PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 147, 710-714 (1974)

Cyclic AMP and Thyroid Hormone: Inhibition of Epiphyseal Cartilage Cyclic 3′,5′-Nucleotide Phosphodiesterase Activity by L-Triiodothyronine (38423)

NATALIE M. THANASSI AND DAVID S. NEWCOMBE

Division of Rheumatology, Department of Medicine, University of Vermont, College of Medicine, Burlington, Vermont 05401

The absence of thyroid hormone results in profound effects on epiphyseal cartilage maturation and growth (1). Moreover, hyperthyroidism in childhood results in advanced bone age and accelerated growth (1, 2) which presumably is related directly to accelerated cartilage maturation. Since thyroid hormones are known to have their effect on certain tissues mediated via the adenylate cyclase–cyclic 3′,5′-nucleotide phosphodiesterase system, the purpose of the present investigation was to determine the effects of the L- and D-isomers of thyroid hormones on soluble cyclic 3′,5′-nucleotide phosphodiesterase activity prepared from the epiphyses of young chickens.

Materials and Methods. Epiphyseal cartilage was obtained from 6- to 8 week-old hybrid Hubbard broiler chickens by the method of Wuthier (3). Articular cartilage was freed completely from epiphyseal cartilage by blunt dissection, and the upper epiphyseal cartilage zones down to the zone of provisional calcification were removed with a special knife as fine shavings. The cartilage shavings were homogenized by a standard technique (4), and the resultant homogenate centrifuged at 100,000*g* for 2 hr. The supernatant of this homogenate was used for all enzyme studies. Protein was determined by the Lowry procedure (5), and cyclic 3′,5′-nucleotide phosphodiesterase (PDE) was assayed by the Thompson–Appleman method (6).

For inhibition experiments, 57 μg of enzyme protein and a 15-min incubation time were used with cyclic AMP as a substrate, and 62 μg of enzyme protein and an identical incubation time were used with cyclic GMP as substrate. One unit of PDE activity was defined as the amount of enzyme required to hydrolyze 1 pmole of cyclic AMP per 15 min at 30°. Specific activity was expressed as units/mg protein. Control incubations contained no enzyme. Thyroid hormones were dissolved in dimethylsulfoxide (DMSO); the final concentration of DMSO in the incubation mixtures was 2.5% (v/v). Appropriate control incubations containing DMSO were used to correct for any effect of the solubilizing agent on the reaction. Other control incubations established that the observed effects of various compounds on cyclic 3′,5′-nucleotide phosphodiesterase activity were not the result of 5′-nucleotidase inhibition. The I_{50} value was obtained by plotting the logarithm of inhibitor concentration as a function of the percentage of enzyme inhibition.

Mean and standard error of the mean were calculated by standard techniques, and a statistical test of the quality versus nonquality (one-tailed test) was carried out using *t* statistics (7).

^{3}H-Cyclic AMP (28 Ci/mmole), D-thyroxine, L-thyroxine sodium pentahydrate, 3,3′,5-triiodo-L-thyronine, and 3,3′,5-triiodo-D-thyronine were obtained from Schwarz–Mann. 3,5-Diiodo-L-thyronine was purchased from Mann Research Laboratories. Labeled cyclic AMP was purified by thin-layer chromatography prior to its use in assays (6), and labeled cyclic GMP was freed from contaminants by the method of Jungas (8).

Results. Inhibition of cyclic 3′,5′-nucleotide phosphodiesterase activity by L-triiodothyronine. As shown in Table I, the enzyme specific activities determined in the presence of all the thyroid hormones with the exception of 3,5-diiodo-L-thyronine and 10^{-6} *M* 3,3′,5-triiodo-L-thyronine were significantly different than the control value ($P < 0.05$). It was also found that L-triiodothyronine at 1×10^{-4} *M* concentration resulted in the most significant difference from the control ($P < 0.0005$). The concentration of

Printed in U.S.A.

L-triiodothyronine that resulted in 50% inhibition of cyclic 3′,5′-nucleotide phosphodiesterase activity was 3.5×10^{-5} *M*.

Under conditions where the enzyme activity was linear with respect to both time and protein concentrations using 1×10^{-6} *M* cyclic GMP as substrate, L-triiodothyronine at a concentration of 1×10^{-4} *M*, resulted in 50% inhibition of cyclic GMP–PDE activity.

Thyroid hormone inhibition of the high- and low-K_m cyclic phosphodiesterase enzyme(s). Since previous work from our laboratory has demonstrated a high and low K_m for epiphyseal cartilage cyclic AMP–phosphodiesterase (4), it was important to determine whether the inhibition by thyroid hormones was restricted to one substrate concentration or included inhibition at both high and low substrate concentrations. At 1×10^{-4} *M* concentrations of cyclic AMP, identical concentrations of inhibitors demonstrated either no inhibition or only slight inhibition of cyclic 3′,5′-nucleotide phosphodiesterase activity as compared to the inhibition seen with 1×10^{-6} *M* cyclic AMP concentrations.

Based on the insignificant inhibition by thyroid hormones where 10^{-4} *M* substrate concentrations were used, we have reported only the inhibition data for 1×10^{-6} *M* cyclic AMP concentrations (Table I).

Effect of divalent cations on triiodothyronine inhibition of phosphodiesterase activity. Thyroid hormones chelate divalent cations (9). To determine whether the inhibition demonstrated with L-triiodothyronine was secondary to chelation of cations such as magnesium, which is necessary for enzyme activity, or calcium, which has been demonstrated to be an activator of PDE (10), enzyme activity was measured in the presence of added magnesium and calcium (Table II). The presence of calcium at a final concentration of 1×10^{-3} *M* in the incubation mixture did not increase the activity as compared to the control, and the addition of calcium at three different concentrations together with L-triiodothyronine did not reverse the inhibitory effect of the hormone. Similarly, the addition of

TABLE I. The Effect of Thyroid Compounds on Soluble Epiphyseal PDE.

Compound	Final concentration in incubation (*M*)	Specific Activity[a] (units/mg protein) 10^{-6} *M* cyclic AMP as substrate	Inhibition (%)
Control	—	489.9 ± 20.7	—
3,3′,5-Triiodo-L-thyronine (L-T_3)	1×10^{-4}	152.1 ± 9.6	69
L-T_3	1×10^{-5}	363.2 ± 22.8	26
L-T_3	1×10^{-6}	470.0 ± 12.2[b]	3
3,3′,5-Triiodo-D-thyronine (D-T_3)	1×10^{-4}	303.5 ± 13.3	38
D-T_3	1×10^{-5}	402.1 ± 10.3	18
3,5-Diiodo-L-thyronine (L-T_2)	1×10^{-4}	393.1 ± 45.4[b]	20
L-T_2	1×10^{-5}	412.5 ± 35.4[b]	16
L-Thyroxine (L-T_4)	1×10^{-4}	283.7 ± 28.8	42
L-T_4	1×10^{-5}	374.6 ± 22.4	24
L-T_4	1×10^{-6}	396.8 ± 18.0	19
D-Thyroxine (D-T_4)	1×10^{-4}	286.9 ± 20.8	41
D-T_4	1×10^{-5}	358.9 ± 18.3	27
D-T_4	1×10^{-6}	432.8 ± 10.6	12

[a] Results are reported as mean ± SEM and are the cumulative data from three separate cartilage preparations. Assays conducted on each preparation were done in triplicate.

[b] These values do not differ significantly from the control ($P > 0.05$). All the other values are significantly different from the control ($P < 0.05$).

TABLE II. The Effect of Divalent Cations on L-Triiodothyronine[a] Inhibition of Cartilage PDE.

Reaction mixture additions	Final concentration of added divalent cation in incubation[b]	Specific activity (units/mg protein) 10^{-6} *M* cyclic AMP as substrate	Inhibition (%)
Control	—	764.6	—
3,3′,5-Triiodo-L-thyronine (L-T_3)	—	233.8	69
$CaCl_2$	1×10^{-3}	595.4	—
L-T_3 + $CaCl_2$	1×10^{-3}	249.2	67
L-T_3 + $CaCl_2$	1×10^{-4}	243.1	68
L-T_3 + $CaCl_2$	1×10^{-5}	221.5	71
$MgCl_2$	1×10^{-3}	667.7	—
$MgCl_2$	2.5×10^{-3}	638.5	—
$MgCl_2$	5×10^{-3}	650.8	—
L-T_3 + $MgCl_2$	1×10^{-3}	244.6	68
L-T_3 + $MgCl_2$	2.5×10^{-3}	241.5	68
L-T_3 + $MgCl_2$	5×10^{-3}	264.6	65

[a] The final concentration of L-triiodothyronine in the incubation mixture is 1×10^{-4} *M*.

[b] All incubation mixtures contain $MgCl_2$ at a final concentration of 5×10^{-3} *M*. The table lists the final concentration of additional $MgCl_2$ in incubation mixtures.

magnesium to incubation mixtures containing L-triiodothyronine did not alter the inhibitory capacity of the hormone.

Discussion. Although both the hypothyroid and hyperthyroid states in children provide direct evidence for an alteration in cartilage growth and maturation, the mechanism for this change in cartilage metabolism remains to be determined (1, 2). The present investigation was undertaken to determine whether cyclic AMP metabolism might be involved in regulating the effects of thyroid hormone-mediated cartilage tissue responses. Studies by Mandel and Kuehl (11) have demonstrated that 10^{-3}–10^{-4} *M* concentrations of L-triiodothyronine inhibit cyclic 3′,5′-nucleotide phosphodiesterase activity in adipose tissue extracts, and Levey and Epstein (12, 13) have shown that L-thyroxine and L-triiodothyronine activate cardiac adenylate cyclase. Since the presence of cyclic 3′,5′-nucleotide phosphodiesterase activity in epiphyseal cartilage would provide indirect evidence for the presence of the complete adenylate cyclase system, and since its inhibition by theophylline would need to be documented for purposes of assaying adenylate cyclase activity, the characterization of cyclic 3′,5′-nucleotide phosphodiesterase activity in epiphyseal cartilage provided an approach both for determining the probable presence of the adenylate cyclase system in epiphyseal cartilage and for examining the relationship between cyclic AMP, thyroid hormones, and cartilage phosphodiesterase activity.

The observation that L-triiodothyronine results in the most significant inhibition of cartilage cyclic 3′,5′-nucleotide phosphodiesterase activity provides evidence that cyclic AMP-mediated reactions may regulate cartilage metabolism in the presence or absence of this thyroid hormone.

The fact that unphysiological concentrations of L-triiodothyronine are necessary to demonstrate an effect on cyclic AMP phosphodiesterase activity and that the L- and D-isomers of thyroxine have a moderate effect on enzyme activity does raise questions as to the specificity of the inhibition and its physiological significance. However, several factors can be cited to suggest that the *in vitro* effect of these hormones may reflect a physiological, *in vivo* action. *First,* broken cell preparations do not always reflect the effects of hormones on whole tissue preparations. Levey (14) has conclusively demonstrated that this is the case for the activation of cardiac adenylate cyclase by catecholamines. *Second,* the delay between the *in vivo* administration of thyroid hormones and the physiological response of end organs has been proposed by Levey and Epstein (13) as evidence that it takes time for sufficient concentrations of active forms of thyroid hormones to reach the target organ and

initiate an end organ response. Clearly, a broken cell preparation permits easy access to the site of hormone action and obviates transport and concentrating phenomena. However, such preparations may not represent the most physiological assay for *in vivo* activity since cell disruption alters the normal intracellular biochemical and structural relationships as has been demonstrated in the case of phospholipids and adenylate cyclase (14). *Third,* the discrepancy between the *in vivo* and *in vitro* potencies of the various thyroid hormone stereoisomers is not unique to the cyclic AMP phosphodiesterase enzyme (13, 15–21). It is known, for example, that D-thyroxine is metabolized more rapidly than its L-isomer, and the tissue distribution of these isomers is quite different (22, 23); thus cell transport systems may be quite different for thyroid isomers. For this reason stereoisomers might as a result of their structural characteristics alone have somewhat comparable capacities to alter enzyme activities *in vitro,* whereas *in vivo* transport differences would prevent enzyme–isomer contact. *Fourth,* the administration of labeled thyroxine to amphibian tadpoles demonstrates a highly specific localization of the label in the perinuclear region of chondrocytes. This fact suggests that the labeled thyroid hormones may be concentrated within a specific metabolic compartment of the chondrocyte (24).

The present investigation provides additional evidence that L-triiodothyronine may account for physiological effects within the chondrocyte. L-triiodothyronine was found to be the most significant inhibitor of cartilage cyclic 3′,5′-nucleotide phosphodiesterase activity, whereas other less potent thyroid hormones demonstrated less significant effects on the enzyme.

Cartilage cyclic 3′,5′-nucleotide phosphodiesterase demonstrates nonlinear Lineweaver–Burk kinetics with resultant high and low Michaelis constants (4). Based on the intracellular and plasma concentrations of cyclic AMP determined for various species and tissues (25), it appears that the low-Michaelis-constant enzyme or the active site with the low Michaelis constant plays a key role under most physiological circumstances. The nonlinear kinetics suggest that either two different enzymes are present, one with a high K_m for its substrate and the other with a low K_m, or one enzyme has two active sites with different K_m values for the same substrate. Therefore, finding that the L-isomer of triiodothyronine has a significant effect at 1×10^{-6} *M* cyclic AMP concentrations suggests that the inhibitory capacity of this hormone is most active against the assumed physiologically active species or active site of cyclic 3′,5′-nucleotide phosphodiesterase. Furthermore, the experiments using added calcium and magnesium in the assay provide evidence that the mechanism of thyroid hormone inhibition of cyclic AMP phosphodiesterase is not the result of cation chelation by these hormones.

In conclusion, the results of these investigations demonstrating the inhibition of cartilage 3′,5′-nucleotide phosphodiesterase by the most potent thyroid hormone, L-triiodothyronine, provides evidence to suggest that cyclic AMP metabolism plays a role in mediating the effect of this thyroid hormone on cartilage metabolism. The alterations in cyclic AMP metabolism which presumably result from L-triiodothyronine inhibition of cyclic 3′,5′-nucleotide phosphodiesterase provide a basis for formulating testable hypotheses concerning the interaction between the adenylate cyclase–cyclic 3′,5′-nucleotide phosphodiesterase system, L-triiodothyronine, and epiphyseal cartilage metabolism.

Summary. The mechanism for the alteration in cartilage growth and metabolism present both in childhood thyroid hormone deficiency states and in hyperthyroid children has not been defined. Since thyroid hormone has a direct effect on the adenylate cyclase–cyclic 3′,5′-nucleotide phosphodiesterase system in certain tissues, the effect of thyroid hormones on soluble cyclic 3′,5′-nucleotide phosphodiesterase (PDE) activity prepared from chicken epiphyseal (growth) cartilage was examined. L-Triiodothyronine at a concentration of 1×10^{-4} *M* resulted in 69% inhibition of soluble cartilage cyclic 3′,5′-nucleotide phosphodiesterase activity. At identical concentrations L-thyroxine, D-thyroxine, D-triiodothyronine, and L-diiodothyronine demonstrated only 42, 41, 38, and 20% inhibition of phosphodiesterase activity, respectively. The concentration of L-triiodothyronine that resulted in 50% inhibition of phosphodiesterase activity was 3.5×10^{-5} *M*. This investigation demonstrates a significant inhibitory effect of L-triiodothyronine on cartilage 3′,5′-nucleotide phosphodiesterase activity, whereas other thyroid analogs tested were considerably less active as inhibitors of this enzyme. These findings suggest that cyclic nucleotide metabolism

may be involved in regulating the effects of the most potent thyroid hormone, L-triiodothyronine, on epiphyseal cartilage.

This investigation was supported in part by National Institutes of Health Grant No. R01 AM 16151 and an Arthritis Clinical Research Center Grant.

1. Krane, S. M., *in* "The Thyroid" (S. C. Werner and S. H. Ingbar, eds.), Chapter 39, p. 763. Harper and Row, New York (1971).

2. Schlesinger, S., MacGilliray, M. H., and Munschauer, R. W., J. Pediat. **83,** 233 (1973).

3. Wuthier, R. E., Calcif. Tissue Res. **8,** 24 (1971).

4. Newcombe, D. S., Thanassi, N. M., and Ciosek, C. P., Jr., Life Sci. **14,** 505 (1974).

5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).

6. Thompson, W. J., and Appleman, M. M., Biochemistry **10,** 311 (1971).

7. Diem, K., and Lentner, C., *in* "Scientific Tables" (J. R. Geigy, ed.), 7th ed., p. 145. Schwabe, Basle (1970).

8. Jungas, R. L., Proc. Nat. Acad. Sci. U.S.A. **56,** 757 (1966).

9. Wolff, E. C., and Wolff, J., *in* "The Thyroid Gland" (R. Pitt-Rivers and W. R. Trotter, eds.), Chapter 1, p. 237. Butterworth, London (1964).

10. Kakiuchi, S., Yamazaki, R., Teshima, Y., and Uenishi, K., Proc. Nat. Acad. Sci. U.S.A. **70,** 3526 (1973).

11. Mandel, L. R., and Kuehl, F. A., Jr., Biochem. Biophys. Res. Commun. **28,** 13 (1967).

12. Levey, G. S., and Epstein, S. E., Biochem. Biophys. Res. Commun. **33,** 990 (1968).

13. Levey, G. S., and Epstein, S. E., J. Clin. Invest. **48,** 1663 (1909).

14. Levey, G. S., Recent Progr. Horm. Res. **29,** 361 (1973).

15. Tapley, D. R., and Cooper, C., J. Biol. Chem. **222,** 341 (1956).

16. Maley, G. F., and Lardy, H. A., J. Biol. Chem. **204,** 435 (1953).

17. Dunne, P. B., and Tapley, D. F., Nature (London) **185,** 622 (1960).

18. Sokoloff, L., and Kaufman, S., J. Biol. Chem. **236,** 795 (1961).

19. Tapley, D. F., J. Biol. Chem. **222,** 325 (1956).

20. Pittman, J. A., Tingley, J. O., Nickerson, J. F., and Hill, S. R., Jr., Metab. Clin. Exp. **9,** 293 (1960).

21. Klebanoff, S. J., J. Biol. Chem. **234,** 2437 (1959).

22. Rall, J. E., Robbins, J., Becker, D., and Rawson, R. W., J. Clin. Invest. **32,** 596 (1953).

23. Tapley, D. F., Davidoff, F. F., Hatfield, W. B., and Ross, J. E., Amer. J. Physiol. **197,** 1021 (1959).

24. Reynolds, W. A., *in* "Hormones in Development" (M. Hamburgh and E. J. W. Barrington, eds.), Chapter 23, p. 315. Appleton-Century-Crofts, New York (1971).

25. Robison, G. A., Butcher, R. W., and Sutherland, E. W., "Cyclic AMP," 531 pps. Academic Press, New York (1971).

Received March 29, 1974. P.S.E.B.M., 1974, Vol. 147.