A COMPARISON OF THYROTROPIN- AND DIBUTYRYL CYCLIC AMP- INDUCED THYROGLOBULIN IODINATION IN CULTURED HUMAN THYROID CELLS

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SUMMARY

The relative degree of 125-I labelling of thyroglobulin - bound mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) in isolated, cultured human thyroid cells has been compared following exposure of 125-I supplemented cells to 100mU/ml of bovine thyrotropin (TSH) or 1.0mM dibutyryl cyclic AMP (dBcAMP) for 96 hours. Pronase digestion of the lysed cells and Sephadex G-10 fractionation of the digested lysates revealed a predominance of [125-I]MIT over [125-I]DIT in both sets of experimental cells as well as in controls. Levels of [125-I]DIT, however, were only enhanced above control values in cells incubated with TSH.

These findings suggest that an increase in availability of intracellular iodide, following cellular exposure to TSH, may facilitate a preferential synthesis of DIT relative to that of MIT. This theory offers an explanation for the differential effects of TSH and dibutyryl cyclic AMP on the levels of newly - synthesised T4 recovered from the cells used in this study, and from the culture medium in a previous investigation.

INTRODUCTION

Previous investigations [1, 2] on the comparative effects of bovine thyrotropin (TSH) and dibutyryl cyclic AMP (dBcAMP) on the release of iodothyronines from cultured, isolated human thyroid cells indicated that 100mU/ml TSH and 1.0mM dBcAMP were equipotent on the release of tri-iodothyronine (T3) and thyroxine (T4) over a period of 96 hours. However, certain differences have been observed between the actions of these two compounds, both on the stimulation of thyroid cell metabolism [3] and on the release of newly - synthesised, as opposed to total T3 and T4 [1, 4].

In order to investigate further the underlying factors responsible for this apparent differential enhancement of the synthesis of T3 and T4, we have investigated the degree of 125-I incorporation into the intracellular iodothyronine precursors mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) as well as on the levels of intracellular [125-I]T3 and [125-I]T4, following treatment of parallel cell cultures with TSH or dBcAMP.

METHODS

Cell culture

Normal human thyroid cells were obtained by trypsinisation of thyroid tissue taken from patients undergoing surgical removal of thyroid cysts. The wide margin of histologically normal tissue excised with each cyst was disaggregated, as described previously [1] in 0.25% (w/v) trypsin in Hank's salt solution (Gibco:Biocult, Ltd., Paisley, Renfrewshire, Scotland). The suspension of single cells produced after 2 hours of trypsinisation at 37°C was collected by centrifugation at 600 x g. Remaining trypsin activity was inactivated by resuspending the pellets in 0.25% (w/v) soyabean trypsin inhibitor (Sigma Chemical Company, Kingston, Surrey, England) for 30 minutes at 37°C, followed by recentrifugation at 600 x g. Cell pellets were then resuspended in Eagle's Basal Medium (Gibco:Biocult Ltd.) containing 10% (v/v) foetal calf serum (Gibco:Biocult Ltd), 200µg/ml gentamicin (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland), 20mM HEPES buffer (Gibco:Biocult Ltd.) and 1µM sodium iodide containing trace 125-I (4mCi/ml, the Radiochemical Centre, Amersham, Bucks, England). The final volume of each culture was 5.0ml.

Three groups of cultures were initiated: Group (i) received 100mU/ml of bovine TSH (Sigma), group (ii) received 1.0mM dBcAMP (Sigma) and group (iii) received a blank sterile saline addition. Cultures were incubated at 37°C in 35 x 10mm cell culture dishes (Gibco:Biocult Ltd.).

Post - culture treatment

After the incubation period, the cell culture dishes were placed on crushed ice, and cell suspensions obtained in situ, by gentle scraping in the presence of the medium. Each suspension was then aspirated and centrifuged at 2000 x g for 10 minutes at 4°C. Cell pellets obtained were resuspended in 2.0ml of cold (4°C) 0.05M sodium phosphate buffer, pH 7.4, containing 3mM methimazole (2-methyl-1-mercaptoimidazole, Sigma) and the suspensions were freeze - thawed twice in a dry - ice / acetone bath to release cellular protein and DNA. The latter was subsequently assayed by the method of Leyva & Kelley [5]. The remaining 1.0ml aliquots of each lysate were incubated with pronase (protease type V, 8.0mg/ml final concentration, Sigma) for 6 hours at 37°C. The resulting digests were frozen to -20°C and stored, prior to subsequent fractionation.

Fractionation of lysate digests

Five - hundred microlitre aliquots of each thawed cell lysate digest were fractionated according to the method of Thomopoulos [6] on 0.9 x 30 cm columns of Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden). Fractions were eluted from the columns in two stages: stage (i); fractions 1 - 100, using 0.5M Tris - Maleic acid, pH 6.0, and stage (ii); fractions 101 - 140, using 0.05M Tris - HCl, pH 9.0 . Eight minute fractions were collected, and the 125-I activity of each fraction was determined using a well - type gamma counter. All counts were expressed in terms of culture DNA content.

RESULTS

The Sephadex G-10 fractionation procedure employed in this study has been shown [6] to effectively separate MIT and DIT from free iodide and iodopeptides in the first stage, and T3 and T4 in the second stage.

Although thyroglobulin digestion has been performed most effectively with pronase and leucylaminopeptidase [7], pronase alone has been found to be satis -

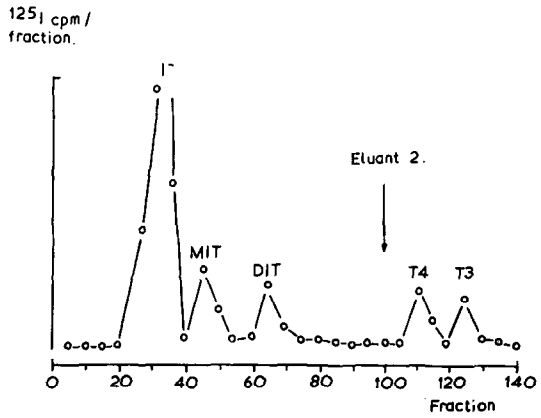

Figure 1. Sephadex G-10 fractionation of a pronase - digested thyroid cell lysate after culturing for 96 hours with 100mU/ml bovine TSH and 1μM sodium iodide, trace-labelled with 125-I. Details of the fractionation procedure are given in Methods.

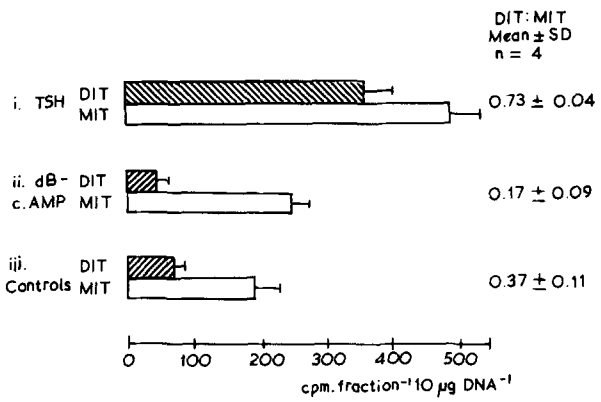

Figure 2. Recovery of MIT and DIT after Sephadex G-10 fractionation of pronase - digested lysates of thyroid cell cultures. Cells were originally supplemented with 100mU/ml of bovine TSH (group i); with 1.0mM dBcAMP (group ii); or with no supplement (group iii)., in addition to 1.0μM sodium iodide, trace - labelled with 125-I.

factory in the release of MIT and DIT residues from iodinated thyroglobulin [3] and therefore this latter method has been used in the present study, for reasons of simplicity and economy.

Figure 1 shows a representative scan for 125-I activity in the Sephadex G-10 column effluent, following fractionation of a pronase - digested lysate of a TSH - supplemented thyroid cell culture, and shows the relative elution positions of 125-I labelled MIT, DIT, T3 and T4. Figure 2 shows the levels of

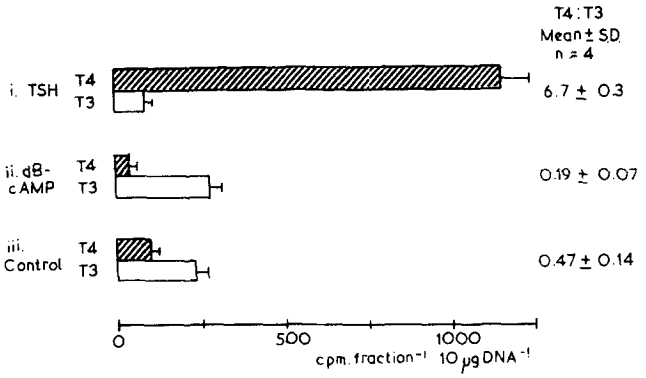

Figure 3. Recovery of T3 and T4 after Sephadex G-10 fractionation of pronase - digested lysates of thyroid cells cultured for 96 hours with 100mU/ml of bovine TSH (group i), with 1.0mM dBcAMP (group ii) or with no supplement (group iii), in addition to 1.0µM sodium iodide, trace - labelled with 125-I.

125-I labelled MIT and DIT released after lysis and pronase digestion of cells which had been incubated with 100mU/ml of bovine TSH, 1.0mM dBcAMP, or with a blank saline addition. Both [125-I]MIT and [125-I]DIT were significantly ($p<0.001$) increased after TSH, but not after dBcAMP. The DIT:MIT ratio was also increased after TSH, although both control and supplemented cells contained more [125-I]MIT than [125-I]DIT. Figure 3 shows the levels of [125-I]T3 and [125-I]T4 eluting in the second stage of the Sephadex G-10 fractionation procedure. In control cultures, and in cells incubated with 1.0mM dBcAMP, [125-I]T3 was predominant. In cells supplemented with 100mU/ml of bovine TSH, however, [125-I]T3 was diminished, whilst levels of [125-I]T4 were very significantly increased ($p<0.001$). Thus in control and dBcAMP supplemented cells, [125-I]T3 was predominant, whilst after TSH, [125-I]T4 was always greater than [125-I]T3.

DISCUSSION

In an earlier investigation [1], [125-I]T4 levels within the medium of human thyroid cell cultures were only increased in response to TSH. The present study confirms that this increased level of [125-I]T4, in response to TSH, is also found within the cells. Moreover, the T4:T3 ratios within the cells were similar to those observed previously in the medium [1], despite probable degradation of both T4 and T3 following release from the cells [8].

Having established that the medium and cellular T4:T3 ratios were comparable, the purpose of the present study was to attempt to relate the levels of newly - synthesised T4 and T3 to those of their intracellular precursors, MIT and DIT.

T4 is formed within the thyroid cell by the coupling of two thyroglobulin - bound DIT residues in close proximity, whereas T3 formation results from the coupling of one MIT and one DIT residue. It may thus be assumed that DIT is more readily utilised in iodothyronine synthesis than MIT.

In contrast to our previous findings [1], control cultures formed newly - iodinated T3 in preference to T4. Since higher levels of intracellular MIT relative to DIT were also observed in controls, we suggest that under conditions in which intracellular iodide availability is limiting, i.e. in non - TSH supplemented cells, MIT may be preferentially formed, with a corresponding increase in T3 synthesis relative to that of T4. A similar observation has been made previously [9] by Tong, Kerkof and Chaikoff.

Incubation of human thyroid cells with bovine TSH has been shown, in a separate study [2] to prolong the duration of inorganic iodide uptake from the medium, with correspondingly less limitation of iodotyrosine synthesis. Thus in the present study, TSH - treated cells contained markedly more MIT and DIT than either dBcAMP or control - treated cells. Moreover, evidence for the more rapid utilisation of DIT in iodothyronine synthesis was provided by the very significant increase in [125-I]T4 recovered from TSH - treated cells (Figure 3). Furthermore, although [125-I]MIT synthesis was also increased, [125-I]T3 was depressed, suggesting the preferential iodination of MIT to DIT, rather than being coupled to a pre-existing DIT residue. With the formation of two suitably - adjacent DIT residues on the thyroglobulin molecule, the rapid formation of T4 would depress DIT levels, and this would provide an explanation for the marked discrepancy between the cellular DIT:MIT and T4:T3 ratios in TSH - supplemented cells (Figures 2 and 3).

The small increase in MIT recovery observed after dBcAMP treatment (Figure 2) was reflected in a comparable increase in [125-I]T3 recovered after lysis of dBcAMP- supplemented cells (Figure 3). The dBcAMP - induced depression of both [125-I]DIT and [125-I]T4 to below basal levels probably reflects this preferential T3 formation.

These studies therefore confirm that the ratio of newly - synthesised T4 to T3 in cellular lysates is directly comparable to the T4:T3 ratio in the medium. Available intracellular iodide appeared to be preferentially utilised for MIT synthesis in controls and dBcAMP - supplemented cultures, and DIT was only increased after treatment with TSH. However, the very much greater levels of 125-I in T4 recovered from TSH - supplemented cells suggests that DIT may be rapidly utilised for T4 synthesis. Levels of T4, markedly increased after TSH, at the expense of T3 and MIT, may reflect the increased availability or flux of intra - cellular iodide after TSH, which would enable further iodination of MIT to DIT. Possible reasons for this apparent shift in iodotyrosine formation towards DIT,

in the presence of TSH, but not with dBcAMP, are not yet clear. However, it seems likely from the results of our own [2] and independent [9] studies that the availability of intracellular iodide may play a role in determining the formation of MIT relative to that of DIT.

It is suggested that the difference may lie in the relative abilities of TSH and dBcAMP to stimulate iodide uptake, over the 96 hour incubation period employed in this study. Lissitzky, Fayet and Verrier [10] found that thyroidal adenylate cyclase was continually activated after 96 hours of in vitro cell exposure to TSH, as a result of permenant saturation by TSH of its cell membrane receptors [11]. Moreover, dBcAMP, although more resistant to degradation than native cyclic AMP [12 may be progressively destroyed during the 96 hour incubation period, resulting in a decreased stimulation of iodide uptake, and a corresponding lack of iodide supply for iodothyronine synthesis. A long - term differential effect of TSH and dBcAMP on iodide uptake over a 96 hour incubation period has already been reported [2]. Under these conditions, available iodide might be channelled into MIT synthesis, with a resultant decrease in the DIT:MIT and T4:T3 ratios. Such a situation has been reported in vivo [13], where iodide depletion results in an increased thyroid production of T3, at the expense of T4 synthesis.

In order to substantiate the theory of a differential period of enhancement of intracellular adenyl cyclase activity and associated iodide uptake in response to TSH and dibutyryl cyclic AMP, we are currently investigating the relative effects of these agents on the rate of turnover of intracellular cyclic AMP and various other intracellular factors which may enhance or repress the adenyl cyclase response to TSH - receptor binding within the human thyroid cell.

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