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Reverse T_3 interacts with αvβ3 integrin receptor and restores enzyme activities in the hippocampus of hypothyroid developing rats: Insight on signaling mechanisms

Juliana Tonietto Domingues [a, b], Daiane Cattani [a], Patricia Acordi Cesconetto [a], Bianka Alzira Nascimento de Almeida [a], Paula Pierozan [c], Karin dos Santos [a], Guilherme Razzera [a], Fátima Regina Mena Barreto Silva [a], Regina Pessoa-Pureur [c], Ariane Zamoner [a, b, *]

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[b] Programa de Pós-Graduação em Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil
[c] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

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ABSTRACT

In the present study we provide evidence that 3,3′,5′-triiodothyronine (reverse T_3, rT_3) restores neurochemical parameters induced by congenital hypothyroidism in rat hippocampus. Congenital hypothyroidism was induced by adding 0.05% propylthiouracil in the drinking water from gestation day 8 and continually up to lactation day 15. In the *in vivo* rT_3 exposure, hypothyroid 12-day old pups were daily injected with rT_3 (50 ng/kg body weight) or saline until day 14. In the *ex vivo* rT_3 treatment, hippocampal slices from 15-day-old hypothyroid pups were incubated for 30 min with or without rT_3 (1 nM). We found that *ex vivo* and/or *in vivo* exposure to rT_3 failed in restoring the decreased ^{14}C-glutamate uptake; however, restored the phosphorylation of glial fibrillary acidic protein (GFAP), $^{45}Ca^{2+}$ influx, aspartate transaminase (AST), glutamine synthetase (GS) and gamma-glutamate transferase (GGT) activities, as well as glutathione (GSH) levels in hypothyroid hippocampus. In addition, rT_3 improved ^{14}C-2-deoxy-D-glucose uptake and lactate dehydrogenase (LDH) activity. Receptor agonists/antagonists (RGD peptide and AP-5), kinase inhibitors of p38MAPK, ERK1/2, CaMKII, PKA (SB239063, PD98059, KN93 and H89, respectively), L-type voltage-dependent calcium channel blocker (nifedipine) and intracellular calcium chelator (BAPTA-AM) were used to determine the mechanisms of the nongenomic rT_3 action on GGT activity. Using molecular docking analysis, we found rT_3 interaction with αvβ3 integrin receptors, nongenomically activating signaling pathways (PKA, CaMKII, p38MAPK) that restored GGT activity. We provide evidence that rT_3 is an active TH metabolite and our results represent an important contribution to elucidate the nonclassical mechanism of action of this metabolite in hypothyroidism.

1. Introduction

Thyroid hormones (TH), 3,5,3′-triiodo-L-thyronine (T_3) and thyroxine (T_4), are involved in metabolic and physiological functions of several tissues, being crucial for neural development and function (Bernal, 2007; Bernal et al., 2003). The classical mechanism of hormone action involves the modulation of gene expression. However, several evidence point to nongenomic mechanisms of TH in different cell types, including neural cells (Dusart and Flamant, 2012; Morte and Bernal, 2014; Galton et al., 2007; Zamoner et al., 2005; Manzano et al., 2007; Gilbert et al., 2007; Madeira et al., 1988; Zamoner et al., 2007a, 2011; Schmohl et al., 2015; Zamoner et al., 2011; Lischinsky et al., 2016; Zanatta et al., 2013a; Bernal et al., 2003). The nongenomic actions of TH might be associated with surface receptor-, as well as kinase- and/or calcium-activated signaling pathways (Morte and Bernal, 2014; Gilbert et al., 2007; Zamoner et al., 2007a; Schmohl et al., 2015;

* Corresponding author. Departamento de Bioquímica, Centro de Ciências Biológicas, UFSC, Campus Universitário, Bairro Córrego Grande, Lab 101, bloco C, térreo, CEP: 88040-970, Florianópolis, Santa Catarina, Brazil.
E-mail addresses: ariane.zamoner@ufsc.br, arianezps@gmail.com (A. Zamoner).

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Bernal et al., 2003; Davis et al., 2010; Bergh et al., 2005).

In the central nervous system (CNS), TH are essential for myelination (Bauer and Whybrow, 2001), neuritogenesis (Fernandez-Lamo et al., 2009), maturation of neurotransmitter systems (Bauer and Whybrow, 2001), synaptic plasticity (Vallortigara et al., 2008, 2009; Zamoner et al., 2007b), intermediate filament phosphorylation (Gilbert et al., 2007; Zamoner et al., 2007b; Schmohl et al., 2015) as well as regulation of ion channels (Madeira et al., 1988).

Besides T_3, known as the classical TH, TH metabolites exist, including 3,3′,5-triiodothyroacetic acid (triac); tetraiodothyroacetic acid (tetrac); 3,3′,5′-triiodo-L-thyronine (reverse T_3, rT_3) and 3,5-diiodo-L-thyronine (T_2).

Triac is produced by the liver and other tissues (Siegrist-Kaiser and Burger, 1994) and several reports support that it is a biologically active metabolite (Medina-Gomez et al., 2003; 2004; Dumas et al., 1982; Kunitake et al., 1989).

Tetrac binds to the TH receptor on the extracellular domain of the plasma membrane integrin αvβ3 protein and modulates multiple intracellular activities regulated by the receptor (Bergh et al., 2005; Cody et al., 2007).

Otherwise, both rT_3 and T_2 were considered inactive metabolites produced by TH deiodination. The iodothyronine deiodinase 3 (D3) degrades T_4 to rT_3 and T_3 to T_2 (Araujo and Carvalho, 2011; Sibilio et al., 2012). However, several reports have recently shown that they may have relevant biological effects in different cell types (Plow et al., 2000; Farwell et al., 2005; Zanatta et al., 2013). In this context, T_2 was found to be a peripheral mediator of several TH metabolic effects (Moreno et al., 2002) and findings previously reported in hypothyroid rats suggest that T_2 has specific actions on resting metabolic rate (Lanni et al., 1996).

The evidence supporting rT_3 as a metabolically active TH comes from several reports on the restoring activity of this TH on the cytoskeleton of hypothyroid rat brain challenging previous understanding that rT_3 is a biologically inactive molecule. In this context, T_4 and rT_3, but not T_3, were able to restore the levels of filamentous actin (F-actin) into cerebellar cells of hypothyroid neonate rats (Faivre-Sarrailh and Rabie, 1988; Farwell et al., 2006; Leonard, 2008), in which rT_3 acted as a potent initiator of actin polymerization in astrocytes.

In hypothyroid rodents, neurons and astrocytes develop poor actin cytoskeletons that T_3 replacement cannot rescue. However, rT_3 initiates reappearance of filamentous actin within minutes, without altering total actin mRNA or protein content (Farwell et al., 1990; Siegrist-Kaiser et al., 1990). This rT_3 property was attributed to TRΔα1, a native thyroid receptor (TR) isoform that lacks a nuclear localization signal and is present in the extra nuclear compartment of astrocytes and neurons. This isoform has the ligand affinity and specificity required for actin polymerization by rT_3. Astrocytes of the developing mouse cerebellum deprived of both TRs showed that TRΔα1 rescued the actin cytoskeleton response to rT_3 (Flamant and Samarut, 2003).

The brain is the predominant D3-expressing tissue in adult animals, and may thus be the main site for the clearance of plasma T_3 and for the production of plasma rT_3 (Peeters and Visser, 2000).

Hypothyroidism is a condition in which the serum levels of THs are below that necessary to carry out physiological functions in the body. Central congenital hypothyroidism is a rare disorder in which inadequate TH biosynthesis occurs due to defective stimulation of a normal thyroid gland by thyroid stimulating hormone (TSH) (Schoenmakers et al., 2015). Thyroid hormone deficiency during fetal and postnatal development results in several biochemical and morphological changes in the neonatal rat brain, such as altered number of neurons and astrocytes, reduced synaptic connectivity, delayed myelination, disturbed neuronal migration, modifications in neurotransmission leading to deleterious effects for central nervous system development (Bernal, 2015).

Hypothyroidism may decrease the ability to induce long-term potentiation (LTP), compromising learning and memory formation. In this context, we previously demonstrated that congenital hypothyroidism decreases the astrocyte protein levels of glial fibrilary acidic protein (GFAP) and glutamate transporters (GLAST and GLT-1) in cerebral cortex of immature rats. These events clearly demonstrate misregulated astrocyte function and glutamate homeostasis (Zamoner et al., 2008a). Corroborating our findings, Alva-Sanchez and colleagues have demonstrated that the neuronal damage in the hippocampus of hypothyroid animals requires activation of the ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) (Alva-Sanchez et al., 2009). Moreover, it has been demonstrated that hypothyroid rats are extremely vulnerable to proconvulsant neurotoxic effects of kainite (Gine et al., 2010). These events might be related to glutamatergic excitotoxicity, corroborating recent results of our research group, which demonstrated that congenital hypothyroidism decreases glutamate uptake, increases Ca^{2+} influx and induces oxidative stress in rat hippocampus (Cattani et al., 2013).

In addition, the TH actions in brain might be triggered by either genomic or nongenomic signaling mechanisms. Accordingly, the αvβ3 integrin dimer has been suggested as a putative plasma membrane receptor for TH, with higher affinity for T_4 and rT_3 than for T_3 (Plow et al., 2000; Bergh et al., 2005). On the other hand, the nuclear receptor for TH interacts preferentially with T_3, being less responsive for T_4 and rT_3 (Davis et al., 2008).

Considering our previous data and evidence in the literature that rT_3 could play biological roles in hypothyroid rat brain, in the present study we addressed the ability of rT_3 in restoring the homeostasis of glutamate metabolism in hypothyroidism, assessing signaling pathways and the role of integrin receptor αvβ3 in the rT_3 actions. In addition, we searched for the implication of rT_3 on the energetic metabolism and the homeostasis of the intermediate filament (IF) cytoskeleton of astrocytes. We used an experimental model of congenital hypothyroidism in rats focusing on genomic and nongenomic mechanisms of action of rT_3 through short-term *ex vivo* and classical *in vivo* actions of rT_3 in the hippocampus of immature pups. Special attention was given to the signaling pathways implicated in the action of rT_3 on gamma-glutamyl transferase (GGT) activity. We used molecular docking analysis to propose that the actions of rT_3 could be downstream of αvβ3 integrin receptor.

2. Experimental procedures

2.1. Radiochemicals and compounds

[^{32}P]Na_2HPO_4 was purchased from CNEN, São Paulo, Brazil. L-[^{14}C] glutamic acid ([^{14}C] glutamate) (specific activity 49 Ci/mmol), $^{45}CaCl_2$ (specific activity of 321 kBq/mg of Ca^{2+}), [U-^{14}C]-2-deoxy-D-glucose (^{14}C-DG), specific activity 9.25 GBq/mmol and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Waltham, MA). Nifedipine, 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester) (Bapta-AM), N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89), D (−)-2-Amino-5-phosphonopentanoic acid (AP5), KN93, 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), benzamidine, leupeptin, antipain, pepstatin, chymostatin, acrylamide, bis-acrylamide, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), Triton X-100, 3,5′,3′-triiodo-L-thyroinine (reverse T_3, rT_3) and Arg-Gly-Asp peptide (RGD) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All

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