Novel Thyroxine Derivatives, Thyronamine and 3-iodothyronamine, Induce Transient Hypothermia and Marked Neuroprotection Against Stroke Injury

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Background and Purpose—Mild hypothermia confers profound neuroprotection in ischemia. We recently discovered 2 natural derivatives of thyroxine, 3-iodothyronamine (T1AM) and thyronamine (T0AM), that when administered to rodents lower body temperature for several hours without induction of a compensatory homeostatic response. We tested whether T1AM- and T0AM-induced hypothermia protects against brain injury from experimental stroke.

Methods—we tested T1AM and T0AM 1 hour after and 2 days before stroke in a mouse model of focal ischemia. To determine whether T1AM and T0AM require hypothermia to protect against stroke injury, the induction of hypothermia was prevented.

Results—T1AM and T0AM administration reduced body temperature from 37°C to 31°C. Mice given T1AM or T0AM after the ischemic period had significantly smaller infarcts compared with controls. Mice preconditioned with T1AM before ischemia displayed significantly smaller infarcts compared with controls. Pre- and postischemia treatments required the induction of hypothermia. T1AM and T0AM treatment in vitro failed to confer neuroprotection against ischemia.

Conclusions—T1AM and T0AM are potent neuroprotectants in acute stroke and T1AM can be used as antecedent treatment to induce neuroprotection against subsequent ischemia. Hypothermia induced by T1AM and T0AM may underlie neuroprotection. T1AM and T0AM offer promise as treatments for brain injury. (Stroke. 2007;38:2569-2576.)

Key Words: hypothermia ■ neuroprotection ■ stroke

Thyroxine (T4) is the principal secreted form of thyroid hormone (TH), constituting 95% of all TH found in human circulation.1 In target tissues (adipose, heart, brain), T4 is deiodinated to 3,5,3,-triiodothyronine, a more active form of TH displaying a higher affinity for the TH nuclear receptors TRα and TRβ. T1 and 3,5,3,-triiodothyronine control a wide range of physiological processes, including metabolism, thermogenesis, brain development, and cardiac function. Individuals with elevated blood levels of TH display high metabolic rates, body temperatures, and heart rates. T1 and 3,5,3,-triiodothyronine are slow acting, taking hours to days to affect these physiological processes by altering gene transcription.

It has been proposed that TH is further deiodinated and decarboxylated to 3-iodothyronamine (T1AM) and thyronamine (T0AM).3 These molecules are present in the mammalian brain, peripheral organs, and blood.3 The nonselective enzyme, aromatic amino acid decarboxylase, promotes the decarboxylation of a wide variety of aromatic amino acids and, coupled with the deiodinases, has been proposed to convert T1 into T1AM and T0AM.3

Recently, we showed that synthetic T1AM and T0AM injected intraperitoneally into adult male C57Bl/6 mice have the opposite effect of T1 and 3,5,3,-triiodothyronine, rapidly inducing hypothermia through a mechanism independent of gene transcription.3 T1AM and T0AM are not ligands for traditional nuclear TH receptors but are agonists of trace amine associated receptor 1 (TAAR1), a G-protein coupled receptor activated by phenylethylamine, tyramine, methamphetamine, and its congeners.4 Although T1AM and T0AM can dose-dependently couple TAAR1 to the production of cAMP, it is not yet clear whether TAAR1 is an endogenous receptor for these molecules.

A single injection of T1AM and T0AM (50 mg/kg) will induce hypothermia in mice within 30 minutes of intraperitoneal administration. This hypothermic response is maintained for 6 hours and is lost by 10 hours postinjection. Hypothermia occurs with no apparent long-term adverse effects and, importantly, with no evidence of shivering or piloerection, suggesting that T1AM/T0AM-induced hypothermia is not opposed by the body’s natural homeothermic response to cold temperatures.3
Hypothermia is well known to induce profound neuroprotection in the setting of stroke. This protective effect depends on the duration and depth of hypothermia and its timing relative to stroke onset. The mechanisms by which hypothermia protects against ischemic injury are numerous and include reductions in metabolic rate, free radical formation, reperfusion injury, and glutamate release. No single action of hypothermia appears to be responsible for its marked neuroprotective actions.

Specific molecular mechanisms also contribute to ischemic protection in the hypothermic animal. After focal cerebral ischemia, mild hypothermia has been shown to increase Bcl-2 expression, decrease Bax expression, and attenuate cytochrome c release from mitochondria. Hypothermia also prevents responses to ischemia that would otherwise promote apoptosis such as increased expression of p53 and attenuation of Akt activity.

Hypothermia is currently achieved through methods that force temperature below the internal homeostatic set point. Such methods include the use of intravascular cooling devices or external cooling by application of ice, cold water, or alcohol to the subject’s body. Forced cooling induces compensatory responses such as shivering, piloerection, and vasoconstriction. Pharmacologic induction of hypothermia using cryogens that alter the subject’s homeostatic temperature set point, without inducing compensatory responses, are promising alternatives to external physical cooling methods. Hence, T1AM and T0AM may be attractive candidate neuroprotectants in ischemia.

We investigated the potential of synthetic T1AM and T0AM to confer neuroprotection acutely in a rodent model of stroke. The ability of these compounds to induce hypothermia could benefit most patients with stroke for whom treatment is only possible after the onset of symptoms. We also tested whether antecedent treatment with T1AM or T0AM preconditions the brain against injury from subsequent exposure to ischemia. Previous studies have shown that brief exposure to hypothermia before ischemia induces tolerance to brain injury. Such pretreatment offers hope to individuals in whom brain ischemia is anticipated such as those undergoing surgery (eg, coronary artery bypass grafting, arterectomy) and other “at-risk” populations.

**Materials and Methods**

3-iodothyronamine and Thyronamine

T1AM and T0AM (Figure 1) were chemically synthesized as previously described and dissolved in 75% saline/25% DMSO (vehicle) at a concentration of 12.5 mg/mL.

**Neuronal Cell Culture**

Cerebral cortices were dissected from E16 C57BL/6 mice and incubated in 0.05% (wt/vol) trypsin-EDTA for 15 minutes at 37°C. Tissue was triturated and cells plated on poly-L-ornithine-coated 96-well plates or 25-mm² glass coverslips at 110⁵ cells per well or 110⁶ cells per coverslip. Cells were cultured in Neurobasal medium supplemented with L-glutamine and B27. Cultures consisted of 50% to 60% neurons as assessed by NeuN staining on coverslips.

**Oxygen and Glucose Deprivation**

OGD was performed by washing cells with phosphate-buffered saline and placing them in an anaerobic chamber for 180 minutes (Coy Laboratories, 85% N₂, 5% H₂, 10% CO₂; 35°C). OGD was terminated by removing cells from the chamber, replenishing with media, and placing them back into the normoxic incubator. For acute administration, T1AM or T0AM was added to the culture media immediately post-OGD and remained present for 24 hours, at which point cell viability was assessed. A peptide based on amino acids 109 to 153 of the neuroprotective protein osteopontin was used as a positive control (Invitrogen; dose: 5 nmol/L) (Doyle K, unpublished data, 2006). The osteopontin peptide sequence is: S(p)DES(p)HHS(p)DES(p)DE-TVTASTQADTFPIPVPTVDVPVNGRDLSAYGLR. For preconditioning administration, T1AM and T0AM were added to the culture media 2 days before OGD. T1AM and T0AM were removed at the time of OGD by washing with phosphate-buffered saline.

**Cell Viability Assay**

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Briefly, 24 hours post-OGD, cells in 96-well plates were incubated in 200 μL, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (0.5 mg/mL in media) for 1 hour at 37°C followed by removal of media and addition of 100 μL of DMSO. Absorbance was read at 550 nm. Data are expressed as percentage of cell death compared with control cultures that did not undergo OGD.
Mice
Adult (8 to 10 weeks) male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All procedures met National Institutes of Health guidelines with the approval of the Oregon Health & Science University Institutional Animal Care and Use Committee.

Neurological Assessment
To determine the behavioral consequences of T1AM and T0AM administration, mice were intraperitoneally injected with 100 μL of vehicle, T1AM (50 mg/kg), or T0AM (50 mg/kg) and scored for neurological deficits, physical appearance, and behavior hourly for the first 6 hours postinjection and at 24 and 48 hours postinjection (N=8 each group). Mice were scored on each of the following: hair/grooming, spontaneous activity, posture, epileptic behavior, and gait. Each mouse was scored from 0 (no deficit) to 4 (severely affected) in each category.

Surgery
Cerebral focal ischemia was induced by middle cerebral artery occlusion (MCAO) as previously published.15 Mice were anesthetized with 4% halothane and subjected to 40 minutes of MCAO. The middle cerebral artery was blocked by threading silicone-coated 7-0 monofilament nylon surgical suture through the external carotid to the internal carotid, blocking its bifurcation into the middle cerebral artery and anterior cerebral artery. After 40 minutes, the filament was removed, thereby restoring blood flow. Cerebral blood flow was monitored throughout surgery by laser Doppler flowmetry. During surgery, body temperature was maintained at 37°C with a thermostat-controlled heating pad. To facilitate recovery after stroke, mouse cages were kept on a heating pad that maintained cage temperature at 28°C, thereby reducing the thermoregulatory burden on the recovering animals.

Temperature Measurement
Mouse temperature was measured using a Raytek MX2 infrared thermometer (Infra-red-USA.com) (emissivity=0.98).16 In a control study, readings from this thermometer showed a high correlation with rectal thermometer readings taken from mice killed acutely or from mice injected with 50 mg/kg T1AM. The 2 methods showed an average difference in recorded temperature of 0.48°C as the mice reached 31°C within 30 minutes of injection (Figure 2A). The 2 methods showed an average difference in recorded temperature of 0.48°C as the mice reached 31°C within 30 minutes of injection (Figure 2A).

Acute Administration of 3-iodothyronamine and Thyronamine
Mice were injected intraperitoneally with 100 μL of vehicle, T1AM (50 mg/kg), or T0AM (50 mg/kg) 1 hour after termination of ischemia and placed in a cage on a heating pad. Cage temperature was maintained at 28°C, thereby allowing the temperature of the drug-treated animals to fall. For the T1AM and T0AM “hypothermia-blocked” group of animals, mice were kept in a separate cage on an adjustable heating pad, which was continually adjusted to keep drug-treated animals at the same temperature as vehicle-injected animals, which were closely monitored by infrared thermometry.

Preconditioning Administration of 3-iodothyronamine and Thyronamine
Mice were injected intraperitoneally with 100 μL vehicle, T1AM (50 mg/kg), or T0AM (50 mg/kg) 2 days before MCAO. Injections took place between 9:00 AM and 10:00 AM. After injection, mice were maintained at ambient temperature (22°C) and their temperature recorded for 6 hours postinjection. For the T1AM “hypothermia-blocked” group of animals, mice were kept in a separate cage on an adjustable heating pad, which was continually adjusted to keep drug-treated animals at the same temperature as vehicle-injected animals, which were closely monitored by infrared thermometry, as previously described. After surgery, all preconditioned mice were kept in cages maintained at 28°C and temperature was recorded every hour for the first 6 hours postsurgery (48 to 54 hours postinjection) and immediately before being euthanized (72 hours postinjection).

Infarct Measurement
Coronal brain sections (1 mm) were placed in 1.5% 2,3,5-triphenyl-tetrazolium chloride ( TTC) in phosphate-buffered saline for 15 minutes at 37°C.18 Stained sections were scanned and measured using ImageJ by a technician blinded to treatment group.19 Measurements were multiplied by the section thickness (1 mm) and summed over the entire brain to yield volume measurements. The percent infarct was calculated as: 100(infarct volume)/(ipsilateral hemisphere volume).

Statistical Analysis
Data are shown as means±SEM of number determinations. Data from cell viability assays and in vivo stroke experiments were analyzed by one-way analysis of variance followed by Bonferroni multiple comparison test using Graphpad Prism version 4.0 (Graphpad Software).

Results
Acute Administration of 3-iodothyronamine and Thyronamine Induces Hypothermia and Significant Neuroprotection Against Stroke Injury
To investigate whether acute administration of T1AM or T0AM protects against ischemic injury, mice were injected intraperitoneally with 50 mg/kg of T1AM or T0AM 1 hour after MCAO. This dose was selected based on our previous finding that the level and duration of hypothermia induced is similar to that induced by physical means in neuroprotective trials.3,20 This dose is well tolerated, causing a temporary reduction in spontaneous activity without other discernable behavioral changes (Table). Administration of T1AM and T0AM caused a reduction in rectal temperature to an average of 31°C within 30 minutes of injection (Figure 2A). The
reduction in body temperature occurred in the absence of shivering or piloerection. By 24 hours postinjection, the body temperature of T1AM- or T0AM-treated mice had returned to the same level as vehicle-injected mice.

Animals administered T1AM and T0AM had significantly reduced infarct volumes, showing 35% and 32% reductions, respectively, compared with vehicle-injected mice (Figure 2B). To determine whether the neuroprotective effect of T1AM and T0AM required a reduction in body temperature, animals were injected with T1AM or T0AM and body temperature was maintained by placing the animals on an adjustable heating pad after MCAO. Body temperatures were monitored hourly and the heating pad was adjusted to maintain temperature of the T1AM/T0AM-injected animals at the same level as vehicle-injected animals. Blockade of T1AM- or T0AM-induced hypothermia eliminated the protective effects of these drugs (Figure 2C) indicating that the neuroprotective effect of these molecules requires the induction of hypothermia.

**Acute Administration of 3-iodothyronamine and Thyronamine In Vitro Does Not Confer Neuroprotection Against Ischemic Injury**

We next examined the acute effects of T1AM and T0AM on primary neuronal cultures using modeled ischemia in vitro. Escalating doses (5 nM to 500 µmol/L) of T1AM or T0AM were applied to primary mouse neuronal cultures maintained at 37°C immediately after exposure to 3 hours of OGD. In these in vitro conditions, T1AM and T0AM do not induce hypothermia and failed to provide protection from OGD-
induced cell death (Figure 3). Furthermore, high doses (50 μmol/L, 500 μmol/L) of T1AM and T0AM were cytotoxic. Treatment with 5 nM of a neuroprotective peptide (osteopontin peptide) was used as a positive control. Collectively, these in vivo and in vitro experiments suggest that acute administration of T1AM and T0AM confers neuroprotection by a temperature-dependent mechanism.

**Preconditioning With 3-iodothyronamine, but Not Thyronamine, Confers Protection Against Stroke Injury**

T1AM and T0AM were tested for their ability to precondition the brain against ischemic injury through an antecedent stimulus of brief hypothermia. Mice were preconditioned by an intraperitoneal injection of vehicle, T1AM (50 mg/kg), or T0AM (50 mg/kg) 2 days before MCAO. Temperature was measured for 6 hours postinjection. The body temperature of mice injected with T1AM or T0AM dropped within 30 minutes (Figure 4A) and animals remained hypothermic for 6 hours. Importantly, mice were normothermic by the time of MCAO challenge. Mice preconditioned with T1AM showed a significant reduction in infarct size (34%) compared with vehicle-treated animals. Mice preconditioned with T0AM showed modest neuroprotection, which did not reach significance (Figure 4B). To test whether the effect of preconditioning with T1AM depends on hypothermia, the T1AM-induced decrease in body temperature was blocked by maintaining the mice on a heating pad. Under these conditions, T1AM administration failed to protect mice from subsequent ischemic challenge. Thus, preconditioning with T1AM appears to depend, in part, on hypothermia. Further support for the requirement of hypothermia in T1AM preconditioning was evinced from neuronal cultures treated with T1AM before exposure to OGD. Primary mouse neuronal cultures pretreated with escalating doses of T1AM or T0AM (5 nM to 500 μmol/L) 2 days before exposure to 3 hours OGD failed to show enhanced protection compared with vehicle-treated cultures (Figure 5).

**Discussion**

These studies show that the thyronamines T1AM and T0AM confer protection against ischemic brain damage when administered acutely after stroke. In addition, this is the first demonstration that a cryogen (T1AM) may be prophylactically administered in situations of anticipated ischemic injury. T1AM and T0AM induce hypothermia—a condition required for the therapeutic neuroprotective effect of these compounds in both acute therapy and preconditioning. Our studies using modeled ischemia in vitro support the requirement for hypothermia in T1AM- and T0AM-induced neuroprotection.

We and others found that after MCAO, mice regulate their temperature to a cooler 34°C to 35°C. In this study, all vehicle-injected mice were mildly hypothermic after stroke, as seen in Figures 2A and 4A, in which vehicle-treated animals show a temperature of between 34°C and 35°C after stroke.

On the basis of the structural similarity between thyronamines and dopamine, it is conceivable that T1AM and T0AM activate a cognate thyronamine G-protein coupled receptor such as TAAR1, thus constituting a new signaling pathway that rapidly results in hypothermia. TAAR1 is a 7-transmembrane G-protein coupled receptor related to catecholamine and 5-hydroxytryptophan receptors. TAAR-1 is an excellent candidate receptor for T1AM and T0AM given the chemical similarity between thyronamines and biogenic amines and because TAAR-1 belongs to the biogenic amine G-protein coupled receptor subfamily whose endogenous agonist remains to be established. Our studies do not address the specific ligand/receptor interactions of T1AM or T0AM; however, such information is under active pursuit and is of great interest to this field.

Preclinical tests with hypothermic durations of 6 hours or more have demonstrated that postischemic hypothermia is effective and that the therapeutic window expands as the duration of hypothermia is increased; when cooling lasts for 48 hours, significant long-term protection of CA1 hippocampal neurons can be achieved even when cooling is delayed by 12 hours. We have shown that T1AM and T0AM can confer neuroprotection when treatment is delayed by 1 hour. We are currently pursuing the full time window available for postischemic administration of these molecules and anticipate that the time window available for T1AM and T0AM administration will mirror the time window available for other hypothermia treatments.

Postischemic hypothermia can also be used to extend the time window available for administering additional neuroprotective agents. Mild postischemic hypothermia has been used successfully in rats to prolong the therapeutic window for Bcl-2 gene therapy from 1.5 to 5 hours. Thus, acute administration of T1AM and T0AM could be a means of...
extending the time window available for administration of other neuroprotectants.

We have shown that T1AM provides antecedent protection to ischemia, an effect that requires hypothermia. This suggests that T1AM triggers hypothermia-induced tolerance to ischemia. Hypothermia-induced ischemic tolerance is initiated within 6 hours, peaks at approximately 1 or 2 days, and is reversed by 7 days after preconditioning stimulation. It is likely that a hypothermic threshold must be reached for successful preconditioning to occur. Our findings support this

Figure 4. Preconditioning with T1AM but not T0AM induces delayed tolerance to ischemic injury. A, Mice were injected with 50 mg/kg T1AM or T0AM 2 days before MCAO. Temperature was measured for 6 hours postinjection. In a separate group (T1AM hypothermia-blocked), mice were maintained on an adjustable heating pad to prevent hypothermia induction. Temperature was also measured for 6 hours postsurgery (48 to 54 hours postinjection) and immediately before being euthanized (72 hours postinjection). B, Infarct volume after antecedent treatment with T1AM, T0AM, and T1AM with hypothermia blocked. N=8 each group. Error bars represent SEM. *P<0.05 compared with vehicle-injected.

Figure 5. T1AM and T0AM preconditioning in vitro. Mouse primary neuronal cultures were treated with the indicated doses of T1AM and T0AM 2 days before OGD. Cell viability assays were performed 24 hours post-OGD (N=3). Error bars represent SEM. *P<0.05 compared with sham wash.
possibility because T1AM failed to induce the same depth and duration of hypothermia as T0AM and failed to confer antecedent protection to ischemia. This is also supported by a recent study by Yunoki et al, in which the magnitude of tolerance induced by hypothermic preconditioning was dependent on the depth and duration of the hypothermic stimulus.12

The mechanism by which hypothermic preconditioning protects the brain almost certainly overlaps with the mechanism by which acute induction of hypothermia protects the brain but is unlikely to be identical. Acute administration of hypothermia can directly protect neurons postsischemia by reducing metabolic rate.25 It has been demonstrated that each 1°C decrease in temperature results in a 10% reduction in tissue metabolic requirements and free radical production (the Q10 effect).25 This process can benefit neurons when hypothermia is induced during or after ischemia but not when hypothermia is induced before ischemia, because it is too temporally remote to the injury process. Instead, the protective effect of hypothermic preconditioning may rely on cellular reprogramming and biochemical changes in the intracellular and extracellular milieu.26

In support of this postulate, hypothermia-induced preconditioning depends on de novo protein synthesis, and in SHSY5 cells, it requires modification of proteins by SUMOylation.13,27 Transcription factors are the main targets of SUMO conjugation and SUMOylation of these proteins generally causes transcriptional suppression. Whether administration of T1AM and T0AM causes widespread SUMOylation of transcription factors is currently under investigation. SUMOylation could in part explain the suppression of gene transcription common to multiple preconditioning stimuli.26,28

There is precedence for studying the neuroprotective effect of drug-induced hypothermia, but our studies are the first to apply endogenous molecules to induce hypothermia as a treatment for stroke. The synthetic cannabinoid HU-120 has been used to induce hypothermia and protect against ischemia in rats.29 However, at hypothermia-inducing doses, HU-120 has toxic side effects that limit its application for humans. Other studies with hypothermia-inducing cannabinoids have been more encouraging. The synthetic cannabinoid WIN 55,212-2 can induce therapeutic hypothermia with fewer side effects than HU-120; however, effective treatment with WIN 55,212-2 requires continuous intravenous infusion for the hypothermic effect to be maintained.30 A recent study demonstrates that hydrogen sulfide can be used to reduce temperature in rodents;31 however, this approach may have limited benefit in brain ischemia because hydrogen sulfide enhances NMDA receptor function, which could lead to increased excitotoxicity.32

HU-120 and WIN 55,212-2 appear to induce hypothermia by binding directly to hypothalamic CB1 receptors and altering the homeostatic temperature set point.30 Hydrogen sulfide likely induces hypothermia less specifically by inhibition of complex IV in the electron transport chain, which causes global hypometabolism.31 We postulate that T1AM and T0AM induce hypothermia in a manner that more closely resembles the synthetic cannabinoids, working through a specific receptor such as TAAR1 and inducing anaplexia (regulated hypothermia).

Humans have greater thermal inertia than smaller animals such as mice. Thus, T1AM and T0AM should be tested in larger species such as nonhuman primates to determine the duration and depth of hypothermia that can be achieved. If such studies reveal a hypothermic effect similar to that seen in rodents, T1AM and T0AM may be considered for future testing as neuroprotectants in humans.

In conclusion, T1AM and T0AM can be used to induce a therapeutic level of hypothermia and, because both are endogenous metabolites of thyroxine, they may be tolerated with fewer side effects than artificially engineered compounds. These unique molecules have developmental potential as cryogens for the treatment of stroke, in which rapid and prolonged cooling offers outstanding therapeutic benefit to patients.

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Disclosures

None.

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