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TRIIODOTHYRONINE INDUCES A TRANSFERABLE FACTOR WHICH SUPPRESSES TSH SECRETION IN CULTURED MOUSE THYROTROPIC TUMOR CELLS*

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SUMMARY

Modulation of TSH release from mouse thyrotropic tumor cells was studied. T_3 (1 nM) inhibited basal TSH release, while 6 nM T_3 blocked TRH-induced TSH release. Prior exposure of cells to actinomycin or cycloheximide prevented T_3 from suppressing basal and TRH-induced TSH release. The TSH-suppressive activity from T_3-treated cells was extracted and exposure of untreated thyrotropic cells to this material resulted in suppression of TSH release. The data suggest that T_3 suppression of TSH is mediated by formation of an inhibitory protein in thyrotropic cells.

Although thyroid hormones and TRH have been shown to regulate TSH secretion *in vitro* (1), the mechanism of T_3-induced inhibition of basal TSH release and of TRH-induced TSH stimulation is not yet clear. Earlier studies had suggested that T_3 may be responsible for the formation in the pituitary of an inhibitory protein which blocks the TRH-induced stimulation of TSH. When actinomycin D was injected into hypothyroid mice, T_3 could not inhibit TSH secretion (2). Subsequently, two inhibitors of protein synthesis, actinomycin and cycloheximide, were also shown to prevent the inhibition of TRH-induced TSH stimulation by T_3 (3). Using incubated hemipituitaries, actinomycin and cycloheximide did not alter basal or TRH-induced TSH release, but did prevent T_3 from inhibiting these TSH responses (4). This *in vitro* study was performed prior to the advent of the TSH radioimmunoassay and employed relatively high doses of cytotoxic agents.

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In order to gain further insight into the mechanism of T_3 suppression of basal and TRH-induced TSH secretion, we examined the effects of cytotoxic antibiotics on the modulation of TSH release by cultured mouse thyrotropic tumor cells. These cells were shown to be responsive to physiologic concentrations of T_3 and TRH.

MATERIALS AND METHODS

Thyrotropic tumor cultures.

Six to 9-week old mice of the LAF1/J strain were purchased from Jackson Laboratories, Bar Harbor, Maine. They were maintained on an iodide-free diet for 4 weeks and subsequently radiothyroidectomized with 200 μCi of ^{131}I i.p. A mouse bearing a thyrotropic tumor (TtT) originally generated by Dr. Jacob Furth was kindly provided by Dr. Martin Surks, Montefiore Hospital, New York. The tumor was transplanted subcutaneously into thyroidectomized female mice, as previously described (5). Mice were sacrificed and tumors (weight 7-8 grams) were excised and immediately washed in Ham's F-10 medium (Irvine Scientific Co., Santa Ana, CA.) containing 20% thyroidectomized calf serum (Rockland Farms, Gilbertsville, PA.), glutamine 5 mM, penicillin (5 U/ml) and streptomycin (5 μg/ml, Irvine Scientific Co.). The tumor was finely minced until the fragments were 1-2 mm. Tissue fragments were then transferred to a petri dish containing fresh medium, and subjected to repeated trituration through an 18 gauge needle, followed by serial triturations until the resultant cell suspension passed through a 23 gauge needle. After this mechanical dispersal of the cells over 20-30 minutes, the cell suspension was centrifuged at 100 x g for 10 minutes and the cells washed twice in fresh culture medium. An aliquot of the resuspended cells was counted, and their viability assessed by their ability to exclude trypan blue. Equal aliquots of cell suspension were seeded into experimental wells, and the cells were incubated at 37° in a humidified atmosphere of 95% air - 5% CO_2 for 48 hours before being used for experiments. At the end of 48 hours, all the medium and any floating tissue debris were carefully aspirated from each well, and fresh medium was added to the wells containing the confluent monolayer of cells. Each well contained 0.4 to 0.5 x 10^6 cells, and 2 ml of medium were used for each incubation. Neither T_3 (<7 ng/dl) nor T_4 (<0.5 μg/dl) could be detected in the culture medium by radioimmunoassay (RIA).

Experimental procedure.

Basal TSH release into the culture medium was measured after timed incubations ranging from 2 to 72 hours. Using tissue obtained from 4 separate tumors, cells were exposed to either varying concentrations of T_3 (Sigma), thyrotropin-releasing hormone (TRH, Abbott Laboratories, Chicago, IL.) or antineoplastic agents, alone or in combination. Antineoplastic agents used were dactinomycin (Merck, Sharp and Dohme), cycloheximide (Upjohn), and methotrexate (Lederle). When antineoplastic agents were used in combination with other agents, these drugs were added to the wells 30 minutes prior to addition of T_3 and/or TRH. All the reagents were dissolved in the culture medium and brought up to the required concentration. At the end of each incubation, medium was aspirated and immediately centrifuged at 100 x g for 10 minutes in order to separate any floating cells. The medium supernatant was frozen at -20° C for hormone RIA.

Transfer of TSH-inhibiting activity.

In order to gain further insight into the nature of the T_3-induced suppression of TSH, we examined the possibility of transferring the T_3-induced suppressing factor from T_3-treated thyrotropic cells to thyrotropic cells which had not been exposed to T_3. Thyrotropic cells were incubated in 75 cm^2 flasks (Falcon) as described above. Each flask contained 30-35 x 10^6 cells. After 48 hours, the culture medium was aspirated and replaced with 10 cc of culture medium containing either T_3 (6 nM), T_3 and dactinomycin (10 ng/ml), or dactinomycin (10 ng/ml). Control flasks contained 10 cc of culture medium only. At the end of 12 hours of incubation, the medium in each flask was aspirated, and the cells taken up in serum-free Ham's F-10 medium (10 cc). The cell suspensions were centrifuged at 800 x g for 20 minutes, and the supernatant discarded. The cells were resuspended, washed and spun twice more. The resultant cell pellet in each test tube was resuspended in 0.5 cc serum-free Ham's F-10 medium and subjected to ultrasonication, using a Biosonik III (Bronwill Scientific, Rochester, NY) at 35 Mhz for 45 seconds. The cell sonicate was centrifuged at 800 x g for 30 min, and the supernatant (0.5 cc total volume from each flask) designated "suppressive factor." Cells which had not previously been exposed to T_3 were then incubated in the presence of aliquots of this suppressive factor and their TSH release was compared to concurrently incubated control wells.

Radioimmunoassays.

TSH released by the cells was measured by a double antibody RIA. Purified rat TSH, anti-rat TSH serum and rat TSH reference standard were supplied by the NIAMDD and Dr. A. F. Parlow. The tracer was prepared, using rat TSH standard, and was labelled with ^{125}I as previously described (6). The tracer (s.a. about 150 $\mu Ci/\mu g$) was purified by gel filtration on a Sephadex G 100 column. Standard curves were generated using the NIH rat TSH standard, as well as a partially purified arbitrary mouse thyrotropic tumor (Tt) standard. This tumor standard was prepared from a mixture of several mouse thyrotropic tumors from previous generations. This arbitrary standard (Fig. 1) was not parallel with the rat TSH standard. Dilutions of fresh tumor extract obtained from mice just sacrificed, as well as serial dilutions of culture medium in which the thyrotropic cells had been incubated, were both parallel to the arbitrary Tt Standard. The 50% B/Bo dose of the rat TSH standard was 250 $\mu U/ml$, while 50% B/Bo of the Tt standard was 10 $\mu g/ml$. All TSH results are expressed as $\mu g/ml$ of arbitrary mouse Tt standard. Aliquots of culture medium were assayed in duplicate at two different dilutions. All data are presented as means ±SEM. Differences were assessed by means of the non-paired t test or by analysis of variance where appropriate.

RESULTS

Basal hormone secretion.

The time-course of basal TSH secretion by the thyrotropic cells is illustrated in Fig. 2. Significant suppression of basal TSH secretion by T3 (6 nM) was seen after the first 2 hours of incubation. This suppression remained evident for the 48 hours. The cells remained adherent to the bottom surface of the wells in a monolayer, and hormone production did not begin falling off

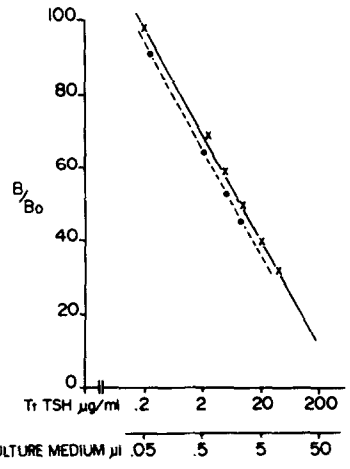


Figure 1. Displacement of ^{125}I-rat TSH by arbitrary extract of mouse thyrotropic tumor standard (T_tTSH). (x——x). Dilutions of thyrotropic tumor cell culture medium (·- - -·) were identical, as assessed by parallel line analysis.

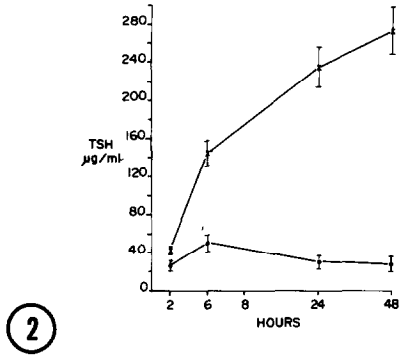
until after 7-10 days of culture. Thereafter, the floating clumps of cells were epithelial in appearance, while the cells remaining adherent in the monolayer appeared predominantly fibroblastic. All experiments were performed during the third day after culture.

Effects of T_3.

The cells were exposed to doses of T_3 ranging from .01 to 60 nM (Fig. 3). One nM T_3 suppressed hormone release over 24 hours. The apparent enhancement of TSH secretion by .01 nM T_3 was not significant.

Effects of inhibitors of protein synthesis on T_3-induced suppression of TSH release.

The suppression by T_3 of both basal and TRH-stimulated TSH release was blocked by exposing the cells to inhibitors of protein synthesis for 30 minutes prior to addition of T_3. Table 1 shows the antibiotics tested and doses employed. Neither actinomycin nor cycloheximide alone inhibited basal or TRH-induced TSH release. Exposure of the cells to both these agents effectively prevented T_3 from suppressing TSH release. Methotrexate, however, did appear to suppress basal TSH release at the doses employed (100 and 250 ng/ml).



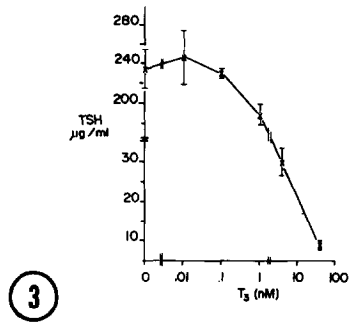


Figure 2. Time course of basal TSH release by thyrotropic tumor cells grown in thyroidectomized calf serum (10%). Cells were plated at time 0, and each point represents mean of 4 wells. x____x = no T_3 added to the culture medium. ·____· = 6 nM T_3 added to the medium.

Figure 3. Dose-response suppression by T_3 of TSH release during 24 hours of incubation. Each point is the mean of 4 wells.

Transfer of T_3-induced thyrotropin suppression.

In order to gain further insight into the possible induction of TSH inhibitory activity by T_3, cells were exposed to the supernatant of the cell-free sonicate of tumor cells which had been treated with T_3. Table 2 shows that the sonicate of cells previously treated with T_3 suppressed basal TSH secretion. T_3 was undetectable (<7 ng/dl) in the sonicate. Concurrent exposure of the cells to both T_3 and actinomycin prevented the subsequent cell-free inhibitory activity of the sonicate supernatant.

DISCUSSION

The data shown here demonstrate that 1 nM T_3 suppresses basal TSH secretion and that 6 nM T_3 prevents TRH-induced TSH stimulation in monolayer cultures of mouse thyrotropic tumor cells. Actinomycin (10 ng/ml) and cycloheximide (100 ng/ml) both did not suppress basal or TRH-stimulated TSH release. These two cytotoxic agents did, however, prevent T_3 from suppressing TSH release. Methotrexate, which suppressed basal TSH release, also partially reversed the T_3-induced inhibition of TSH release. Actinomycin inhibits protein synthesis by binding directly with DNA (7), and cycloheximide inhibits transfer

Table 1

Modulation of TSH Release by T_3, TRH and Cytotoxic Agents[1]

Experiment		Basal				TRH-Stimulated			
		Control	Control	T_3	T_3	Control	Control	T_3	T_3
I	Actinomycin (ng/ml)	0	10	0	10				
	TSH release	40 ±7.8	65 ±13	26* ±2.6	66 ±18				
II	Actinomycin (ng/ml)	0	50	0	50	0	50	0	50
	TSH release	39 ±.6	38[2]	32* ±2.6	40 ±2.9	63 ±.8	60 ±9	53* ±.3	68 ±4
III	Methotrexate (ng/ml)	0	100	0	100				
	TSH release	55 ±.8	36* ±.3	35* ±.2	39 ±.3				
IV	Methotrexate (ng/ml)	0	250	0	250				
	TSH release	41 ±0.7	33 ±2	13* ±0.2	20* ±.42				
V	Cycloheximide (ng/ml)	0	100	0	100	0	100	0	100
	TSH release	50 ±0.3	54 ±0.2	29* ±0.3	51 ±0.6	63 ±0.4	62 ±1.7	47* ±3	60 ±1.7

[1]Cells were exposed to antibiotics 30 minutes prior to addition of T_3 (6 nM) or TRH (1 nM). Each TSH response is mean (μg/ml)±SEM of 4 wells.

[2]Mean of 2 wells.

*$p < 0.025$ versus controls.

Table 2

Transfer of T_3-induced Thyrotropin Suppression Activity†

Treatment	TSH response induced by treated cell sonicate (μg/ml)
Control	72 ± 5
T_3 1 nM	45 ± 2.5*
T_3 1 nM + actinomycin 10 ng/ml	81 ± 6.5
Actinomycin 10 ng/ml	52 ± 6.

*$p < .05$ versus control.

†Cells not previously exposed to T_3 were treated with cell-free sonicates derived from cells receiving the indicated treatment. The accumulation of TSH in the medium was measured after 2 hours.

RNA action (8). Methotrexate, however, acts more proximally in inhibiting protein synthesis by blocking dihydrofolate reductase (9). This may explain why inhibition of basal TSH secretion was found with methotrexate but not with the other two inhibitors of protein synthesis.

These results extend and confirm those of previous workers (2-4) who had suggested that T_3 suppression of TSH may be due to formation of an "inhibitory protein" by T_3. Recent work has also shown that cycloheximide blocked T_3 suppression of TRH-induced TSH release by rat pituitary fragments (10).

Bowers et al (3) had previously postulated that the synthesis of an intracellular inhibitory protein is stimulated by T_3 and interacts within the thyrotrope cell to suppress TSH release. Alternatively, the degradation of this inhibitory polypeptide could be prevented by T_3. We have confirmed the existence of an inhibitory factor by showing that TSH-inhibiting activity could be extracted from T_3-treated thyrotropic cells. This suppressive factor contained no detectable T_3, and preliminary evidence from our laboratory suggests that it elutes on a G-25 Sephadex gel column in a region of 20,000-30,000 daltons.

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