Effects of Thyroid Hormone Deficiency on Cyclic Adenosine 3':5'-Monophosphate and Control of Lipolysis in Fat Cells*

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SUMMARY

Isolated fat cells from hypothyroid rats, in contrast with those from normal animals, lack ability to give a lipolytic response to epinephrine or glucagon. However, activation of triglyceride lipolysis was induced with (N\textsuperscript{6}, O\textsuperscript{\'}-dibutyryl cyclic adenosine 3':5'-monophosphate), or a combination of epinephrine and phosphodiesterase inhibitors.

Adenylyl cyclase was activated by catecholamines or glucagon in membrane ghosts prepared from fat cells obtained from hypothyroid rats. The characteristics of the enzyme, and its activation by hormones and fluoride were similar to those observed in fat cell membrane ghosts from normal animals. Thus, adenylyl cyclase appears to be functional in the hypothyroid state, although lipolysis is blocked.

Whole fat cells were incubated in the presence and absence of epinephrine or theophylline, and accumulation of cyclic AMP over 10 min was measured. Basal, unstimulated levels were found to be similar in cells from normal and hypothyroid animals. In the presence of theophylline alone, similar levels of cyclic AMP were again observed. An increase in cyclic AMP accumulation in response to epinephrine stimulation, however, was not obtained in cells from hypothyroid rats except in the presence of the phosphodiesterase inhibitor.

Cyclic nucleotide phosphodiesterase activities were fractionated by discontinuous sucrose gradient centrifugation. The soluble, low affinity form of phosphodiesterase activity did not differ in the normal and hypothyroid states. The particulate, high affinity forms of cyclic AMP phosphodiesterase activity were elevated in fat cells from hypothyroid rats.

Thyroid hormones may thus exert a modulating effect on cyclic AMP-mediated responses by regulating the activity of a membrane associated, high affinity, cyclic AMP phosphodiesterase.

Hypothyroid animals have lost the ability to mobilize fat from adipose tissue in response to other hormones. Conversely, the hyperthyroid animal displays an exaggerated lipolytic response to such hormones as epinephrine and glucagon (1-4). An understanding of how these modulating effects are brought about would be helpful in elucidating the biochemical mechanism of action of thyroid hormones. Explanations have been proposed, including a deficiency of adenylyl cyclase as a result of possible changes in the rate of protein synthesis (5). Definitive evidence to support any interpretation has been lacking, and the present study using isolated fat cells and fat cell outer membrane ghosts from hypothyroid rats, was carried out to determine where the metabolic block occurs which prevents normal activation of triglyceride lipolysis in response to epinephrine and glucagon.

EXPERIMENTAL PROCEDURE

Hypothyroid Rats—Surgical or radioiodide-induced thyroidectomy can lead to complications of calcium metabolism due to imbalances in thyrocalcitonin or parathyroid hormone, often necessitating supplementation of the diet with calcium lactate (6). In order to avoid these problems and utilize procedures similar to those described by others (1, 2, 4), the animals were treated with propylthiouracil.

Male Sprague-Dawley rats weighing about 100 g were placed on a Remington iodine-deficient diet, TD-88221, containing 0.15% propylthiouracil obtained from General Biochemicals (Chagrin Falls, Ohio). A marked reduction in weight gain was observed by the 3rd day (6 to 8 g per week as compared with about 30 g for normal animals). Thyroid goiters were produced with a 3- to 4-fold increase in gland weight after 10 days. Goiter formation could be suppressed by administration of thyroxine. Rats were maintained for a minimum of 2 weeks on this diet before being killed. The number of days the animals were on the diet is described for each experiment.

Preparation of Isolated Fat Cells and Fat Cell Membrane Ghosts—Fat cells and membrane ghosts were prepared by the procedure described by Harwood et al. (7).

Incubation of Fat Cells with Hormones and Phosphodiesterase Inhibitors—Fat cells obtained from three or more rats were pooled and distributed into plastic vials to give a final concentration of 2.5 × 10\textsuperscript{4} cells in 4 ml per vial of Krebs-Ringer-Tris buffer containing 2% bovine serum albumin. Hormones were added as described in the text.

The abbreviations used are: propylthiouracil, 6-n-propyl-2-thiouracil; dibutyryl cyclic AMP (B\textsubscript{2}ACAMP), N\textsuperscript{6}, O\textsuperscript{\'}-dibutyryl cyclic adenosine 3':5'-monophosphate; cyclic AMP, cyclic adenosine 3':5'-monophosphate; cyclic GMP, cyclic guanosine 3':5'-monophosphate; T\textsubscript{3}, triiodothyronine; T\textsubscript{4}, thyroxine.

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indicated in the figures. Incubations were for 30 min at 37° in a metabolic shaker, followed by the addition of ice-cold hexane. Glycerol production was used as a measure of lipolysis and was assayed by the fluorometric procedure of Chernick (8).

In the experiments designed to measure cyclic AMP accumulation, 1 x 10⁶ cells in 4 ml of the above medium were incubated in each vial. Incubations were stopped at the end of 10 min by the addition of 4 ml of ice-cold 2 M perchloric acid. Blank values were obtained from vials containing all constituents except the fat cells. Collagenase (type II), epinephrine bitartrate, theophylline, BtcáMP, thyroid hormones and propylthiouracil used in the incubations were from Sigma. Pentex V bovine serum albumin was obtained from Miles Laboratories. The phosphodiesterase blocking agent, methylisobutylxanthine, was a gift from the G. D. Searle Co.

**Cyclic AMP Measurement**—Cyclic AMP was measured by radioimmunoassay procedures according to Steiner et al. (9) as modified by Williams et al. (10). This modified procedure uses cyclic [³H]AMP as the radioactive ligand in place of iodinated succinyl-cyclic AMP tyrosine methyl ester. The sensitivity of the cyclic AMP immunoassay was in the range of 0.2 to 40 pmoles of cyclic AMP. The characteristics of the antisera (kindly donated by Dr. R. H. Williams, University of Washington, Seattle, Washington) were as previously described (10). Determinations included at least three dilutions of each sample which showed parallelism to the standard curves. Cyclic[³H]AMP (specific activity, 28 Ci per mmole), obtained from Schwarz-Mann, was purified before radioimmunoassay (11).

Cyclic[³H]AMP (4000 cpm) was added to each incubation vial after perchloric acid precipitation. The supernatants from the perchloric acid precipitation were neutralized with 3 ml Tris and applied to Dowex 2 (Cl⁻ form) columns (6 x 30 mm), previously washed with 20 ml of glass distilled water (pH 5.5). After an additional 20 ml of water, cyclic AMP was eluted with 6 ml of 0.05 M HCl, the acid eluate lyophilized to dryness, and redissolved in 0.25 ml of 200 mM Tris-acetate, pH 8.0.

**Assay of Adenyl Cyclase in Membrane Ghosts**—Adenyl cyclase activities were determined according to Ramachandran (12) as modified by Thompson et al. (13), using membrane preparations from four normal and four hypothyroid rats in each experiment. Membrane pellets were suspended in 1.2 ml of enzyme grade sucrose (0.32 M) and dispersed to a homogeneous suspension in an ice-cold Duall homogenizing vessel. Protein was determined by the method of Schacterle et al. (14). [α-³²P]ATP (2.5 Ci per mmole) was obtained from Amersham-Searle and other reagents were from Sigma.

**Cyclic Nucleotide Phosphodiesterase Analysis**—The radioisotopic method of Thompson and Appleman (15) was used to determine cyclic GMP phosphodiesterase (20 μM cyclic GMP substrate) and cyclic AMP phosphodiesterase activities (0.5 μM cyclic AMP substrate). The method of Weiss et al. (16) was employed for cyclic AMP phosphodiesterase activity (200 μM cyclic AMP substrate).

Cyclic nucleotide phosphodiesterase enzyme forms were fractionated by a modification of the method of Thompson et al. (17). About 2 or 3 million fat cells from normal or hypothyroid rats were homogenized in 3.0 ml of 0.32 M sucrose-40 mM Tris-acetate, pH 8.0, containing 3.75 mM mercaptoethanol. Hand homogenization involved nine up and down strokes of a Teflon pestle in a Duall glass homogenizer. The homogenate (1.5 ml) was layered on a discontinuous sucrose gradient and centrifuged at 105,000 X g in a Beckman SW 41 rotor for 45 min, 0.4 ml fractions were collected.

**RESULTS**

**Effects of Lipolytic Agents and Hormones on Fat Cells from Normal and Hypothyroid Rats**—Fat cells obtained from propylthiouracil-treated rats were totally unresponsive to epinephrine or glucagon as shown in Figs. 1 and 2. The lipolytic effect of various concentrations of epinephrine and BtcáMP (Fig. 3) illustrates the typical response of cells from normal animals. Lipolysis could be activated in both the normal and hypothyroid state by BtcáMP, although the enhancement over basal levels was consistently less in the hypothyroid animal. The activation of lipolysis by BtcáMP serves as a control and also indicates, in agreement with others (4), that hormone-sensitive lipase is present in the hypothyroid state and can be converted to the active form by an agent which bypasses the adenyl cyclase step. Further evidence to support this is seen in the observation (Fig. 4) that a combination of epinephrine and phosphodiesterase inhibitors also stimulate lipolysis in the hypothyroid rat at concentrations where each alone is ineffective. Thus, in the hormonally insensitive cells the enzymatic system distal to the protein kinase is functional.

**Reversal of Antilipolytic Effect by Administration of Triiodothyronine**—The antilipolytic effect of the propylthiouracil diet can be reversed by the administration of thyroid hormones (Fig. 5). This demonstrates that the observed hormonal effects are a result of thyroid hormone deficiency, and not the diet.

**Lack of Direct Inhibitory Effect of Propylthiouracil on Epinephrine-stimulated Lipolysis**—An additional control experiment to test whether propylthiouracil itself might cause direct inhibition is described in the results shown in Table 1. Even a very high concentration of propylthiouracil added in vitro was unable to cause more than a small effect on epinephrine-stimulated lipolysis in normal cells.
Adenylyl Cyclase Activity in Fat Cell Outer Membrane Ghosts Obtained from Normal and Hypothyroid Rats—It has been reported that adipose tissue homogenates obtained from hypothyroid rats show diminished adenylyl cyclase activity in response to catecholamines, and the conclusion was drawn that this is due to a primary effect of thyroid hormones on protein synthesis (5). It thus became important to determine the adenylyl cyclase activity, if any, which might be present in our lipolytically insensitive fat cells. The additional question of whether or not there had been a loss in the ability of epinephrine or glucagon to effectively bind to receptors in the membrane and stimulate enzymatic production of cyclic AMP was also considered. The results shown in Fig. 6 clearly demonstrate that the metabolic block in the hypothyroid state cannot be due to the absence of adenylyl cyclase as has been presumed by these earlier studies. The basal specific activity of the enzyme from hypothyroid rats is reduced when compared with normal animals in agreement with Krishna et al. (5), but the apparent affinity and maximal stimulatory effect of isoproterenol is similar in both cases (Fig 6). Neither adenylyl cyclase from normal rats nor hypothyroid rats was stimulated by isoproterenol to full maximum capacity as indicated by fluoride activation. At 5 mM NaF the enzyme activity of the same membrane preparation from normal cells reached a level of 579 ± 4 pmol/min/mg of protein, and that obtained from hypothyroid rats, 520 ± 15 pmol/min/mg of protein. Additional experiments showed both membrane ghost preparations to be responsive to glucagon to the extent of about 2- to 3-fold activation over basal levels. Thus, "coupling" of catecholamine and glucagon receptors to adenylyl cyclase is not appreciably affected by thyroid status under these experimental conditions.

**Table I**

| Addition of propylthiouracil in vitro | A     | B     |
|-------------------------------------|-------|-------|
|                                     | nmoles glycerol/10⁶ cells |       |
| Cells only                          | 410 (380, 420) | 390 (320, 450) |
| Epinephrine 1 x 10⁻⁶ M              | 3000 (2900, 3100) | 3100 (3000, 3100) |
| Epinephrine 1 x 10⁻⁴ M and propylthiouracil | 2400 (2300, 2400) | 2400 (2400, 2400) |
| Bt₂cAMP 2.5 mM                      | 3500 (3300, 3700) | 2600 (2600, 2700) |

Fig. 3. Lipolytic response of fat cells from normal rats. Fat cells were obtained from three male rats, average weight 120 g. Hormones were added as indicated. The values for glycerol production are the means of either two or three incubations. Vertical lines indicate range. EPI, epinephrine; dcAMP, Bt₂cAMP.

Fig. 4. Effect of phosphodiesterase inhibitors on lipolytic sensitivity of fat cells from hypothyroid rats. Fat cells were obtained from three male rats, average weight, 150 g, which had been fed the goitrogenic diet for 27 days. Hormones were added as indicated. The values for glycerol production are the means of either two or three incubations. Vertical lines indicate range. EPI, epinephrine; THEO, theophylline, MIX, 1-methyl-3-isobutylxanthine, dcAMP.

Fig. 5. Restoration of epinephrine (EPI) sensitivity of fat cells of propylthiouracil-treated rats by in vivo T₄ administration. Three propylthiouracil-treated male rats, average weight 145 g, which had been on the propylthiouracil diet for 34 days, were given subcutaneous injections of T₄, at a dose of 50 µg/100 g body weight per day for 4 days. The animals were maintained on the propylthiouracil diet during this time. Rate was killed 3 hours following the last injection. The fat cells were then isolated and incubated in the presence of epinephrine and also with Bt₂cAMP (dcAMP) at the concentrations indicated. The values for glycerol production are the means of three incubations. Vertical lines indicate range.
Cyclic AMP Levels in Whole Fat Cells in Response to Hormones and Theophylline—Table II illustrates the results obtained from experiments carried out to determine (a) whether basal levels of cyclic AMP differ significantly in fat cells from normal and hypothyroid rats; and (b) whether we could observe changes in the concentration of cyclic AMP in response to hormonal stimulation both in the presence and absence of theophylline. Basal cyclic AMP levels were found to be the same. With 1 mM theophylline, similar amounts of cyclic AMP accumulated over the 10-min incubation period. These results serve to reconfirm the intact nature of the adenyl cyclase enzyme seen in our experiments with the membrane ghost preparation.

Epinephrine stimulation of whole fat cells from normal rats showed a marked difference in total cyclic AMP content (cells plus medium), in comparison with fat cells from hypothyroid rats. These data are generally consistent with the impaired hormone sensitivity seen in the hypothyroid animals. Appreciable elevation of cyclic AMP concentrations in the hypothyroid state only occurs in the presence of both epinephrine and theophylline.

Cyclic Nucleotide Phosphodiesterases in Normal and Hypothyroid Rat Fat Cells—Adenylyl cyclase appeared to be intact in fat cells from the hypothyroid animals. Nevertheless, epinephrine-stimulated cyclic AMP levels were much lower in these cells after a 10-min incubation. The level and distribution of cyclic nucleotide phosphodiesterases (Fig. 7) were therefore investigated. Discontinuous gradient fractionation studies indicate cyclic GMP phosphodiesterase activity is predominantly soluble as has been shown for other tissues (17, 18). Both soluble and particulate cyclic AMP hydrolyzing activities were detected by using a 0.5 mM substrate concentration. Cyclic AMP phosphodiesterase (measured at 200 mM substrate concentration) appears in the soluble fraction (19).

Previous studies indicate the fat cell soluble phosphodiesterase activity to be predominantly high Km activity and the particulate to be the low Km enzyme form displaying negative cooperativity (20). The particulate forms of low Km activity were elevated in fat cells from hypothyroid rats when calculated either on the basis of the number of cells applied to the gradient (Fig. 7), or specific activity of each particulate fraction. The gradient patterns shown represent experiments with four separate groups of propylthiouracil-treated rats. The specific activity of soluble, high Km cyclic AMP phosphodiesterase was unaffected, and that of the soluble cyclic GMP phosphodiesterase activity was only slightly elevated in the hypothyroid animals. The protein distribution patterns and total enzyme activity recoveries were similar in all cases. Additional experiments will be required to characterize more fully the altered pattern of phosphodiesterase activities associated with hypothyroid status.

DISCUSSION

A physiologically significant, specific biochemical process in which thyroid hormones directly participate has been difficult to establish. It is now apparent that many of the responses to T3 or T4 represent secondary metabolic adjustments, and are only remotely related to the immediate molecular events occurring when these hormones interact with cellular components. Considering the wide diversity of physiological actions attributed to these hormones (21), it is probable that these responses may share common interdependent relationships rather than the hormone having different, multiple direct effects. Many of these effects appear to be associated with known cyclic AMP-dependent processes which may partially explain the frequently noted but still poorly defined relationship between thyroid hormones and catecholamines (22). Impairment of the lipolytic response to epinephrine occurs after surgical or pharmacological thyroidectomy, and conversely, administration of thyroid hormones causes an enhanced sensitivity to epinephrine (1-5, 23-26). Rapid effects of thyroid hormones on cardiovascular function are also well known and are, in some respects, similar to those produced by the catecholamines (21, 22). Stimulation of cardiac adenylyl cyclase by T3 and T4 in vivo has been observed (27), although whether this can account for the effects of these hormones on cardiac function seems problematic at present (6, 27-30). Other presumed direct effects of T3 or T4 administration on cyclic AMP levels have been described (31). In general agreement with the concept of an involvement of cyclic AMP, glycogen depletion was observed by Tata et al. (31) to be one of the most immediate consequences of thyroid hormone administration.

The primary mode of action of thyroid hormones has been thought to be upon protein synthesis (32-36), and Krishna et al. (5) suggested that the effect of these hormones on adipose tissue might be mediated through synthesis of adenylyl cyclase. However, such an effect could not be seen in other tissues (29, 37, 38), and Caldwell and Fain (25) failed to observe any increase in adenylyl cyclase activity in rat cell membrane ghosts prepared from T3-treated rats. These observations are consistent with our experiments with hypothyroid animals (Figs. 4 and 6) which contradict the proposal that the metabolic blockade is due to a
deficiency of the enzyme, adenylyl cyclase. It is apparent that events associated with the binding of epinephrine and glucagon and importantly, the coupling of receptor to adenylyl cyclase in the membrane preparation are unaffected by thyroid status.

Not only is lipolytic activity in response to hormones blocked in the hypothyroid state, but cyclic AMP also does not accumulate. While it is conceivable that these two events are totally independent of one another, the experimental evidence suggests that this is improbable. Blockade can be overcome by 8B2cAMP and by a combination of epinephrine and phosphodiesterase inhibitors (Fig. 3) leading also to an increase in endogenous cyclic AMP levels (Table II).

Close coupling of adenylyl cyclase activity with lipase activity has been proposed by Manganiello et al. (39). Linkage of these two enzyme activities would then depend on the rate of turnover of lipolytically significant cyclic AMP. If cyclic AMP metabolism is related to the loss of hormonal response in the hypothyroid state, then the modulating effect of thyroid hormones could involve an alteration in the turnover rate of total or compartmentalized cyclic AMP.

Defective adenylyl cyclase activity is not likely to provide the explanation for the total lack of hormone induced lipolysis, since we have shown that adenylyl cyclase is equally responsive in membrane ghost preparations from both normal and hypothyroid animals. Furthermore, cyclic AMP levels in both types of intact cells can be elevated in response to hormonal stimulation if phosphodiesterase inhibitors are present. These observations lead directly to the possibility that an important factor differentiating the hypothyroid from the normal state may be an altered capacity for hydrolysis of the cyclic AMP formed in response to epinephrine or other hormonal stimulation. Inhibition of phosphodiesterase in vitro by addition of thyroid hormones has been reported (40–44), but these experiments have been largely discounted because of the excessively high levels of hormone required, and the lack of structural specificity.

The results of our studies suggest that activation of membrane-bound cyclic AMP phosphodiesterase held in close proximity to an adenylyl cyclase-lipase complex could be responsible for the insensitivity to epinephrine and glucagon, and may represent the site of the lesion in the thyroid-deficient state. Such localized changes in specific phosphodiesterases would not be readily detected in homogenates or in the supernatant fraction after centrifugation, and this may partially account for the inability of previous workers (5, 25, 44) to find a correlation between thyroid status and phosphodiesterase activity in adipose tissue.

Since normal morphogenetic development may be controlled by intracellular changes in cyclic AMP levels through altered membrane-bound phosphodiesterase activities (45), it is tempting to speculate that some of the effects of thyroid hormones on growth and development (21) might be explained in this manner. Regulation of specific phosphodiesterase activities may thus represent one critical aspect of a more general mechanism for the control of cellular metabolism by growth and developmental hormones.

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