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IN VITRO THYROID HORMONE RAPIDLY MODULATES PROTEIN PHOSPHORYLATION IN CEREBROCORTICAL SYNAPTOSOMES FROM ADULT RAT BRAIN

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Abstract—Thyroid hormones induced rapid changes in phosphorylation in a membrane-containing lysate of synaptosomes purified from adult rat cerebral cortex. The *in vitro* addition of 3,5,3′-L-triiodothyronine or L-thyroxine strongly influenced incorporation of label from [γ-^{32}P]-ATP into proteins in a cerebrocortical synaptosomal lysate. Incubation with 3,5,3′-L-triiodothyronine or L-thyroxine had strong biphasic dose-dependent effects on the phosphorylation of 38±1, 53±1, 62±1, and 113±1 kDa proteins (which we termed α, β, γ, and δ, respectively) and several others. Although we observed differing levels of phosphorylation among the four proteins, doses of 3,5,3′-L-triiodothyronine or L-thyroxine ranging from 1 to 30 nM caused significant dose-dependent stimulation of the phosphorylation of all of them, an effect which occurred within three minutes. In each case, the enhancement of phosphorylation diminished with higher concentrations (100 nM–1 μM) of 3,5,3′-L-triiodothyronine. In contrast, incubations with similar doses of 3,3′,5′-L-triiodothyronine (reverse L-triiodothyronine) were without significant effect, indicating a specificity for 3,5,3′-L-triiodothyronine and L-thyroxine. Western blots of synaptosomal lysates incubated with 3,5,3′-L-triiodothyronine (1 nM–1 μM) demonstrated phosphorylation at the serine residues of a 112 kDa protein (matching δ) and phosphorylation at tyrosyl residues of a distinct 95 kDa protein. These data support the contention that thyroid hormones have a variety of rapid nongenomic pathways for regulation of protein phosphorylation in mature mammalian brain. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: thyroid hormone, synaptosome, protein kinase, phosphotyrosine, phosphoserine.

A growing body of evidence indicates a unique mechanism of action of thyroid hormones (THs) in adult mammalian brain. During a critical period of early life, THs are essential for normal growth and development of the CNS (Anderson et al., 2003; Bernal, 2002). The importance of TH effects in mature brain is underscored by the variety of neurological and psychiatric disorders resulting from adult-onset thyroid disturbance, including anxiety, lethargy, sleep disorders, mood disorders, cognitive deficits, and seizures (Bauer et al., 2003; Haggerty and Prange, 1995; Laurberg, 1990; Sarkar, 2002; Utiger, 1987). In contrast to the developmental effects of THs, most of the CNS-related changes occurring with adult dysthyroidism are reversible with the adjustment of circulatory TH levels (Bunevicius and Prange, 2000; Mandel et al., 1993). The developmental effects of THs in brain are thought to be mediated through nuclear TH receptors which regulate gene expression (Konig and Neto, 2002; Oppenheimer, 1999). Although nuclear TH receptors are present in adult mammalian brain (Luo et al., 1988), relatively few effects on CNS gene expression in adulthood are directly ascribed to them (Bernal, 2002; Calza et al., 1997).

THs are concentrated and metabolized in nerve terminal fractions (synaptosomes) of adult rat brain (Dratman et al., 1976; Dratman and Crutchfield, 1978; Mason et al., 1993; Sarkar and Ray, 1994). Furthermore, specific TH-binding sites have been demonstrated in synaptosomal membranes from adult rats (Mashio et al., 1982, 1983; Sarkar and Ray, 1998) and developing chick brain (Giguere et al., 1992), consistent with the existence of one or more membrane receptors for TH. Treatment of rat brain synaptosomes with THs has been shown to inhibit GABA uptake (Mason et al., 1987), decrease Na^+/K^+ ATPase activity (Sarkar and Ray, 1993, 1998) and enhance presynaptic flux of Ca^{2+} (Chakrabarti and Ray, 2000; Mason et al., 1990; Sarkar and Ray, 2003). In synaptoneurosomes, a preparation of sacs composed of both pre- and postsynaptic membrane from rat brain, THs inhibit the stimulation of chloride flux by GABA or muscimol (Martin et al., 1996, 2004). The rapid *in vitro* effects of TH on subcellular fractions of adult rat brain with minimal nuclear contamination are not likely to be mediated by a mechanism regulating gene expression. Therefore, it is frequently hypothesized that in adult brain TH has nongenomic neurotransmitter-like mechanisms of action, distinct from the genomic actions regulating nervous system development during the critical period of early life (Dratman, 1974; Dratman and Gordon, 1996; Martin et al., 1996; Mason et al., 1993; Sarkar, 2002).

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CaM, calmodulin; ECL, enhanced chemiluminescence associated with horseradish peroxidase-catalyzed oxidation of luminol; EGTA, ethylene glycol tetraacetic acid; ERK1 and ERK 2, extracellular signal-regulated protein kinases 1 and 2; G-protein, guanine nucleotide binding protein; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); HRP, horseradish peroxidase; MAPK, mitogen-activated protein kinase; Na_3VO_4, sodium *ortho*-vanadate; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; rT3, 3,3′,5′-L-triiodothyronine (reverse T3); SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5); TBST, Tris-buffered saline containing 0.05% Tween-20; TH(s), thyroid hormone(s); T3, 3,5,3′-L-triiodothyronine; T4, L-thyroxine.

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A variety of nongenomic effects of TH has been documented in non-neural tissues including liver, heart, fat, and blood (Bassett et al., 2003; D'Arezzo et al., 2004; Davis and Davis, 2002; Segal, 1989). L-Thyroxine (T4) has been shown to stimulate the mitogen-activated protein kinase (MAPK) pathway in cultured HeLa and CV-1 cells which do not have functional nuclear TH receptors (Lin et al., 1999). T4-agarose also promotes phosphorylation of MAPK and MAPK substrates in a variety of intact cell lines (Lin et al., 2003; Tang et al., 2004), while guanosine 5′-*O*-(3-thiotriphosphate) or pertussis toxin inhibit T4-induced MAPK phosphorylation (Lin et al., 1999), consistent with a cell membrane mechanism mediated via a guanine nucleotide binding protein (G-protein). T4 and 3,5,3′-L-triiodothyronine (T3) were found to inhibit G-protein activity in synaptosomes from developing chick brain (Giguere et al., 1996). While a direct influence of TH on protein kinase activity has not been previously investigated in tissues from mature brain, hypothyroidism has been associated with decreased levels of phosphorylated MAPK in hippocampus from intact adult rat (Gerges and Alkadhi, 2004).

On the basis of these observations, the current studies investigated the possibility of a metabotropic pathway for rapid neurotransmitter-like actions of TH by testing for *in vitro* effects of T3 on protein phosphorylation in osmotically ruptured synaptosomes purified from adult rat brain.

EXPERIMENTAL PROCEDURES

Materials

Mouse AC-40 monoclonal antibody raised against an epitope on the C-terminal end of actin, purified goat antibody raised against mouse IgG Fab fragment and conjugated to horseradish peroxidase (HRP), bovine serum albumin (BSA), calmodulin (CaM), disodium ATP, EGTA, 2-mercaptoethanol, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), sodium dodecyl sulfate (SDS), sodium *ortho*-vanadate (Na_3VO_4), sodium salt of T3, T4, 3,3′,5′-L-triiodothyronine (reverse T3; rT3) and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kits for enhanced chemiluminescence (ECL) associated with horseradish peroxidase-catalyzed oxidation of luminol and Hyperfilm ECL X-ray film were obtained from Amersham Biosciences Corp (Piscataway, NJ, USA). Perkin Elmer Life and Analytical Sciences (Boston, MA, USA) was the source of [γ-^{32}P]-ATP (specific activity: 10 Ci/mmol). Polyvinylidene difluoride (PVDF) membrane and electrophoresis supplies were purchased from Bio-Rad Life Science Research (Hercules, CA, USA).

Mouse monoclonal antibodies against phospho-tyrosine (independent of surrounding amino acid sequence) and phosphoserine [surrounded by Pro at the+2 position and Arg/Lys at the −3 position (14-3-3 binding motif)] were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

Animals

Young adult male Sprague–Dawley rats (~3 months old) were purchased from Hilltop Laboratory Animals (Scottsdale, PA, USA) and were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals in a vivarium accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Temperature was controlled (20–24 °C) and the light phase of the 12-h light/dark cycle began at 8:00 AM. The procedures were approved by the Rutgers University Institutional Review Board, which determined that the experiments appropriately minimized the number of animals used and their suffering.

Preparation of purified synaptosomal fraction

Rats were decapitated and their brains were immediately dissected in ice-cold isotonic saline solution. Synaptosomes were purified from cerebral cortical tissue by discontinuous density gradient centrifugation (Hajos, 1975). The cerebral cortex was homogenized (10% w/v) in 0.32 M sucrose (pH 7) and centrifuged at 1000×*g* for 10 min at 4 °C to remove cell debris and nuclei. The pellet was resuspended in 5 ml of sucrose solution and re-centrifuged at 1000×*g* for another 10 min. The supernatant obtained from the second centrifugation was further centrifuged at 8000×*g* for 20 min. The P2 pellet obtained from the second centrifugation was suspended in 5 ml of 0.32 M sucrose solution, layered over 20 ml of 0.8 M sucrose and centrifuged at 9000×*g* for 30 min. The 0.8 M fraction containing the synaptosomes was collected carefully, discarding the top layer and the bottom pellet. The 0.8 M fraction was immediately diluted with a calculated amount of ice-cold bi-distilled water to bring the concentration of the fraction to 0.4 M and centrifuged at 12,000×*g* for 20 min. The final synaptosomal pellet was suspended in 5 ml of 0.32 M sucrose and repelleted at 12,000×*g* for 20 min to get the purified synaptosomal fraction. This final pellet was lysed with hypotonic shock and assayed for protein (Vera, 1988). The synaptosomal lysate, including cell membrane and soluble content, was diluted to one mg/ml, and frozen in aliquots at −70 °C.

Phosphorylation reactions

Synaptosomal lysate was incubated at a final concentration of 0.33 mg protein/ml in a reaction mixture containing 50 mM HEPES, 10 mM $MgCl_2$, 0.1 mM EGTA at pH 7.4 with and without various concentrations of T3 (1 nM–1 μM) or other additions as indicated. T3 was dissolved in a minimum volume of 0.01 M NaOH, diluted with reaction buffer and the pH was adjusted to 7.4. Control solutions and all hormone dilutions were prepared with the same concentrations of NaOH and reaction buffer and were protected from light. In some cases, 1 mM Na_3VO_4, a tyrosine-specific phosphatase inhibitor (Gordon, 1991) was used as a positive control (Fig. 1). Na_3VO_4 was activated by boiling and adjusting the pH to 10, and then stored at −20 °C for later use (Gordon, 1991).

Except in time-response experiments, T3 was pre-incubated with the synaptosomal lysate for 1 h at 0 °C and then for 5 min at 37 °C. The phosphorylation reaction was initiated by addition of a final concentration of 20 μM unlabeled ATP for Western blot analysis or 20 μM [γ-^{32}P]-ATP (3 μCi) for autoradiographic experiments at 37 °C. Reactions were terminated after the indicated intervals by addition of 1:3 (v/v) of 4× SDS-sample buffer containing 12% SDS, 40% glycerol, 0.2 M Tris–HCl and 0.004% Bromophenol Blue at pH 7.0.

Analysis of proteins

SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. Samples were denatured by heating at 98 °C for 3 min, and then subjected to 7.5% SDS PAGE at a constant current (Laemmli, 1970). The gel was stained with silver nitrate, dried and exposed to X-ray film at −80 °C for 48 h. The optical densities (OD) of protein bands in the gel and the phosphorylated bands in the autoradiogram were quantified using the ONE-DSCAN program (Scanalytics, Inc., Fairfax, VA, USA). The data were then transformed, quantified and graphed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). From all experiments, the same four most distinctly influenced bands were quantitated and are referred to as α, β, γ, and δ, respectively.