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Biochemistry

Thyroid Hormone T3 Regulates NOX2 Activity and BDNF Secretion in Differentiated PC-12 Cells during Hypoxia via αvβ3 Integrin

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There is growing evidence that the Rac1-activated NADPH oxidase system stimulates ROS production, increases the BDNF expression, and regulates neurogenesis. Thus, moderate ROS amount could have beneficial effects on signaling and neurogenesis. Thyroid hormones (TH) are involved in mechanisms of neuronal plasticity and function of glial cells after ischemic stroke. Our findings suggest that T3 regulates the neuronal actin cytoskeleton dynamics via αvβ3-integrin, which increases neuronal cell viability through the Rac1/NADPH oxidase pathway during hypoxia. We hypothesized that TH via αvβ3-integrin could regulate NADPH oxidase activity through Rac1/NOX2 interactions, or NOX4 expression, and consequently stimulate BDNF production. We analyzed the T3 and T4 effects on Rac1 binding to NOX2 in differentiated PC-12 cells and revealed that T3 causes increases of the Rac1/NOX2 interaction, and this effect was significantly elevated after blocking of αvβ3 integrin by anti- αvβ3integrin antibodies in hypoxia. In contrast to NOX2, αvβ3-integrin inhibitory antibody significantly decreased NOX4 expression in differentiated PC-12 cells and abolished the T3-induced elevation of the BDNF secretion during hypoxia. We suggest that T3 regulates the moderate activity of NOX2 via αvβ3-integrin-mediated Rac1 binding to NOX2, which stimulates the BDNF production and contributes to its neuroprotective signaling pathway activity during hypoxia. *© 2022 Bull. Georg. Natl. Acad. Sci.*

αvβ3-integrin, Rac1, NOX2, NOX4, hypoxia, PC-12

Thyroid hormones (TH), 3,5,3′,5′-tetraiodo-L-thyronine (T4) and 3,5,3′-triiodo-L-thyronine (T3) have a prominent role in the development and function of the central nervous system (CNS) during embryonic and fetal stages, and throughout the entire life [1]. Cellular effects of TH in the brain can be mediated by nuclear receptors/transcriptional activity and also by non-genomic actions [2,3]. Non-genomic actions of TH are fast and include several interactions of TH with the cellular membrane, cytoskeleton, and mitochondria, modulating several intracellular pathways. In contrast to T4, the non-genomic action of T3 regulates the neuronal actin cytoskeleton dynamics via αvβ3 integrin, which increases neuronal cell viability during hypoxia via the Rac1/NADPH oxidase/ cofilin-1 pathway [4].

All known NADPH oxidases (NOXs) are transmembrane proteins which catalyze the production of superoxide and a host of radical and non-radical molecular species collectively called reactive oxygen species (ROS). There is accumulating evidence for an involvement in an increasing number of biologically important signal transduction processes [5]. NOX enzymes are involved in CNS development, neural stem cell biology, and the function of mature neurons. In most mammals, there are seven NOX isoforms, but only NOX2 and NOX4 were demonstrated as the major types involved in brain tissue ROS generation [6]. Under physiological conditions, NOX enzymes likely generate only low levels of ROS in the CNS. The most likely function of such a low-level ROS generation is cellular signaling [7]. It should be underlined that cell fate decision in response to ROS exposure is determined by the sensitivity of CNS cells to ROS, which is a key factor for the signaling versus cell death decision [8]. Data from literature indicate that modulation of ROS levels plays a role in the expression of brain-derived neurotrophic factor (BDNF). Oxidative stress can interact with the BDNF system to modulate synaptic plasticity and cognitive function. BDNF's role in brain development and function has been very well documented [9]. and its function has been associated with TH levels in the brain. [10] Based on these observations we hypothesized that αvβ3-integrin-mediated T3-induced BDNF elevation is the result of the specific NADPH oxidase activation during hypoxia. Thus, identifying the involvement of the specific NADPH oxidase in αvβ3 integrin-mediated T3 effects on BDNF production during hypoxia is crucial for further thera-peutic purposes in ischemia-induced pathological conditions.

Materials and Methods

Cell line. Pheochromocytoma cells (PC-12, ATCC® CRL-1721™) were cultured in a humidified atmosphere containing 5% CO_2 at 37°C in a high-glucose Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), and 100 unit/mL penicillin as well as 50 μg/mL gentamicin sulfate. To induce differentiation, PC-12 cells (5×10^6 cells per sample) were incubated in low serum-containing DMEM (1% HS and 1% FBS) supplemented with 100 ng/mL nerve growth factor (NGF) for 5 days. The cells were scored as differentiated if one or more neurites were longer than the cell body diameter. The experiments were performed under two settings: 1. T3/T4/αvβ3 integrin inhibitor pre-treatment of differentiated PC-12 cells for 24 h before hypoxia, followed by exposure to hypoxic conditions for 1 h without any treatment; and 2. Pre-incubation of differentiated PC-12 cells for 24 h with T3/T4/αvβ3 integrin inhibitor before hypoxia, followed by exposure to hypoxic conditions for 1 h with T3/T4/αvβ3 integrin inhibitor treatment. The physiological concentrations of thyroid hormones, 10 nM T3 and 100 nM T4, were used in the experiments. The αvβ3 blocking antibody (23C6) was used as an αvβ3 integrin inhibitor (1 μg/mL) to evaluate the involvement of αvβ3 integrin in thyroid hormone-induced effects. Hypoxic conditions (0–1% oxygen) were maintained using nitrogen gas in a BioSpherix C-Chamber placed in a CO_2 incubator and controlled by a ProOx Model P110 controller (BioSpherix, USA).

Cell lysate preparation. After 1 h of exposition to hypoxia, PC-12 cells were removed from the cell culture flasks using 0.025% trypsin/EDTA containing phosphate-buffered saline (PBS) buffer (incubation for 1 min), scraped, and pelleted by centrifugation at 300 × g. Trypsin inactivation was performed using aprotinin-containing PBS (1 μg/mL). Incubated PC-12 cells were lysed using a lysis buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, and PI cocktail and passed through a 25 Ga needle 10 times using a 1 mL syringe. After cell lysis, the

nuclei and intact cells were sedimented at 720 × g for 5 min, and the supernatant was subjected to electrophoresis and western blotting.

Immunoprecipitation and Western blotting. Supernatants were incubated with the anti-NOX2 antibodies for 60 min at 4°C. In immunoprecipitation experiments, proteinA/G-agarose (20 μg) was added and the incubation continued for 2 h. Samples were centrifuged at 2500 × g and the pellets were washed four times with TEE buffer (50 mM Tris–HCl, pH 7.4, 1 mm EDTA, and 1 mM EGTA). The combined supernatant obtained after immunoprecipitation was boiled at 90°C for 5 min with 2x sample buffer, resolved by SDS-PAGE on 7.5–15% gels, and transferred to nitrocellulose membranes for the analysis of NOX2, NOX4, and Rac1 proteins. After blocking with 5% bovine serum albumin and 0.05% Tween 20 in Tris–HCl-buffered saline (TBST), the nitrocellulose membranes were incubated with the corresponding primary antibodies (Santa Cruz Biotechnology) in blocking solution. Subsequently, the membranes were incubated with secondary antibodies. Immunolabeled bands were visualized using enhanced chemiluminescence and analyzed by Image J.

BDNF assay. The BDNF content was measured in the medium using the BDNF Human BDNF ELISA Kit (abcam), according to the manufacturer's protocol.

Statistical Analysis. The significance level was set at *$p < 0.05$, **$p < 0.01$, and n.s. (not significant) versus serum-free hypoxic control (control, H), #$p < 0.05$, ##$p < 0.01$ versus T3, and &$p < 0.05$,

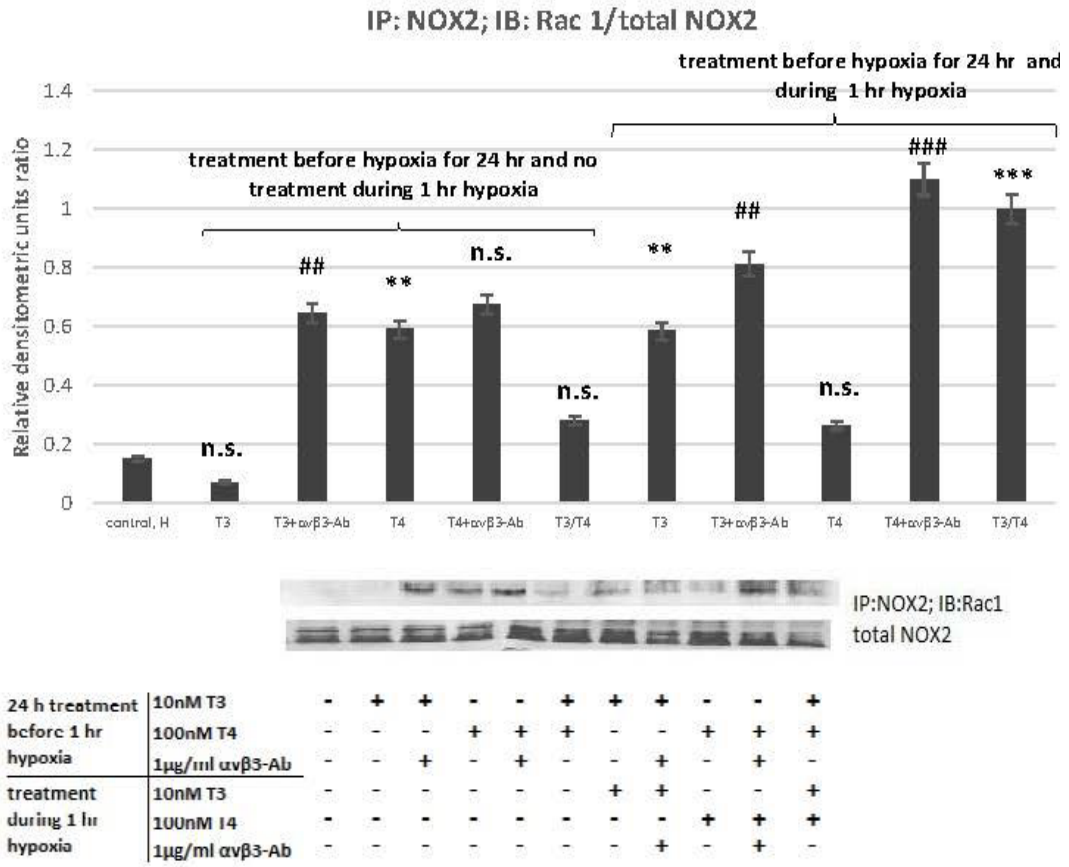

Fig. 1. Immunoblotting densitometric units ratio of immunoprecipitated NOX2 and immunostained with anti-Rac1 primary antibody, and total NOX2.

&&p<0.01 versus T4. All data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Scheffe's post hoc comparison test.

Results and Discussion

In order investigate the αvβ3 integrin-mediated mechanism of TH in hypoxia we used NGF-treated differentiated rat PC-12 cells and compared T3/T4/αvβ3 integrin inhibitory antibody pre-treated differentiated PC-12 cells for 24 h before hypoxia, followed by exposure to hypoxic conditions for 1 h without any treatment, with pre-incubated differentiated PC-12 with T3/T4/αvβ3 integrin inhibitory antibody cells for 24 h before hypoxia, followed by exposure to hypoxic conditions for 1 h with T3/T4/αvβ3 integrin inhibitor treatment. We analyzed the effects of T3 and T4 on

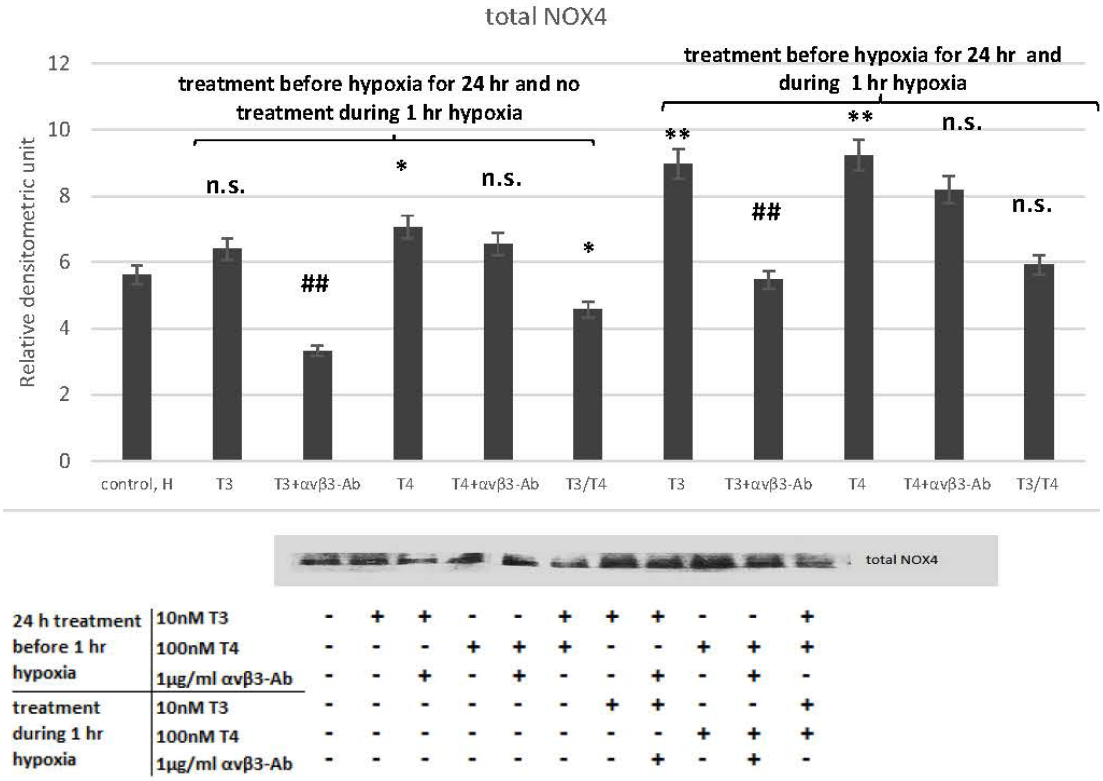

Fig. 2. Immunoblotting densitometric units of total NOX4.

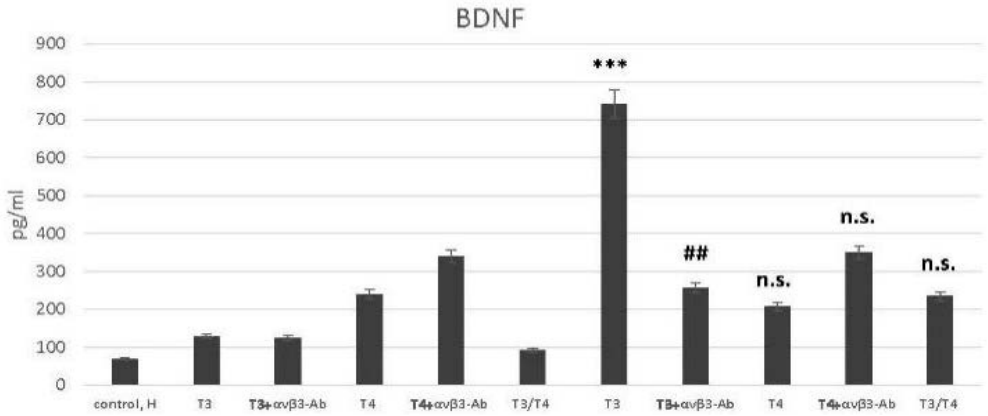

Fig. 3. BDNF release in the cell culture medium of differentiated PC-12 cells.

Rac1 binding level to NOX2 and total NOX4 expression level in differentiated PC-12 cells during hypoxia. Our experiments revealed that TH elevates the Rac1 interaction with the NOX2 enzyme exactly during the hypoxia and this interaction is increased by the αvβ3 integrin inhibitory antibody (Fig. 1). In contrast to T3, T4 increases the Rac1/NOX2 complex level only during the pre-incubation of the PC-12 cells with T4, whereas the presence of the T4 in the incubation medium does not change the Rac1 and NOX2 interaction level. Unlike T4, the simultaneous action of T3/T4 also increases the Rac1/NOX2 interaction, which emphasizes the predominant role of T3 in hypoxia. Previously, we found that T3 regulates the neuronal actin cytoskeleton dynamics via αvβ3 integrin, which increases neuronal cell viability during hypoxia through Rac1/NADPH oxidase activation. Thus, it is possible, that NOX2 participates as a signaling molecule during hypoxia. It is reported that the cytosolic subunit p40 (phox) of the NOX2 complex is partially associated with F-actin in neuronal growth cones, while ROS produced by this complex regulates F-actin dynamics and neurite growth [11]. Because, NOX2 is activated by NOX4 derived ROS, in the next, we decided to measure the NOX4 expression level in the cells. We found that T3 and T4 together and independently increase the expression of NOX4, and this effect is abolished by αvβ3 integrin inhibitory antibody (Fig. 2). We did not find a correlation between the NOX2 and NOX4 expression levels. Both Nox2 and Nox4 are reported to be involved in various agonist-stimulated signal transduction pathways, but their specific roles in modulating such pathways is remaining unclear [12]. We analyzed the BDNF release in the culture medium. Our results showed that treatment of cells with T3 during hypoxia increased the BDNF level. The impact of T3 was abolished by the addition of an anti-αvβ3 integrin antibody. Notably, T4 did not change the BDNF production during hypoxia (Fig. 3).

NOX-generated ROS have emerged as important regulators of the actin cytoskeleton and cytoskeleton-supported cell functions. The effects of NOX-derived ROS on cytoskeletal remodeling may be largely attributed to the ability of ROS to directly modify proteins that constitute or are associated with the cytoskeleton. Additionally, NOX-derived ROS may participate in signaling pathways governing cytoskeletal remodeling [13]. In recent years, it is showed BDNF-mediated structural plasticity including regulation of cytoskeleton membrane dynamics [14]. The protein content of BDNF is up-regulated by oxidative stress. Low levels of ROS might cause insufficient gene expression for redox homeostasis and, therefore, impaired response to oxidative challenge. On the other hand, high levels of ROS exceed the adaptive tolerance of cells, resulting in significant oxidative damage, apoptosis, and necrosis. So, the moderate level of ROS is crucial in the ROS signaling pathway and cell survival. Our findings suggest that T3 regulates the moderate activation of NOX2 via αvβ3 integrin-mediated Rac1 binding to NOX2, which stimulates the BDNF production and contributes to its neuroprotective signaling pathway during hypoxia.

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ბიოქიმია

თიროიდული ჰორმონი T3 არეგულირებს αvβ3 ინტეგრინით გაშუალებულ NOX2-ის გააქტივებას და BDNF-ის სეკრეციას დიფერენცირებულ PC-12 უჯრედებში ჰიპოქსიის დროს

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NADPH ოქსიდაზა – წარმოებული ROS-ი მნიშვნელოვანია ცნს-ის პათოფიზიოლოგიურ და ნორმალურ პროცესებში. Rac1-ით გააქტიურებული NADPH ოქსიდაზური სისტემა ასტიმულირებს ROS-ის წარმოქმნას, რაც იწვევს BDNF-ის ექსპრესიას და, თავის მხრივ, არეგულირებს ნეიროგენეზს. ფარისებრი ჯირკვლის ჰორმონები (TH; T3/T4), მონაწილეობენ იშემიური ინსულტის შემდეგ ნეირონების პლასტიკურობისა და გლიური უჯრედების ფუნქციონირებაში. ჩვენი წინა კვლევები მოწმობს, რომ T3 არეგულირებს ნეირონული აქტინის ციტოჩონჩხის დინამიკურობას αvβ3 ინტეგრინის საშუალებით, რაც ზრდის ნერვული უჯრედების სიცოცხლისუნარიანობას Rac1/NADPH ოქსიდაზას გზის მეშვეობით ჰიპოქსიის დროს. ჩვენ ვივარაუდეთ, რომ TH-მა αvβ3 ინტეგრინის საშუალებით შეიძლება დაარეგულიროს NADPH ოქსიდაზას აქტივობა NOX2-თან Rac1-ის დაკავშირების გზით, ან NOX4-ის ექსპრესიის ცვლილებით და, შესაბამისად, ასტიმულიროს BDNF ექსპრესია. კვლევით დადგინდა, რომ T3 იწვევს Rac1-ის NOX2-თან შეკავშირების ზომიერ მატებას და ეს ეფექტი მნიშვნელოვნად იზრდება αvβ3-ინტეგრინის ინჰიბირებით ჰიპოქსიის დროს. NOX2-გან განსხვავებით, αvβ3-ინტეგრინის ინჰიბიტორული ანტისხეული მნიშვნელოვნად ამცირებს NOX4-ის ექსპრესიას დიფერენცირებულ PC-12 უჯრედებში და ხსნის T3-ით გამოწვეულ BDNF სეკრეციის მომატებას ჰიპოქსიის დროს. ჩვენ ვვარაუდობთ, რომ T3 არეგულირებს NOX2-ის ზომიერ აქტივობას, Rac1-ის NOX2-თან დაკავშირების გზით, ეს ეფექტი გაშუალებულია αvβ3 ინტეგრინით, რაც ასტიმულირებს BDNF-ს წარმოებას და ხელსუწყობს ნეიროპროტექტორული სასიგნალო გზის გააქტივებას ჰიპოქსიის დროს.

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