tween 20 (P1379, Sigma-Aldrich, USA), then nitrocellulose membranes were incubated with the appropriate primary antibodies dissolved in the blocking solution: anti-cofilin1 at ratio 1:500 (sc-53934, Santa Cruz Biotechnology, USA), anti-p-cofilin1at ratio 1:500 (sc-271921, Santa Cruz Biotechnology, USA), anti-Fyn at ratio 1:500 (sc-434, Santa Cruz Biotechnology,USA), anti-p-Fyn at ratio 1:500 (sc-377555, Santa Cruz Biotechnology, USA). This step was done according to the manufacturing protocol. Subsequently, the membranes were incubated with secondary antibodies at ratio 1:2000 (ab112458, Abcam, USA). Immunolabeled bands were visualized using enhanced chemiluminescence (sc-2048, Santa Cruz Biotechnology, USA) and analysed by the Image J (1.53k, National Institute of Health, USA). After the chemiluminescence visualisation the p-cofilin1, nitrocellulose membrane was stripped using stripping buffer [1 L Stripping Buffer: 15 g glycine (3187.2, Roth, Germany), 1 g sodium dodecyl sulphate (SDS, 11667289001, Sigma-Aldrich, USA), 10 mL Tween 20 (P1379, Sigma-Aldrich, USA), pH=2.2] and re-probed for cofilin1 analyzing. After the chemiluminescence visualisation the p-Fyn, nitrocellulose membrane was stripped using stripping buffer (1 L Stripping Buffer: 15 g glycine, 1 g SDS, 10 mL Tween 20, pH=2.2) and re-probed for Fyn protein detection using the stripping method. Briefly, after the first chemiluminescence visualisation, the membranes were incubated with fresh stripping buffer using a volume that will cover the membrane, incubated at room temperature for 10 minutes, buffer was discarded and incubation with fresh

stripping buffer was repeated. After this step, buffer was discarded and membrane was washed for 10 minutes in PBS. Washing procedure in PBS was repeated. Finally, membrane was washed twice in TBST for 5-5 minutes. All procedures were performed at room temperature. The stripped and washed membrane was blocked in 5% bovine serum albumin TBST solution for the next western blotting.

NADPH oxidase assay

NADPH oxidase was analysed using lucigenin, according to the method described by Zhang et al. (27), with modifications by Wang and Lou (22). Differentiated PC-12 cells (1×10^6) were washed with PBS and disrupted by sonication in ice-cold Krebs buffer [130 mM NaCl (S9888, Sigma-Aldrich, USA), 5 mM KCl (P3911, Sigma-Aldrich, USA), 2 mM $MgCl_2$ (M8266, Sigma-Aldrich, USA), 1.5 mM $CaCl_2$ (C1016, Sigma-Aldrich, USA), 5 mM glucose (G8270, Sigma-Aldrich, USA), 35 mM phosphoric acid (04102, Sigma-Aldrich, USA), and 20 mM HEPES (H3375, Sigma-Aldrich, USA), pH=7.4]. After centrifugation at 1000 x g, the pellet was resuspended with Krebs buffer, contained 0.5 mM lucigenin (sc-202698, ChemCruz, USA). NADPH oxidase produces free radicals in the result of addition of NADPH (0.1 mM), as the substrate, and in the presence of lucigenin induces luminescence, which intensity was measured using microplate fluorescence reader (Twinkle LB979, Berthold, USA) with bottom optics in the clear-bottomed plates. Luminescence readings increased linearly within 5 minutes and were expressed as Relative Light Units (RLU/minute).

Rac1, activation assay

Rac1 activation assay was performed using the Rac1,2,3 G-LISA Activation Assay Kit (BK125, Cytoskeleton, USA), according to the manufacturer's protocol. The cytosolic fraction of PC-12 cells was used. The results were expressed as optical density (OD) per mg of total protein.

Cytotoxicity assay

Cytotoxicity was analysed by monitoring the release of lactate dehydrogenase (LDH) into the culture medium from hypoxia-exposed damaged PC-12 cells. LDH was assayed using the LDH assay kit (88954, Pierce™, USA). Released LDH from damaged PC-12 cells was analysed using a microplate spectrophotometer reader (ELx808, BioTek, USA) with an optical density at a wavelength of 490 nm and 680 nm.

Statistical analysis

Statistical analysis was performed by SPSS software (SPSS v. 25, IBM, USA). One-Way analysis of variance and Tukey's Supplementary Test statistical method were used to evaluate the significance of the experimental data. A value of $P<0.05$ was considered as statistically.

Results

To study the non-genomic αvβ3 integrin-mediated effects of T3 thyroid hormone during hypoxia, we treated differentiated PC-12 cells with T3 and αvβ3-integrin inhibitor (αvβ3 blocking antibody), and exposed to hypoxic condition for one hour. We found, that T3 thyroid hormone increased the viability of PC-12 cells (P=0.0050) during hypoxia. This pro-survival effect of T3 hormone was abolished by the αvβ3 antibody (P=0.0153), which underlined the integrin-mediated regulation of cell viability during hypoxia (Fig.1).

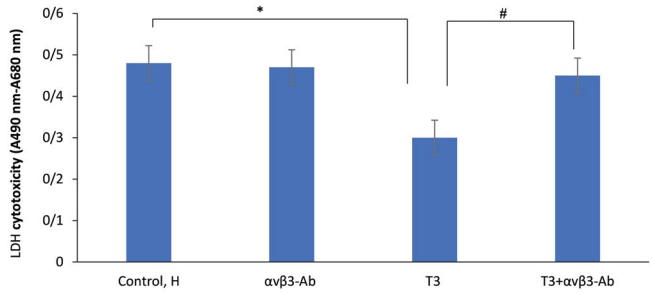

Fig.1: Analysis of the cytotoxicity in differentiated PC-12 cells under hypoxia with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 µg/ml) during 1 hour hypoxia by the LDH test as described in "Materials and Methods. Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and asterisk and hash indicate a statistically significant difference. LDH; Lactate dehydrogenase, *; P=0.0050 vs. control and #; P=0.0153 vs. T3 hormone treatment.

Next, we analysed the actin cytoskeletal contents, monomer-G actin and filament polymer-F actin, and determined the G/F actin ratio. Our results showed that treatment of cells with T3 thyroid hormone increased the G/F actin ratio (P=0.0010) during hypoxia. The impact of T3 hormone was abolished by the addition of an anti-αvβ3 integrin antibody (P=0.0020, Fig.2A, 2B).

Furthermore, we have found that T3 hormone treatment increased the cofilin1/p-cofilin1 ratio (P=0.0045) during hypoxia by decreasing phosphorylation of cofilin1 (reduced p-cofilin content), which was abolished by the αvβ3-integrin blocking antibody (P=0.0058, Fig.3A, B). To evaluate the integrin downstream regulator of the cofilin1/p-cofilin1 ratio, we further analysed the effect of T3 hormone on the phosphorylation of the Fyn kinase (a member of the Src-family of kinases) in the differentiated PC-12 cells during hypoxia. In the hypoxic cells, we found that T3 hormone reduced the content of the phosphorylated Fyn kinase and accordingly decreased the p-Fyn/Fyn ratio (P=0.0010, Fig.4A, B). This ratio is restored to the control level by an αvβ3-integrin blocking antibody (P=0.0138).

In addition, we analysed the Rac activity and found that in the hypoxic cells, addition the T3 hormone increased the Rac activity (P=0.0069), and this effect was eliminated after the treatment the cells with αvβ3-integrin blocking antibody (P=0.0078, Fig.5A). As one of the most important targets of Rac, we assessed the NADPH oxidase activity and found that the T3 hormone adding to the hypoxic cells induces an elevation of NADPH oxidase activity (P=0.0010). This effect was abolished after treating the cells with anti-αvβ3 integrin (P=0.0014, Fig.5B).

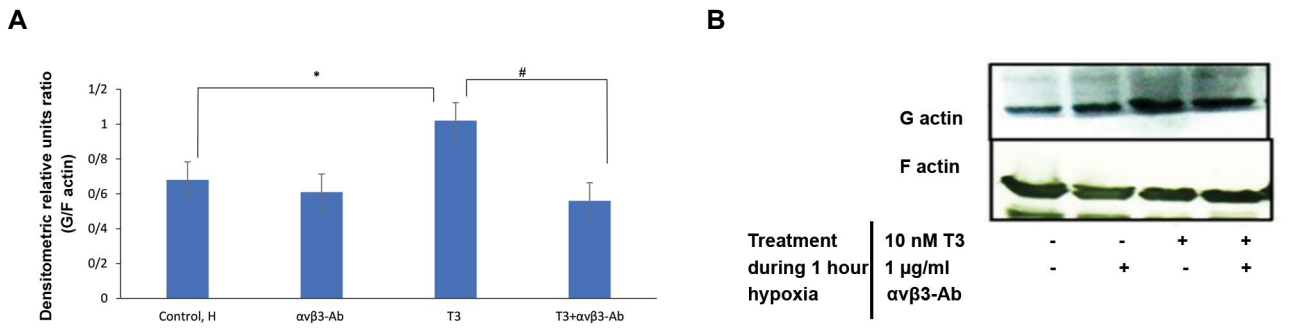

Fig.2: Immunoblotting analysis. **A.** Diagram Analysis of the G- and F-actin content in differentiated PC-12 cells under hypoxia with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 μg/ml) during 1 hour hypoxia. Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and asterisk and hash indicate a statistically significant difference. *; P=0.0010 vs. control and #; P=0.0020 vs. T3 hormone treatment. **B**. Western blots of the G and F actins in the differentiated PC-12 cells with/without 10 nM T3 hormone treatment and αvβ3 antibody (αvβ3-Ab, 1 μg/ml) during 1 hour hypoxia. Bands were quantified by Image J.

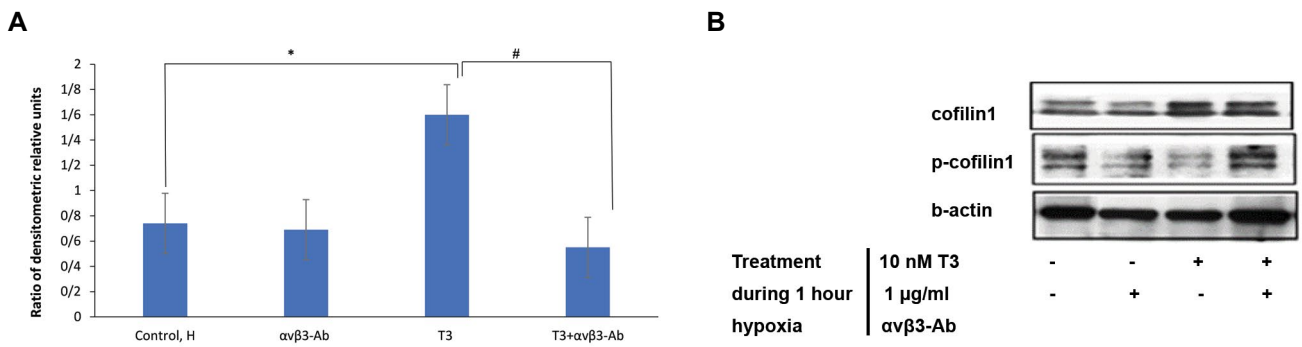

Fig.3: Immunoblotting analysis. **A.** Ratio of cofilin1/p-cofilin1 in the differentiated PC-12 cells with/without 10 nM T3 and αvβ3 antibody (αvβ3-Ab, 1 μg/ml) during 1 hour hypoxia. Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and asterisk and hash indicate a statistically significant difference. *; P=0.0045 vs. control and #; P=0.0058 vs. T3 treatment. **B.** Western blots of cofilin1 p-cofilin1, and beta-actin (loading control) in differentiated PC-12 cells with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 μg/ml) during one-hour hypoxia. Bands were quantified by Image J.

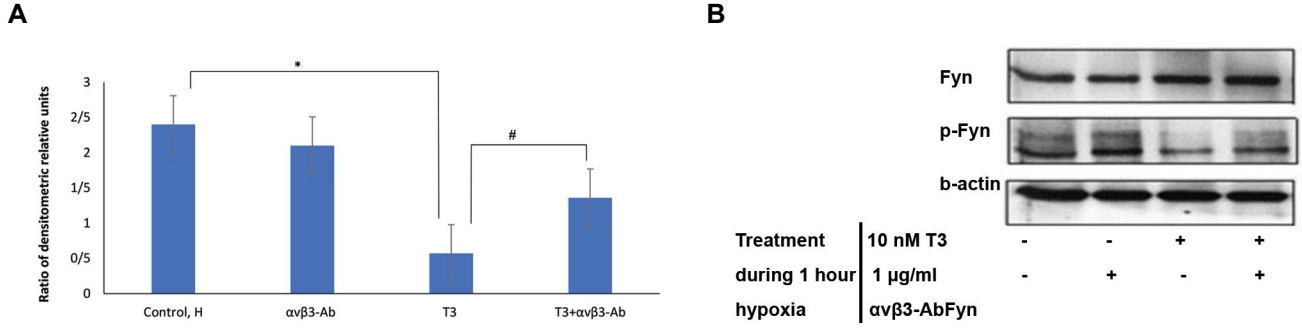

Fig.4: Immunoblotting analysis. **A.** Diagram of the p-Fyn/Fyn ratio in differentiated PC-12 cells with/without 10 nM T3 and αvβ3 antibody (αvβ3-Ab, one μg/ml) during 1 hour hypoxia. Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and asterisk and hash indicate a statistically significant difference. *; P=0.0010 vs. control and #; P=0.0138 vs. T3 treatment. **B.** Western blots of p-Fyn, Fyn and beta-actin (loading control) in the differentiated PC-12 cells with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 μg/ml) during one-hour hypoxia. Bands were quantified by Image J.

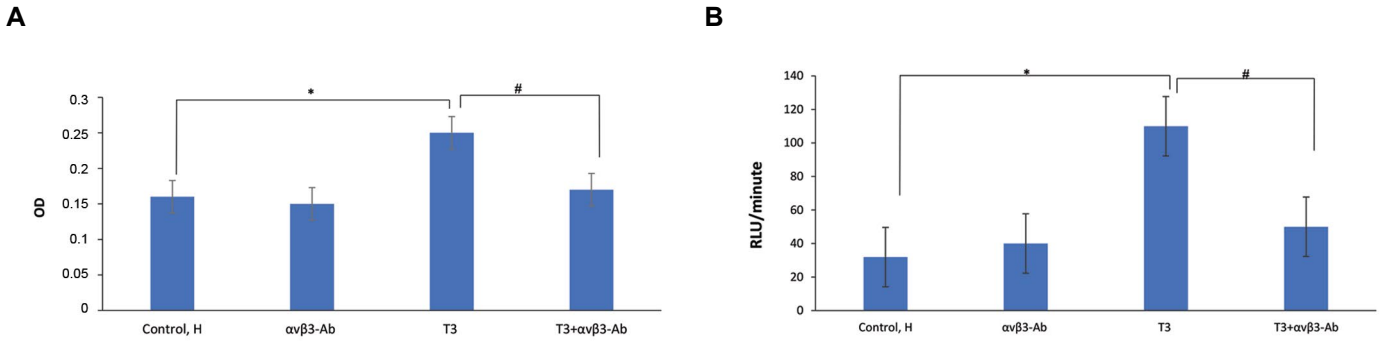

Fig.5: Analysis of the Rac 1 and NADPH oxidase activity in the differentiated PC-12 cells with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 µg/ml) during 1 hour hypoxia. **A.** Level of Rac1 in the differentiated PC-12 cells with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab,1 µg/ml) during one h hypoxia. Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and the asterisk and hash indicate a statistically significant difference *; P=0.0069 vs. control and #; P=0.0078 vs. T3 hormone treatment. **B.** NADPH oxidase activity in the differentiated PC-12 cells with/without 10 nM T3 hormone and αvβ3 antibody (αvβ3-Ab, 1 µg/ml) during 1 hour hypoxia. NADPH oxidase activity was measured as Relative Light Unit per minute (RLU/minute). Results of triplicate experiments are shown as mean ± SEM. Error-bars denote one standard error of the mean and asterisk or hash indicates a statistically significant difference. *; P=0.0010 vs. control and #; P=0.0014 vs. T3 hormone treatment.

The schematic representation of the results is described in Figure 6.

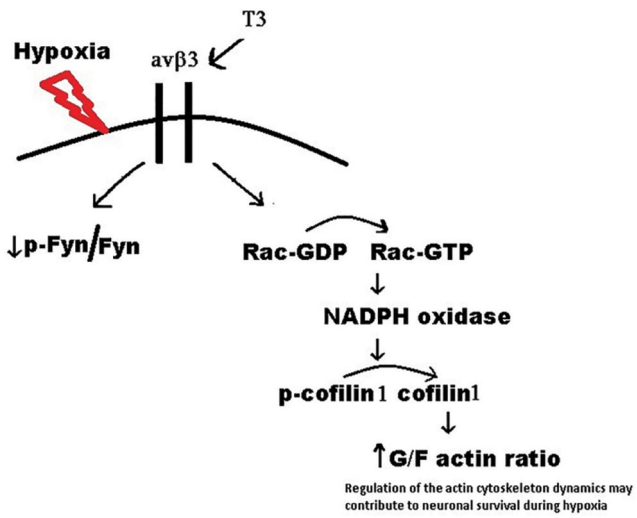

Fig.6: Schematic representation of the role of T3 hormone in actin cytoskeletal dynamic in the differentiated PC-12 cells during hypoxia. T3 hormone via αvβ3 integrin suppresses Fyn activation and, on the other hand, through the Rac1/NADPH oxidase/cofilin1 pathway increases the G/F actin ratio. In this way, extracellular T3 hormone regulates the neuronal actin filament dynamic via the αvβ3 integrin receptor during hypoxia.

Discussion

Thyroid hormones (T3 and T4) are crucial agents for normal brain development that contribute to recovery and neuronal regeneration after brain injury. These hormones act mainly through the nuclear receptors, but nongenomic actions of the T3 hormone were also found in the brain (28). We found that the T3 hormone treatment for one hour during hypoxia increased the viability of PC-12 cells. This pro-survival effect of the T3 hormone treatment was abolished by the αvβ3 antibody, which underlined the integrin-mediated T3-dependent modulation of the cell viability during hypoxia. This finding suggests that cell viability mainly depends on the presence of a T3 hormone in the extracellular medium, which contributes to the αvβ3-mediated pro-survival signalling during hypoxia.

Hypoxia induces F-actin filament accumulation due to the inactivation of actin-binding and severing protein, cofilin, by LIM kinase-mediated phosphorylation (17). Therefore, maintaining the typical G/F actin ratio is crucial for the actin filament dynamic regulation in the neuronal cell viability. In neurons, cofilin-1 may contribute to degenerative processes by forming cofilin-actin rods (23, 29). Based on these observations, we analysed the cytoskeletal content of actin, actin monomer-G actin, and filament polymer-F actin and determined the G/F actin ratio, a marker of cytoskeletal dynamics (30). According our results, the increased G/F actin ratio is mainly regulated through the αvβ3 integrin receptor and this effect is correlated with the reduction of p-cofilin1 levels. Thus, changes in actin dynamics appear to be the result of the cofilin1 dephosphorylation.

Hypoxia induces an actin rearrangement in several cell types and tissues, presumably via the Rho GTPase signalling (17). The Rho family GTPases play an important role in the control of cellular morphology. There are several studies on the role of the Fyn, a member of the Src family of kinases, in the inactivation of the Rho and activation of the Rac, which triggers morphological alterations. Also, Rho and Rac exhibit contrasting effects on the cell morphological complexity (31, 32). On the other hand, the Fyn activation has an undesirable effect on neurons, that makes them more vulnerable to a synaptotoxicity. The opposite effect is achieved by reducing the Fyn activation, and while it may be neuroprotective, an excessive inhibition could lead to poor long-term potential and hypothetically may influence the cognitive function in humans. Thus, a

therapeutic aim to maintain a delicate balance between activation and inhibition of the Fyn is likely to optimize the neural networks function (33, 34). Overactivation of the Src family protein tyrosine kinases, including the Fyn, has been implicated in the pathogenesis of cerebral ischemia and can initiate apoptosis (35). Based on these observations, we analysed the effect of thyroid hormones on the phosphorylation of the Fyn in differentiated PC-12 cells during hypoxia. We found that the T3 hormone treatment decreased and maintained a moderate p-Fyn/Fyn ratio, suggesting that the T3 hormone regulates the actin cytoskeleton dynamic via αvβ3 integrin through the dephosphorylation of the Fyn. Thus, our results agree with the observation that suppression of the Fyn kinase phosphorylation switches on the anti-apoptotic signalling cascade, especially in dopaminergic cells, and confirms the suggestion that pharmacological inhibitors directed at the Fyn activation could prove to be a therapeutic target in the treatment of dopaminergic degeneration during various neurological disorders including Parkinson's disease (36).

Several studies have shown that the Rac activation promotes the formation of lamellipodia and filopodia. Simultaneously, the Rho activity prevents neurite initiation, while inducing neurite retraction (37). It is demonstrated that Rac-mediated ROS production downregulates Rho activity (38). There is evidence that the Rac1 GTPase also can promote multiple pathological events and signalling pathways that collectively contribute to the neuronal damage and cognitive dysfunction following the cerebral ischemia (19). The Rac1 participates in a ROS formation via an NADPH oxidase activation in different cell lines. According to our findings, T3 hormone induced a moderate NADPH oxidase activation through binding the Rac1 to NADPH oxidase 2 (NOX2) in the differentiated PC-12 cells during the hypoxia condition. This effect is regulated through αvβ3 integrin (39).

In our opinion, non-genomic mechanisms, initiated by T3 thyroid hormone via αvβ3 integrin could contribute to the recovery of lost neurological functions after an ischemic stroke. Nevertheless, quantitative live cell imaging is needed for a more precise analysis of the non-genomic-dependent cytoskeleton rearrangement during ischemia.

The thyroid hormones level is significantly associated with post-stroke outcomes (40). According to our observations, one of the most critical targets of thyroid hormones is the αvβ3 integrin, which can participate in the hypoxic brain repair processes and have a pro-survival role in the neuronal cells during hypoxia. This receptor plays a central and complex role in the cell-cell and cell-matrix interactions that mediate cell adhesion, migration, and invasion. Additionally, αvβ3 integrin exerts intracellular effects on the actin cytoskeleton organization by regulating various signalling processes. As a cell surface receptor, this integrin has a central role in the pathophysiology of many brain diseases and is a potential target of new neurological drug development. Modulation of the thyroid hormone signalling in the post-ischemic brain may be a promising therapeutic strategy that may regulate endogenous repair mechanisms.

Conclusion

In conclusion, our results show that T3 can regulate the actin filament dynamics by the increasing the alteration of the G/F actin ratio via the Rac1 GTPase/NADPH oxidase/cofilin1 pathway and αvβ3-integrin-dependent suppression of Fyn kinase phosphorylation. These findings suggest, that the T3 regulates the neuronal actin cytoskeleton dynamics via αvβ3 integrin, which increases neuronal cell viability during hypoxia. In the future, we suppose to study the precise pathways of Fyn kinase regulation by T3 through αvβ3 integrin during hypoxia.

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Authors' Contributions

E.K., J.B., T.B.; Contributed to all experimental work, and reviewed the literature for the manuscript. T.B., J.S., D.M.; Participated in study design, data collection and evaluation, drafting, and statistical analysis. D.M Performed editing and approved the final version of this manuscript for submission. All authors approved the manuscript for submission.

References

1. Tan ZS, Vasan RS. Thyroid function and Alzheimer's disease. J Alzheimers Dis. 2009; 16(3): 503-507.
2. Frye RE, Wynne R, Rose S, Slattery J, Delhey L, Tippett M, et al. Thyroid dysfunction in children with autism spectrum disorder is associated with folate receptor α autoimmune disorder. J Neuroendocrinol. 2017; 29(3).
3. Alevizaki M, Synetou M, Xynos K, Pappa T, Vemmos KN. Low triiodothyronine: a strong predictor of outcome in acute stroke patients. Eur J Clin Invest. 2007; 37(8): 651-657.
4. Bunevicius A, Iervasi G, Bunevicius R. Neuroprotective actions of thyroid hormones and low-T3 syndrome as a biomarker in acute cerebrovascular disorders. Expert Rev Neurother. 2015; 15(3): 315-326.
5. Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S, et al. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology. 2005; 146(7): 2864-2871.
6. Davis PJ, Leonard JL, Lin HY, Leinung M, Mousa SA. Molecular basis of nongenomic actions of thyroid hormone. Vitam Horm. 2018; 106: 67-96.
7. Cohen K, Flint N, Shalev S, Erez D, Baharal T, Davis PJ, et al. Thyroid hormone regulates adhesion, migration and matrix metalloproteinase 9 activity via αvβ3 integrin in myeloma cells. Oncotarget. 2014; 5(15): 6312-6322.
8. Barbakadze T, Natsvlishvili N, Mikeladze D. Thyroid hormones differentially regulate phosphorylation of ERK and Akt via integrin αvβ3 receptor in undifferentiated and differentiated PC-12 cells.

Cell Biochem Funct. 2014; 32(3): 282-286.
9. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. Endocr Rev. 2010; 31(2): 139-170.
10. McKayed, KK, Simpson JC. Actin in action: imaging approaches to study cytoskeleton structure and function. Cells. 2013; 2(4): 715-731.
11. Tojkander S, Gateva G, Lappalainen P. Actin stress fibers – assembly, dynamics and biological roles. J Cell Sci. 2012; 125(Pt 8): 1855-1864.
12. Eira J, Silva CS, Sousa MM, Liz MA. The cytoskeleton as a novel therapeutic target for old neurodegenerative disorders. Prog Neurobiol. 2016; 141: 61-82.
13. Pollard TD, Cooper JA. Actin, a central player in cell shape and movement. Science. 2009; 326(5957): 1208-1212.
14. Davis PJ, Goglia F, Leonard JL. Nongenomic actions of thyroid hormone. Nat Rev Endocrinol. 2016; 12(2): 111-121.
15. Farwell AP, Dubord-Tomasetti SA, Pietrzykowski AZ, Stachelek SJ, Leonard JL. Regulation of cerebellar neuronal migration and neurite outgrowth by thyroxine and 3,3',5'-triiodothyronine. Brain Res Dev Brain Res. 2005; 154(1): 121-135.
16. Berrier AL, Martinez R, Bokoch GM, LaFlamme SE. The integrin beta tail is required and sufficient to regulate adhesion signaling to Rac1. J Cell Sci. 2002; 115(Pt 22): 4285-4291.
17. Zieseniss A. Hypoxia and the modulation of the actin cytoskeleton - emerging interrelations. Hypoxia (Auckl). 2014; 2: 11-21
18. Wong WT, Faulkner-Jones BE, Sanes JR, Wong RO. Rapid dendritic remodeling in the developing retina: dependence on neurotransmission and reciprocal regulation by Rac and Rho. J Neurosci. 2000; 20(13): 5024-5036.
19. Raz L, Zhang QG, Zhou CF, Han D, Gulati P, Yang LC, et al. Role of Rac1 GTPase in NADPH oxidase activation and cognitive impairment following cerebral ischemia in the rat. PLoS One. 2010; 5(9): e12606.
20. Mizuno K. Signaling mechanisms and functional roles of cofilin phosphorylation and dephosphorylation. Cell Signal. 2013; 25(2): 457-469.
21. Wang W, Mouneimne G, Sidani M, Wyckoff J, Chen X, Makris A, et al. The activity status of cofilin is directly related to invasion, intravasation, and metastasis of mammary tumors. J Cell Biol. 2006; 173(3): 395-404.
22. Wang Y, Lou MF. The regulation of NADPH oxidase and its association with cell proliferation in human lens epithelial cells. Invest Ophthalmol Vis Sci. 2009; 50(5): 2291-2300.
23. Bamburg JR, Bernstein BW. Actin dynamics and cofilin-actin rods in alzheimer disease. Cytoskeleton (Hoboken). 2016; 73(9): 477-497.
24. Goloshvili G, Barbakadze T, Mikeladze D. Sodium nitroprusside induces H-Ras depalmitoylation and alters the cellular response to hypoxia in differentiated and undifferentiated PC12 cells. Cell Biochem Funct. 2019; 37(7): 545-552.
25. Scarlett A, Parsons MP, Hanson PL, Sidhu KK, Milligan TP, Burrin JM. Thyroid hormone stimulation of extracellular signal-regulated kinase and cell proliferation in human osteoblast-like cells is initiated at integrin alphaVbeta3. J Endocrinol. 2008; 196(3): 509-517.
26. Bhambhvani HP, Mueller TM, Simmons MS, Meador-Woodruff JH. Actin polymerization is reduced in the anterior cingulate cortex of elderly patients with schizophrenia. Transl Psychiatry. 2017; 7(12): 1278.
27. Zhang W, Wang Y, Chen CW, Xing K, Vivekanandan S, Lou MF (2006) The positive feedback role of arachidonic acid in the platelet-derived growth factor-induced signaling in lens epithelial cells. Mol Vis. 2006; 12: 821-831.
28. Liu YY, Brent GA. Thyroid hormone and the brain: Mechanisms of action in development and role in protection and promotion of recovery after brain injury. Pharmacol Ther. 2018; 186: 176-185.
29. Hoffmann L, Rust MB, Culmsee C. Actin(g) on mitochondria – a role for cofilin1 in neuronal cell death pathways. Biol Chem. 2019; 400(9): 1089-1097.
30. Menon S, Gupton SL. Building blocks of functioning brain: cytoskeletal dynamics in neuronal development. Int Rev Cell Mol Biol. 2016; 322: 183-245.
31. Arthur WT, Noren NK, Burridge K. Regulation of Rho family GTPases by cell-cell and cell-matrix adhesion. Biol Res. 2002; 35(2): 239-246.
32. Liang X, Draghi NA, Resh MD. Signaling from integrins to Fyn to Rho family GTPases regulates morphologic differentiation of oligodendrocytes. J Neurosci. 2004; 24(32): 7140-7149.
33. Nygaard HB, van Dyck CH, Strittmatter SM. Fyn kinase inhibition as a novel therapy for Alzheimer's disease. Alzheimers Res Ther. 2014; 6(1): 8.
34. Matrone C, Petrillo F, Nasso R, Ferretti G. Fyn tyrosine kinase as harmonizing factor in neuronal functions and dysfunctions. Int J Mol Sci. 2020; 21(12): 4444.
35. Du CP, Tan R, Hou XY. Fyn kinases play a critical role in neuronal apoptosis induced by oxygen and glucose deprivation or amyloid-β peptide treatment. CNS Neurosci Ther. 2012; 18(9): 754-761.
36. Saminathan H, Ghosh A, Zhang D, Song C, Jin H, Anantharam V, et al. Fyn kinase-mediated PKCδ Y311 phosphorylation induces dopaminergic degeneration in cell culture and animal models: implications for the identification of a new pharmacological target for parkinson's disease. Front Pharmacol. 2021; 12: 631375.
37. Govek EE, Newey SE, Van Aelst L. The role of the Rho GTPases in neuronal development. Genes Dev. 2005; 19(1): 1-49.
38. Nimnual AS, Taylor LJ, Bar-Sagi D. Redox-dependent downregulation of Rho by Rac. Nat Cell Biol. 2003; 5(3): 236-241.
39. Kvergelidze E, Barbakadze T, Mikeladze D. Thyroid Hormone T3 Regulates NOX2 activity and BDNF secretion in differentiated PC-12 cells during hypoxia via αvβ3 integrin. Bull Georg Natl Acad Sci. 2022; 16(2): 115-121.
40. Talhada D, Santos CRA, Gonçalves I, Ruscher K. Thyroid hormones in the brain and their impact in recovery mechanisms after stroke. Front Neurol. 2019; 10: 1103.