In Vitro Actions of Thyroid Hormone on Tyrosine-Directed Phosphorylation of Proteins in a Nucleus-free Subcellular Fraction from Adult Rat Brain.

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Introduction

Several converging lines of evidence point to a unique mechanism of action of thyroid hormones (THs) in adult brain. Developmental effects of THs in brain are thought to be mediated through nuclear TH receptors regulating gene expression (Oppenheimer 1999). While nuclear TH receptors occur in adult mammalian brain, few effects on CNS gene expression in adulthood are directly ascribed to them (Bernal, 2002). In adult brain, THs are concentrated in nerve terminals , suggesting a synaptic action of TH (Dratman, 1974; Mason, et al., 1993; Sarkar and Ray, 1994; Dratman and Gordon, 1996). Actions of TH on mitogen-activated protein kinase have been shown in peripheral tissues (Shih et al., 2001). Furthermore, decarboxylated TH metabolites are potent ligands of a trace amine receptor known to be present in brain (Scanlan et al., 2004). Neurotransmitter-like (or neurosteroid-like) actions of THs in mature neurons have been frequently suggested (Dratman, 1974; Mason, et al., 1993; Martin et al., 1996; Martin et al., 2004; see Dratman and Gordon, 1996; Sarkar, 2002). The present study provides further details of a nongenomic action of L-T3 to modulate protein phosphorylation in synaptosomes from adult rat brain (Sarkar et al., 2003).

Methods

Animals

Young adult male Sprague-Dawley rats (250-300 g) were decapitated and their brains immediately dissected in ice-cold isotonic saline.

Preparation of Synaptosomal Lysate

Synaptosomes were purified from cerebral cortical tissue using the method of Hajos (1975). A P_2 pellet was prepared by differential centrifugation in 0.32 M sucrose and layered on 0.8 M sucrose. Subsequent to an additional centrifugation at 9,000 x g for 30 min, the 0.8 M layer was diluted to 0.4 M. The pellet sedimenting after 20 min at 12,000 x g (purified synaptosomes) was lysed with hypotonic shock. The resulting suspension of soluble and membrane protein (synaptosomal lysate) was the preparation used in all experiments.

Incubation

The synaptosomal lysate was incubated in 10 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM HEPES (pH 7.4) with various concentrations of 3,5,3'-L-triiodothyronine (T3). Except for the time-response experiment, L-T3 was pre-incubated with the synaptosomal lysate for 1 h at 0ºC and for 5 min at 37ºC. The phosphorylation reaction was initiated by addition of a final concentration of 20 μM of unlabeled ATP [or 3 μCi of γ-^{32}P-ATP for autoradiographic experiments] at 37°C. The reaction was terminated by addition of SDS-Sample Buffer (Laemmli, 1970).

SDS-PAGE and Autoradiography

The samples were denatured for 3 minutes at 100°C, subjected to electrophoresis on 7.5 % SDS-PAGE at constant current, and stained with silver nitrate. The dried gels were then exposed to x-ray film at -80°C for 48 hours and the optical densities of bands in the gel and the resulting autoradiogram were quantified using the ONE-DSCAN program (Scanalytics, Inc., Fairfax, VA).

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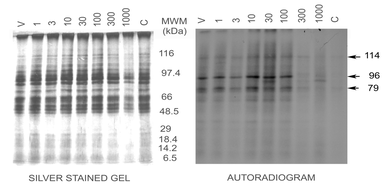
Methods (Continued)

Western Blot

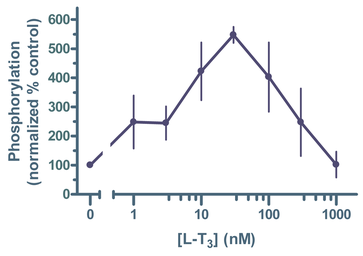
Proteins were transferred from the gel onto PVDF membrane by electrophoresis in a buffer containing 20% methanol, 0.192 M glycine, and 0.025 M Tris (pH = 8.0) using the semi-dry method at 10 V for 25 min per gel. Membranes were incubated in 5% BSA overnight at 4°C to block nonspecific binding, washed 3 times for 10 min each with Tris-Buffered Saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) and the membrane was probed with monoclonal anti-mouse anti-phosphotyrosine antibody (1:1500 dilution) overnight at 4°C in TBS. The membranes were washed with gentle shaking at room temperature in TBST refreshed 8-10 times at 10-min intervals, and incubated for 60 min with secondary antibody conjugated with HRP (1:2500). After three more 10-min TBST washings, the anti-phosphoprotein bands were visualized with the use of enhanced chemiluminesence reagent followed by exposure to X-ray film and quantitation.

Figure 1. SDS-PAGE of Synaptosomal Proteins incubated *In Vitro* with γ-^{32}P-ATP and Varying Concentrations of T3



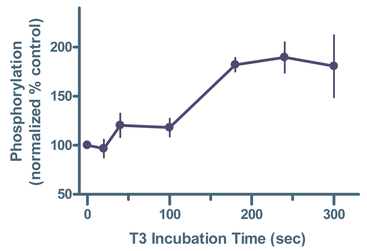
Synaptosomal lysate was preincubated with various doses of T3 and then with γ-^{32}P-ATP for 1 min at 37°C as described in Methods. The proteins were subjected to SDS-PAGE. A silver-stained gel is depicted on the left and the corresponding autoradiogram on the right. Lanes are labeled according to the concentration of T3 in nM. The middle column indicates the positions of the molecular weight markers (MWM).

Figure 2. Influence of Concentration of L-T3 on *In Vitro* Modulation of Protein Phosphorylation



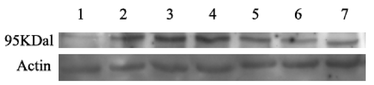
Synaptosomal lysate was preincubated with various doses of T3 and then with γ-^{32}P-ATP for 1 min at 37°C (see Methods). The proteins were subjected to SDS-PAGE followed by autoradiography. The integrated OD from bands on the autoradiograms corresponding to a 96 kDal protein were quantitated and corrected for the integrated lane density from the corresponding silver-stained gel. Results are the mean normalized values from three separate experiments (± SEM).

Figure 3. Time Course of Influence of L-T3 on *In Vitro* Modulation of Protein Phosphorylation



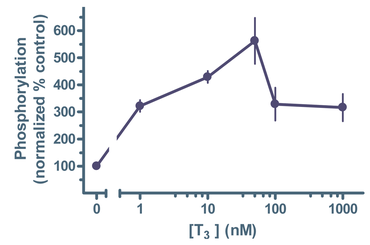
Synaptosomal lysate was incubated with γ-^{32}P-ATP for a total of 5 minutes at 37°C (see Methods). At varying times, 10 nM T3 was added to the incubation so that the incubation time with hormone varied as indicated. The proteins were subjected to SDS-PAGE followed by autoradiography. The integrated OD from bands on the autoradiograms corresponding to a 96 kDal phosphorylated protein were quantitated and corrected for the integrated lane density from the corresponding silver-stained gel. Results are the mean normalized values from three separate experiments (± SEM).

Figure 4. Anti-Phosphotyrosine Western Blot of Synaptosomal Lysate Incubated with T3



Synaptosomal lysate was preincubated with various concentrations of T3 as described in Methods and then incubated with for 2 minutes at 37°C with 20 μM ATP. Following SDS-PAGE and western blotting (see Methods), a 95 kDal protein showed the most pronounced increases of anti-phosphotyrosine antibody binding as a function of in vitro T3 concentration. Incubates were loaded into lanes as follows: (1) Control, (2) 1 nM T3, (3) 10 nM T3, (4) 50 nM T3, (5) 100 nM T3, (6) 1 μM T3, (7) 10 μM T3. To document comparable loading of protein by lane, the original blot was also analyzed following stripping and reprobing with anti-actin antibody (bottom).

Figure 5. Western Blot Analysis of Effects of T3 on Tyrosine Phosphorylation in Synaptosomal Lysate



Synaptosomal lysate was preincubated with various concentrations of T3 as described in Methods and then incubated with for 2 minutes at 37°C with 20 μM ATP. Following SDS-PAGE and western blotting (see Methods), a 95 kDal protein showing the most pronounced increases of anti-phosphotyrosine antibody binding was quantitated. The normalized data derived from the ratio of the integrated OD of the autoradiograms from anti-phosphotyrosine immunoblot to that of anti-actin immunoblot of the same bands (see Fig. 4) were combined for three experiments Results are the mean normalized values from three separate experiments (± SEM).

Summary

(1) Following incubation with concentrations of T3 from 10-100 nM, a dramatic enhancement of incorporation of ^{32}P per unit amount of protein was demonstrated in several proteins, including a prominent band of molecular weight 96 kDa, Physiological concentrations of T3 in nerve terminals are ~15 nM (Mason et al., 1993; Sarkar and Ray, 1994). However, the higher doses of T3 (300-1000 nM) showed a lesser stimulation of phosphorylation, indicating a biphasic action.

(2) During a fixed 5-min incubation with γ-^{32}P-ATP, the optimal incubation time with 10 nM T3 to maximally stimulate phosphorylation of the 96 kDa band was approximately 3 minutes, a time which would be inconsistent with extensive metabolism of the TH and suggestive of a relatively direct mechanism of action of the hormone.

(3) In western blot analysis, a 95 kDal protein showed a pronounced increase in binding of anti-phosphotyrosine antibody following in vitro incubation of lysed synaptosomes with T3. Phosphorylation of tyrosyl residues was demonstrated with in vitro incubation of synaptosomal lysate with concentrations of the hormone as low as 1 nM, and again a biphasic effect of hormone concentration was evident, with higher concentrations of T3 being less effective.

Conclusion

In vitro incubations with brain physiological concentrations of T3 rapidly enhanced protein phosphorylation in synaptosomal lysate, a nucleus-free subcellular fraction including membrane and soluble proteins from cerebral cortex of young adult male rats. In the case of a prominent 96 kDal protein, at least, the site of phosphorylation is likely to be tyrosyl residues. This finding suggests a novel nongenomic signal transduction pathway for T3 in adult mammalian brain.

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