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Reverse triiodothyronine (rT3) attenuates ischemia-reperfusion injury

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Abstract

Hypothyroidism has been associated with better recovery from cerebral ischemia-reperfusion (IR) injury in humans. However, any therapeutic advantage of inducing hypothyroidism for mitigating IR injury without invoking the adverse effect of whole body hypothyroidism remains a challenge. We hypothesize that a deiodinase II (D2) inhibitor reverse triiodothyronine (rT3) may render brain specific hypometabolic state to ensue reduced damage during an acute phase of cerebral ischemia without affecting circulating thyroid hormone levels. Preclinical efficacy of rT3 as a neuroprotective agent was determined in rat model of middle cerebral artery occlusion (MCAO) induced cerebral IR and in oxygen glucose deprivation/reoxygenation (OGD/R) model in vitro. rT3 administration in rats significantly reduced neuronal injury markers, infarct size and neurological deficit upon ischemic insult. Similarly, rT3 increased cellular survival in primary cerebral neurons under OGD/R stress. Based on our results from both in vivo as well as in vitro models of ischemia reperfusion injury we propose rT3 as a novel therapeutic agent in reducing neuronal damage and improving stroke outcome.

Keywords

rT3; Stroke; MCAO; Thyroid hormone; Cerebral ischemia

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

LR performed the experiments, LR, MMG, RAS and SP designed the study and wrote the manuscript.

Disclosures

None.

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1 Introduction

Low Thyroid hormone (TH) levels attenuate and its high levels aggravate ischemic damage in animal model and human subjects [1–6]. Hypothyroidism though neuroprotective is unachievable to attain rapidly without risk of severe bradycardia and ethical considerations. In the brain, most bioactive TH, 3,3', 5-triiodothyronine (T3) is derived locally by deiodination of Thyroxine (T4) to T3 catalyzed by the type II iodothyronine deiodinase (D2) [7] in the glial cells. Cerebral T3 concentration is also regulated by the neuronal type III deiodinase (D3), which catalyzes its degradation [7]. Previous reports have implicated TH inactivation as a physiological adaptive response to limit hypoxic-ischemic injuries in mammalian tissues [8]. Based on this knowledge we hypothesized agents that can rapidly decrease T3 signaling in the brain may constitute a protective mechanism that may limit/reduce damage caused by a brain stroke. Reverse triiodothyronine (3,3', 5'-triiodothyronine, reverse T3, or rT3) is an isomer of T3, however due to its inability to bind nuclear TH receptors it is usually considered biologically inactive. Interestingly, rT3 is known to negatively regulate nuclear T3 action via its ability to reduce T4 to T3 conversion in D2 expressing tissues such as brain [9–11]. Therefore the combined abilities of rT3 to cross the blood/brain barrier and competitively inhibit D2 mediated T4 to T3 conversion without altering circulating T3 levels, prompted us to investigate whether it might be able to mitigate the damage in post-ischemic brain after reperfusion injury.

2 Material & methods

2.1 Chemicals

Chemicals and culture reagents were procured from Sigma-Aldrich and Life Technologies, USA.

2.2 Animals

Male Sprague-Dawley albino rats (weight 250–350 g) were housed at a temperature of 25 ± 2 °C with alternating 12 h light and dark cycles and free access to standard food pellets and water. Levels of TH were measured using RIA from Diagnostic Product Company (New York, USA) in blood samples drawn from animal tail. The institutional animal ethics committee CPCSEA, New Delhi, approved all animal procedures performed. Sham operated rats retained the nomenclature “C” and ischemia was introduced in male Sprague-Dawley rats by occlusion of middle cerebral artery and retracting the same gently to allow reperfusion for next 3 and 7 days. Post ischemic rats were divided equally in two groups and those who underwent ischemia/reperfusion for 3 and 7 days were labeled as IR3, IR7 group respectively. The second group receiving the daily intravenous dose of rT₃ (50ng/100 gm body weight) were labeled as IR3+rT₃ and IR7+rT₃ while rats in first group continued without any intervention. Body weights of all the animals were recorded at weekly intervals as well as at the beginning of occlusion/reperfusion. The femoral artery was cannulated to record blood pressure and heart rate through a Statham P23 DC pressure transducer and P44 tacograph respectively on different channels of a Grass model 7 polygraph, (Grass Instruments Co., Quincy, Mass, USA). Body temperature was monitored in each group of

rats before surgery, and at the time of sacrifice. It was recorded manually by thermo-probe temporarily inserted 2 cm into the rectum.

2.3 Transient focal ischemia

Rats were fasted overnight and anaesthetized by an intra peritoneal injection of 300 mg/kg chloral hydrate. Right middle cerebral artery (MCA) was occluded by advancing a 4.0 nylon filament from right external carotid up to middle cerebral artery for 2 h and retracting the same gently to allow the reperfusion for next 3 and 7 days. In equal number of rats the sham operation was performed. Body temperature was maintained 36–38 °C by using a heating lamp.

2.4 Blood collection and tissue sampling

Following 3 and 7 days of occlusion/reperfusion or sham procedure, rats were anaesthetized and blood was collected. Subsequent to blood sampling, rats were sacrificed by decapitation followed by tissue collection.

2.5 Neurological evaluation

All the animals were subjected to neurological evaluation by using a 6-point postural reflex test [27]. Briefly, scoring was as follows, 0 = no deficit, 1 = failure to extend left forepaw, 2 = circling to the left while pulled by tail, 3 = paresis of the left side, 4 = No spontaneous walking and 5 = death.

2.6 Infarct assessment & quantization

For TTC staining, the fore-brain was quickly removed and sliced into 2 mm thick coronal sections. Seven coronal brain sections were cut from freshly obtained cleaned tissue and stained with TTC. The area of infarction of each section was determined using a computerized image analysis system (Biovis Image plus, India).

2.7 Primary neuronal culture

Embryonic day 16 cerebral cortices were minced in small pieces, trypsinised at 37 °C, 5 min with 0.5 mg/ml trypsin. Following incubation, trypsin was aspirated and tissues were incubated with DMEM-F12 media containing 10% FBS. Cells were then dissociated and the resulting cell suspension were filtered through a 50 µm diameter nylon mesh and pelleted by centrifugation. Approximately, 1×10^5 cells in 12 well plates were plated in each Poly-L-Lysine (PLL) coated wells. More than 90% of the neurons were attached to the PLL coated surface within about 5 min. Unattached cells were discarded. Media (DMEM-F12 containing 10% FBS) was replaced regularly. The cultures were maintained at 37 °C under more than 90% humidity, 5% CO₂ and 95% air, i.e. 21% O₂. The medium was changed every 2–3 days until cells were reached confluence at ~10 days. Experiments were carried on after 10–11 days of seeding. After 10–11 days cultured cells were treated with drugs for indicated times.

2.8 Primary glial culture

Brains were removed from 0 to 1 day old pups and cleaned of meninges and blood vessels. The two cerebral hemispheres were then kept in HBSS with antibiotics. Brain tissue was

minced in small pieces, trypsinised at 37 °C, 5 min with 0.5 mg/ml trypsin. Following incubation trypsin was aspirated and tissues were incubated with DMEM-F12 media containing 10% FBS. Cells were dissociated to form a cell suspension by passing 20–30 times through 1-ml pipette tip and then passing through 200 µl pipette tip. The resulting cell suspension is filtered through a nylon mesh (50 µm pore size) and pelleted by centrifugation. The cell pellet was then suspended in DMEM-F12 media with 10% FBS. Cells were seeded in PLL coated flask (175 cm²) in a density of 30 million cell in 12–15 ml. After 5 min, unattached cells were plated in fresh PLL coated flask. Unattached cells again after 5 min were transferred to fresh PLL coated flask. The medium was changed every 2–3 days until cells were reached confluence at ~10 days. Experiments were planned on or after 21 days of seeding [28].

2.9 OGD/R in primary neuronal/glial culture

At a time of experiment, media was removed and cells were washed with PBS. Glucose free DMEM media was added to experimental flasks and flask was transferred to hypoxic chamber (5% CO₂, 90% air/N₂, 5% O₂, humidity 85%, 37 °C) for 60 min and 320 min for neurons and glial cells respectively. Glucose free DMEM media was replaced with DMEM glucose media and flask kept in normoxic chamber (85% humidity, 5% CO₂ and 95% air, i.e. 21% O₂, 37 °C) [29]. In three set of flask rT3 (final concentration 1, 10, 100 nM) is added at a time of reperfusion and kept in normoxic chamber. After 24 h of reperfusion media was collected for LDH release to assess cell death.

2.10 Tissue processing and measurement of enzymatic activity

Brain tissue stored at –70 °C was slowly brought to 4 °C on ice. A 10% (w/v) brain homogenate was prepared in phosphate buffer (50 mM, pH 7.0) under ice-cold condition using teflon homogenizer. The homogenate was centrifuged at 2000 r.p.m. for 10 min at 4 °C. In the supernatant, levels of protein, MDA, and GSH were measured. Rest of homogenate was centrifuged at 20,000 rpm for 30 min at 4 °C and mitochondrial pellet was obtained and used for the ROS generation in every group.

2.11 LDH assay/release

Measurement of lactate dehydrogenase activity in tissue homogenate. LDH activity in tissue homogenate was estimated with the reagent kit (Boehringer Mannheim, Germany), based on the oxidation of lactate to pyruvate with simultaneous reduction of NAD to NADH. The rate of NAD reduction was measured as an increase in absorbance at 340 nm. Results were expressed as U/min/mg protein. In cell culture media was removed after completion of experiment and stored for estimation of LDH release. LDH release is marker of cell death and estimated by Standard method and expressed as % of OGD.

2.12 Malondialdehyde levels

Measurement of lipid peroxides (MDA) was determined by quantifying the reaction product with thiobarbituric acid in tissue or cell homogenate. The coloured end product was read at 540 nm. Results were expressed as nmoles MDA/mg protein.

2.13 Glutathione levels

Reduced GSH was measured in the tissue/cell supernatant using dithiobis-2-nitrobenzoic acid reagent [2]. The coloured end product was read at 412 nm and the results were expressed as mg GSH/mg protein.

2.14 Reactive oxygen species generation

The intracellular ROS was measured using 2', 7' - Dichlorofluorescein diacetate (DCF-DA) in cortical neurons of primary culture and also from the adult rat brain tissue underwent ischemia reperfusion and rT3 treatment. Data were analyzed using cell Quest software (Becton Dickinson, USA).

2.15 Transference dUTP nick-end labeling (TUNEL) staining

TUNEL staining was performed with ApoAlert DNA Fragmentation Assay Kit (CLONTECH Laboratories, Inc. USA) in paraffin embedded tissue sections according to the manufacturer's instruction.

2.16 RNA isolation and Real-Time PCR

The ipsilateral cerebral cortex were aseptically collected and immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total RNA both from tissue and cultures were extracted using TRIzol Reagent (Life Technologies, NY, USA) according to Manufacturer's guideline. Real-Time PCR analysis of relative gene expression was performed using SYBER green chemistry on a Bio-Rad Cyclor Detection System (Bio-Rad, Hercules, CA, USA). Primer sequences available upon request.

2.17 Cell viability assay by trypan blue dye exclusion test

The culture medium was removed and replaced by 0.1% for trypan blues solution in phosphate –buffered saline for 3 min at room temperature. Viable cells were counted using bright field and phase contrast microscopy for blue cells and total cells respectively.

2.18 Statistical analysis

Statistical analysis was performed using student t-test for comparison between two groups and one-way analysis of variance for more than two groups. To find out group differences Post Hoc analysis of variance was done using Student –Newman-Keul's (SNK) test.

3 Results

3.1 rT3 protects against IR injury in vivo

Post MCAO day 3 & 7 brains sections stained with 2,3,5-triphenyltetrazolium chloride (TTC) showed a clear rT3-dependent decrease in infarct volume (Fig. 1A and B). Consistent with reduction in the infarct size rT3 treated animals also exhibited decreased neurological deficit upon ischemia-reperfusion induction (Fig. 1C). Of note, rT3 had no adverse effect on circulating T4/T3, blood pressure and body temperature (Table 1). Tissue injury markers such as LDH release and TUNEL positivity (apoptosis marker) which are elevated upon IR injury were also reduced upon rT3 treatment (Fig. 1D and E). Moreover, mediators of redox

damage i.e., MDA, ROS levels were reduced and antioxidant GSH levels were increased upon rT3 administration (Fig. 2A–C).

3.2 rT3 protects against IR injury in vitro

To check whether rT₃ protects neurons from in vitro ischemia and reperfusion in a cell autonomous manner we subjected neocortical neuronal to oxygen glucose deprivation (OGD) under nitrogen for 1h, followed by re-oxygenation with medium containing O₂ and glucose±rT₃. At 24 h after OGD and reperfusion, rT₃ increased neuron survival and vitality (Fig. 3A and B), indicating that rT₃ protects neurons against oxidative stress-induced cell death in a dose dependent manner. In consonance with above indicators of peroxidation damage i.e. release of LDH, levels of MDA and ROS were significantly increased in untreated OGD/reoxygenated neurons, and declined significantly in rT₃-treated neuronal cells (Fig. 3C and D), whereas the antioxidant GSH levels were increased upon rT₃ administration (Fig. 3E).

3.3 rT3 alter deiodinase's expression under IR stress

To check whether ischemic/reperfusion injury and its attenuation through rT₃ alters TH homeostasis we measured mRNA expression of both Type II (D2) and Type III (D3) deiodinase by performing Real Time PCR. D2 increased 2-fold on day 3, and further increased several-fold on day 7, suggesting there may be a late phase requirement of T₃ during plausible recovery phase. This response was blunted by rT₃ administration (Fig. 4A). Most importantly, D3 levels during injury phase were unchanged on days 3 & 7 but were increased several fold after rT₃ administration, reducing T₃ availability to the injured brain by possibly inducing a protective hypometabolic state (Fig. 4B). Consistent with the in vivo results, rT₃ significantly reduced glial D2 and increased neuronal D3 levels in vitro (Fig. 4C and D). Although we could not directly measure brain T₃ levels *per se*, in agreement with the presumption that rT₃ reduced T₃ availability in the brain, we observed down-regulation of a classical T₃-responsive target gene, Brain-derived neurotrophic factor (*Bdnf*) [12–14] in rT₃ treated animals (Fig. 4E). These results indicate that alteration in the levels of brain deiodinase enzyme by rT₃ may play a critical role in reducing the neuronal T₃ availability to injured brain thereby acting as the major determinant of ischemia/reperfusion injury attenuation.

4 Discussion

The rationale of our present work is derived from our previous findings that pre-ischemic hypothyroidism reduces the neurological deficits [2] and corroborated by others [1,5,15,16]. Several possible mechanisms have been proposed which relates to favorable outcomes in acute ischemic stroke in hypothyroid patients. Firstly, hypothyroidism is believed to confer a preconditioning to stress leading to reduced sensitivity to adrenergic stimulation prior to stroke [1]. Secondly, the moderated metabolic rate in hypothyroid patients could account for their favorable functional outcomes.

Reported increase in D2 expression leading to high Triiodothyronine (T₃) under ischemic condition [17] along with our published observations that hyperthyroidism augments the

secondary damage resulting from ischemic insult [4] supports the notion that excess of T3 may cause the “burn out” akin to hypermetabolic state and increased production of glutamate and ROS.

Therefore, based on this background we tested the hypothesis that rT3 can produce a brain-specific hypothyroid state that can protect the brain from ischemia. Our results clearly provide evidence for the neuroprotective action of rT3 during ischemic-reperfusion injury under both in vivo as well as in vitro conditions. Oxidative stress, apoptosis and inflammation are central mediators of tissue injury in stroke [18]. In this respect, our results showed that rT3 reduced the induction of oxidative stress and apoptotic signaling after IR insult. Our results also provide evidence that besides acting as a D2 inhibitor to reduce T4 to T3 conversion, rT3 also inhibits D2 transcription. rT3 also increased D3 expression, which together with decreased D2, would lead to a reduced local T3 level in the brain and this effect probably underlines the neuroprotective action of rT3, although additional non-deiodinase mediated signaling by rT3 cannot be ruled out. In this regard, a non-genomic survival signaling has been recently described for rT3 [19].

It is pertinent to point here that although we and other's have shown a negative correlation between T3 levels and stroke outcome, contrasting reports have shown that low circulating T3 is a predictors of poor stroke outcome [20–24]. We believe that this discrepancy may exist due to a differential need of T3 action during the course of IR initial insult followed by recovery. The stroke outcome is essentially governed by three stage process of primary injury through clot formation resulting in oxygen-glucose deprivation, secondary injury subsequent to reperfusion and recovery period that requires restoration of neurogenesis. Given the fact that T3 increase oxidative stress in neurons [25] it is plausible that high T3 levels may be detrimental during the initial hours of IR injury. We believe that hypothyroidism and agents such as rT3 which induce tissue specific T3 reduction could be beneficial at this injury stage. However, during the later recovery/neurogenesis phase T3 action may be beneficial based on earlier reports [26].

In conclusion, this study provides for the first time a proof-of-concept for using deiodinase II inhibitor, rT3 to improve stroke outcome by dampening TH signaling in the brain. Further studies are warranted to further explore the mechanistic basis of rT3 neuroprotective action in ischemic brain and testing its clinical efficacy.

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Fig. 1. rT3 prevents ischemic-reperfusion injury in rats.

(A,B) 2,3,5-Triphenyl tetrazolium chloride (TTC) stained coronal section of rat brain from different group for infarct assessment. Sham operated rats are denoted as “C”. Unstained area represents the infarct area of the brain tissue. Significant decrease in infarct volume in rT3 (50 ng/100 g B.W). treated rats after MCAO. Values are means $\pm$ SD (n = 10), **p < 0.01 vs control (c), #p < 0.05 vs respective IR.(C) rT3 treated rats after MCAO showed better neurological score in vivo. All rats showed normal postural reflex (neurological deficit score = 0) before intervention. Values are means $\pm$ SD (n = 10), #p < 0.05 vs respective IR

control. **(D)** rT3 attenuated brain tissue injury as indicated by LDH activity, Values are means $\pm$ SD (n = 10), **p < 0.01 vs control, ##p < 0.01 vs respective IR control. **(E)** Histogram showing %TUNEL positivity (apoptotic marker) in the cerebral cortex post IR injury $\pm$ rT3 treatment per 100 nuclei in 8–10 different fields, **p < 0.01 vs control, #p < 0.01 vs respective IR control. Values are means $\pm$ SD (n = 5), **p < 0.01 vs control, ##p < 0.01 vs respective IR control.

Fig. 2. rT3 reduces IR induced oxidative stress.

(A) Malondialdehyde (MDA) levels (index of lipid peroxidation) in brain tissue was significantly decreased in rT3 treated rats. Values are means $\pm$ SD (n = 10), **p < 0.01 vs control, ##p < 0.01 vs respective IR control. (B) Antioxidant glutathione (GSH) levels in brain tissue was maintained in rT3 treated rats in vivo, Values are means $\pm$ SD (n = 10), **p < 0.01 vs control, ##p < 0.01 vs respective IR control. (C) Levels of reactive oxygen species (ROS) in viable neuronal preparation obtained from brain tissues of different group of rats. Data were analyzed using cell Quest software (Becton-Dickinson, USA), **p < 0.01 vs control, ##p < 0.01 vs respective IR. Results are expressed as mean $\pm$ SD (n = 10).

Fig. 3. rT3 reduce OGD/R induced toxicity in primary cortical neurons in culture.

(A) Viability of neurons in control and after 24 h OGD (OGD/R) and with rT3 treatment were estimated by trypan blue exclusion test (n = 3), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3/OGD/R vs respective OGD/R alone group. (B) Cell death was assessed by measuring the release of the LDH in the cell media and is expressed as percent of OGD/R (n = 6), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3+OGD/R vs respective OGD/R alone group. (C) Levels of ROS in neurons in arbitrary fluorescence unit (AFU) were obtained from fluorescence of DCFHDA dye in

FACS. (n = 6), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3+OGD/R vs respective OGD/R alone group. (D) Malondialdehyde (MDA) levels (marker of lipid peroxidation) in neuronal cell in vitro. (n = 6), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3+OGD/R vs respective OGD/R alone group. Results are expressed as mean $\pm$ SD. (E) GSH levels (marker of cellular anti-oxidant capacity) in neuronal cell in vitro. (n = 6), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3+OGD/R vs respective OGD/R alone group. Results are expressed as mean $\pm$ SD. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. rT3 alter the expression of D2 and D3 under ischemic-reperfusion injury in animals and *in vitro*.

(A, B) Real-Time PCR analysis show a suppressive and stimulatory effect of rT3 on D2 and D3 expression respectively in animals undergoing IR injury, (n = 6), **p < 0.01 vs control, #p < 0.01 vs. respective IR control. (C, D) rT3 similar to its effect *in vivo* also blunted D2 increase in OGD/R *in vitro*. rT3 further increased the expression of D3 induced under OGD/R, (n = 6), *p < 0.01 denotes OGD/R treated vs untreated control, #p < 0.01 denotes rT3+OGD/R vs respective OGD/R alone group. Results are expressed as mean ± SD. (E)

Real-Time PCR analysis show a inhibitory effect of rT3 on T3 responsive gene *Bdnf* expression respectively in animals undergoing IR injury, (n = 6), **p < 0.01 vs control, #p < 0.01 vs respective IR control. Results are expressed as mean $\pm$ SD.

Table 1

Blood pressure and heart rate were measured in 5 animals in each group. Other parameters were measured in 20 animals in each group. Data were expressed in mean +SD and statistical significance by student's t-test.

Groups	Body weight (g)	Blood pressure (mm mercury)	Heart rate (beats/min)	Thyroid hormone		Body Temperature in C		
				T3	T4	Before	At a time	after reperfusion
				(nM)				
Control	270 + 30	106 + 10	280 + 20	1.05 + 0.13	56 + 10	37 + 0.5	37.3 + 0.4	37 + 0.3
rT3	280 + 20	110 + 8	270 + 15	1.15 + 0.10	60 + 5	37 + 0.3	37.2 + 0.4	37 + 0.5