Effect of Triiodothyronine or Iodide on the Thyroidal Secretion *In Vitro*: Inhibition of TSH- and Dibutyryl-Cyclic-AMP Induced Endocytosis

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Synopsis

T₃ or iodide, at the concentration of 10⁻⁸ M, inhibits TSH- or dibutyryl-cyclic AMP-induced thyroidal intracellular colloid droplet formation, while leaving the TSH-induced increase in cyclic AMP levels intact. The effect of T₃ appeared not to be mediated through the iodide derived from deiodination of T₃. Those results clearly demonstrate that T₃ or iodide acts at the point beyond cyclic AMP generation and prior to the formation of intracellular colloid droplets. This is also the site at which microtubule active reagents exert their influence. Whether T₃ or iodide fits this category or acts in as yet undetermined manner remains to be established.

In our previous study it was described that the large dose of triiodo-L-thyronine (T₃) or thyroxine (T₄), when administered to the mice prepared for McKenzie bioassay reduced the thyroidal responsiveness to TSH or LATS as evidenced by the decrease in thyroidal intracellular colloid droplet formation and subsequent radioiodine release (Shishiba, *et al.*, 1974). To clarify the possible site of action of thyroid hormone itself on thyroid tissue, an *in vitro* extension of the study was required because precise comparison of the responsiveness of thyroid tissue to those reagents was possible at a fixed concentra-

tion of TSH or other stimulators.

For the mechanism of thyroid secretion, it was postulated that TSH stimulates adenylate cyclase activity to increase cyclic AMP levels in the thyroid tissue, the latter being responsible for the stimulation of intracellular colloid droplet formation resulting in the subsequent hormonal release (Pastan and Wollman, 1967). Taking this postulate into account, in the present study, we sought to determine whether the effect of T₃ exists at the site where TSH increases cyclic AMP levels or at the site where increased cyclic AMP enhances the formation of intracellular colloid droplet.

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Materials and Methods

The methods employed were almost the same as described by Williams and Wolff with slight modification for laboratory adoption (Williams and Wolff, 1971). The male mice of ddy strain, purchased at the age of four weeks and kept on low iodine diet for two weeks, were employed all through the experiments. The mice were sacrificed with the dislocation of the neck. The thyroid glands were

removed with trachea and immersed for 30 min in the ice-cold Earl's balanced salt solution containing: bovine serum albumin (BSA) 5 mg/ml, glucose 5 mg/ml, streptomycin 0.5 mg/ml, penicillin 0.5 mg/ml and sodium perchlorate 10^{-3} M to block the reutilization of iodide. The medium was gassed with 95% O₂ and 5% CO₂ 30 min prior to use. Then, thyroid glands were transferred to new flasks and preincubated at 37° for 60 min in the above described medium enriched with T₃, iodide, tyrosine or colchicine as stated in the results. T₃ or tyrosine was dissolved with the minimum amount of 0.1 N NaOH and added to the medium whose pH was then adjusted to 7.4. The medium was continuously gassed with 95% O₂ and 5% CO₂. After pre-incubation, thyroid glands were transferred to flask containing the medium described for pre-incubation without or with the addition of TSH or dibutyrylcyclic AMP (DBC-AMP) and incubated at 37° for 60 min with the continuous gas bubbling as described. In the experiments in which colloid droplet formation and cyclic AMP levels in the tissue were to be examined, one incubation flask contained thyroid tissue from 8 animals. After incubation, three of them were quickly blotted on a moist filter paper, fixed with Bouin's fixative, stained with PAS, counterstained with hematoxylin and examined for intracellular colloid droplets. The number of intracellular colloid droplets were counted on 25 follicles without the knowledge of identity of the slides on the part of the examiner. The remaining five thyroid glands were excised from trachea, quickly blotted and weighed to 0.1 mg. The tissue was instantaneously frozen with acetone-dry-ice liquid. The content of cyclic AMP was measured with the kit distributed by Schwarz/Mann according to the method described by Steiner et al (Steiner, *et al.*, 1969).

T₃ was purchased from Sigma Chemicals. To check the magnitude of iodide contamination, 100 µg of T₃, dissolved in the minimum amount of 0.1 N NaOH and 95% ethanol, was applied on a Whatman 3 MM filter paper and chromatographed in an ascending butanol-acetic acid-ammonia (78:5:17) system overnight. Iodide portion was located by the addition of ¹³¹I, eluted with redistilled water and the content of iodide was measured with the celium-arsenic reaction, with an appropriate control. The contamination of iodide was proved to be less than 1.4%, at least at the beginning of incubation. The magnitude of deiodination of T₃ during the incubation with thyroid tissue was also examined with the addition of ¹²⁵I-T₃, purchased from Daiichi Radioisotope Laboratories, Tokyo. Under the incubation condition described, no significant deiodination of ¹²⁵I-T₃ was observed. Therefore, the content of iodide derived from exogenous T₃ in the medium during the incubation would not exceed 1.4% of the added T₃.

Armour Thytropar was used as a source of TSH

all through the experiments. DBC-AMP was purchased from Sigma Chemicals and other reagents were purchased at the purest grade available on market.

Results

Figure 1 shows the results of the experiment in which effect of concentration of TSH on the number of intracellular colloid droplets in thyroid tissue *in vitro*. As noted in the figure, TSH, even at the concentration of 0.005 mU/ml was effective in inducing a significant rise in intracellular colloid droplets. The effect was plateaued at the concentration of TSH ranging from 0.1 mU/ml to 2.5 mU/ml, and then, decreased at the higher concentration of TSH.

Table 1 summarizes the results of the experiment in which the effect of T₃, iodide or tyrosine on the intracellular colloid droplet formation *in vitro*. As shown, TSH induced intracellular colloid droplets to the level as much as 734. T₃ or iodide, when tested at the concentration of 10^{-4} M or less, did not show any significant effect. When the dose of T₃ or iodide increased to 10^{-3} M, however, significant inhibition of TSH-induced colloid droplet formation was obvious as evidenced by the decrease in the number of colloid droplet to the level approximately half of the control. This inhibitory effect of T₃ or iodide on colloid droplet formation was manifest not only at the dose of TSH 0.1 mU, but also at 3.3 mU/ml as shown in Table 2 and 3. Tyrosine, even at the concentration of 10^{-3} M, was not effective at all in this aspect.

Table 2 summarizes the results of the experiment in which the effect of T₃ or iodide on the response of thyroid tissue either to TSH or DBC-AMP. In the former, tissue levels of cyclic AMP were concomitantly measured with the colloid droplet formation. When TSH (3.3 mU/ml) was added to the medium, intracellular colloid droplets were

Figure 1. The effect of dose of TSH on the intracellular colloid droplet formation *in vitro*.

Table 1. Effect of T₃, iodide or tyrosine on TSH-induced colloid droplet formation *in vitro*.

Dose of test substance	Colloid droplet counts in 25 follicles			
	No stimulator	P	TSH 0.1 mU/ml	P**
None	14 ± 5*		734 ± 120	
T ₃ , 10 ⁻⁵ M	5 ± 2	N. S. §	682 ± 82	N. S.
10 ⁻⁴ M	5 ± 3	N. S.	674 ± 152	N. S.
10 ⁻³ M	16 ± 6	N. S.	241 ± 49	<0.05
I ⁻ , 10 ⁻⁵ M	22 ± 21	N. S.	741 ± 111	N. S.
10 ⁻⁴ M	6 ± 3	N. S.	802 ± 199	N. S.
10 ⁻³ M	7 ± 3	N. S.	98 ± 22	<0.01
Tyrosine, 10 ⁻³ M	8 ± 2	N. S.	730 ± 112	N. S.

* Mean ± SE of the value obtained from three animals.

** Statistical analysis was made for the difference between the number of colloid droplets induced by TSH alone and the addition of test substances. In all the experiments, the difference between no stimulator and the TSH-group with or without addition of test substances is highly significant.

§ N. S. stands for not significant.

Table 2. The effect of T_3 or iodide on TSH- or DBC-AMP-induced colloid droplet formation and cyclic AMP generation.

Test substance	Colloid droplets in 25 follicles						Cyclic AMP in pM/mg			
	No stimulator	P	TSH 3.3m U/ml	P	DBC-AMP 10^{-4} M	P	No stimulator	P	TSH 3.3 mU/ml	P
1) none	4 $\pm$ 2*		989 $\pm$ 87		950 $\pm$ 203		0.255 $\pm$ 0.037**		1.370 $\pm$ 0.538	
2) T_3 , 10^{-8} M	3 $\pm$ 4	N.S. §	146 $\pm$ 118	<0.02	50 $\pm$ 5	<0.02	0.339 $\pm$ 0.197	N.S.	1.616 $\pm$ 0.593	N.S.
3) I^- , 10^{-8} M	3 $\pm$ 3	N.S.	32 $\pm$ 9	<0.01	15 $\pm$ 4	<0.01	0.362 $\pm$ 0.022	N.S.	0.658 $\pm$ 0.346	N.S.

* Mean $\pm$ SE of the value obtained from three animals.** Mean $\pm$ SE of the value obtained from three similar experiments, one of which is composite with colloid droplet study.§ N.S. stands for not significant. Statistical analysis made for the value between 1) and 2) or 3) is listed on the table. That for colloid droplet counts between no stimulator and TSH or DBC-AMP group is also significant. That for cyclic AMP between no stimulator and TSH group is highly significant ($p < 0.01$) by the paired t test for the line 1), 2) and 3).Table 3. The effect of T_3 or colchicine on TSH-induced colloid droplet formation and cyclic AMP production.

Test substance	Colloid droplet counts in 25 follicles				cyclic AMP in pM/mg			
	No stimulator	P	TSH 3.3 mU/ml	P	No stimulator	P	TSH 3.3 mU/ml	P
1) None	23 $\pm$ 4*		1326 $\pm$ 83		0.092 $\pm$ 0.092**		0.692 $\pm$ 0.074	
2) Colchicine, 10^{-5} M	9 $\pm$ 3	N.S.	396 $\pm$ 39	<0.001	0.129 $\pm$ 0.052	N.S.	0.515 $\pm$ 0.269	N.S.
3) T_3 , 10^{-4} M	34 $\pm$ 18	N.S.	814 $\pm$ 218	N.S.	0.124 $\pm$ 0.076	N.S.	0.458 $\pm$ 0.313	N.S.
4) T_3 and colchicine	6 $\pm$ 4	N.S.	122 $\pm$ 31	<0.001	0.126 $\pm$ 0.081	N.S.	0.897 $\pm$ 0.313	N.S.

* Mean $\pm$ SE of the value obtained from three animals.** Mean $\pm$ SE of the value obtained from three similar experiments, one of which is composite with colloid droplet study.§ N.S. stands for not significant. Statistical analysis made for the value between 1) and 2), 3) or 4) is listed on the table. Significance of the difference between 2) and 4) for colloid droplet study with TSH is $p < 0.01$. The statistical analysis for cyclic AMP between no stimulator and TSH-group is highly significant ($p < 0.01$) by the paired t test for the line 1), 2), 3) and 4).

increased to the magnitude as much as 989. Concurrently, cyclic AMP of the tissue was increased from 0.26 pM/mg to the level of 1.37 pM/mg. T_3 , although ineffective in influencing either colloid droplet formation or cyclic AMP levels by itself, inhibited the TSH-induced rise in colloid droplet counts to 146. Cyclic AMP levels in the tissue, however, were normally increased by TSH even in the presence of T_3 . Iodide, at the concentration of 10^{-8} M, inhibited TSH-induced colloid droplet formation in a fashion similar to T_3 . In the presence of iodide, TSH-induced increase in cyclic AMP was less although the difference from that without the addition of iodide was

slightly short of statistical significance. While inhibited by iodide, the increase in cyclic AMP induced by TSH was still significant when compared to the basal level ($P < 0.01$). Although not shown here, the effect of T_3 or iodide in this aspect was reversible when T_3 or iodide was washed out from the medium.

When DBC-AMP, instead of TSH, was added to the medium, the colloid droplet formation was increased to the level of 950. Both T_3 and iodide inhibited the DBC-AMP-induced colloid droplet formation to the level significantly different from the response elicited by DBC-AMP only.

Table 3 compares the effect of T_3 to that

of colchicine. As shown, colchicine, at the concentration of 10^{-5} M, inhibited TSH-induced increase in colloid droplet formation while leaving the increase in cyclic AMP levels intact. T₃, at the concentration of 10^{-4} M, was slightly effective to suppress TSH-induced colloid droplet formation although the magnitude of the effect was less when compared to that of 10^{-3} M. When colchicine and T₃ were employed in combination, the suppression of TSH-induced colloid droplet formation was more pronounced, significantly exceeding the separate effect of T₃ or iodide. The concurrent rise in cyclic AMP levels in the tissue produced by TSH was not affected at all either by colchicine, T₃ or both in combination.

Discussion

The present study demonstrates that T₃ or iodide, at the concentration of 10^{-3} M, inhibits TSH-induced intracellular colloid droplet formation, while those reagents were not effective in inhibiting the concurrent rise in cyclic AMP levels in the tissue. To further confirm the possible site of the action of T₃ or iodide, the effects of T₃ or iodide on DBC-AMP-induced colloid droplet formation was also studied. From the current concepts, TSH stimulates adenylate cyclase activity resulting in an increase in cyclic AMP levels in thyroid tissue, the latter being responsible for an increase in intracellular colloid droplet formation (Pastan and Wollman, 1967). Based on this postulate, it was argued that, if inhibition of colloid droplet formation was due to inhibition of adenylate cyclase, T₃ or iodide should inhibit only the TSH- but not DBC-AMP-induced colloid droplet formation. Our experiments show that T₃ or iodide also inhibits DBC-AMP-induced increase in colloid droplet formation. The present results, thus, demonstrate that an inhibitory locus for T₃ or iodide exists at a

point beyond the generation of intracellular cyclic AMP but prior to colloid droplet formation, while the results obtained for iodide do not preclude the possible inhibition of adenylate cyclase in tissue. This appears to be a critical step in the activation of thyroid hormone secretion which is affected by metabolic inhibitors, membrane stabilizers, microtubule-active reagents such like colchicine, vincristine or D₂O, or by lithium (Wolff and Williams, 1973; Onaya and Solomon, 1969; Williams and Wolff, 1971; Williams, *et al.*, 1971).

Of those reagents cited above, chlorpromazine and lithium were already shown to inhibit TSH-induced increase in cyclic AMP while possessing the inhibitory effect on intracellular colloid droplet formation (Wolff and Williams, 1973). From the present results, iodide may share the similar site with lithium or chlorpromazine in its inhibitory effect, considering the possible presence of inhibitory effect on cyclic AMP generation. As for T₃, the similarity of the effect of T₃ to colchicine was evident in our experiment. Among other possibilities, this similarity is at least compatible with the notion that T₃ may exert its influence directly or indirectly through microtubular system whose function is one of the prerequisite for colloid droplet formation. At any event, whether T₃ or iodide fits any of these categories or acts in as yet undetermined manner remains to be explored.

Although T₃ and iodide show the similar effect on intracellular colloid droplet formation except that the latter may possess an action on cyclic AMP generation, the effect of T₃ and iodide appears to be independent. The concentration of iodide derived from exogenous T₃ at the concentration of 10^{-3} M during the incubation would never exceed 1.4×10^{-5} M as evidenced in the previous description. Iodide, at the concentration of 10^{-4} M or less, never showed any effect on TSH-induced colloid

droplet formation.

Finally, the present result obtained *in vitro* does not necessarily mean that the effect of T_3 or iodide *in vivo* is also at the point beyond cyclic AMP generation and prior to colloid droplet formation. In our *in vivo* study, thyroid hormone administered *in vivo*, reduced the cyclic AMP generation and colloid droplet formation induced by TSH *in vitro* (Shishiba, *et al.*, 1974b).

For the explanation of this discrepancy, it would be argued that a physiological site of T_3 action may be deranged in the artificial *in vitro* condition and the site which is not manifest *in vivo* may be revealed, as stated by Oppenheimer (Oppenheimer, *et al.*, 1973). Another *in vivo* counterpart of the present study is the report by Ohtake *et al* informing the temporal inhibition of colloid droplet formation induced by TSH *in vivo* (Ohtake, *et al.*, 1973). The precise relevancy of the present experiment to their observation remains to be disclosed.

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