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Binding of thyroid hormone to mouse granulosa cell nuclei and its biological relevance

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Abstract. Thyroid hormone showed specific binding ability to mouse granulosa cells from immature mice, primed with post menopausal gonadotropin. Saturation of specific binding sites was reached by 2 nM concentration of the hormone. A Scatchard analysis of thyroid hormone binding exhibited a K_d of 42×10^{-9} M/mg nuclear DNA and a maximum binding capacity of 1 pmol/mg nuclear DNA. Competitive inhibition studies showed thyroid hormone binding to be analogue specific. Addition of 100 ng of thyroid hormone to granulosa cell incubations (1×10^6 cells/well) resulted in a three-fold increase in cellular protein synthesis. Thyroid hormone resulted in a dose dependant increase in progesterone release from granulosa cell. It also stimulated the formation of pregnenolone (83%) and progesterone (81%) from radiolabeled cholesterol as compared to control. This stimulation by thyroid hormone was completely inhibited by cycloheximide. Results indicate a direct effect of thyroid hormone on granulosa cells, its binding to nuclei causing an increase in steroidogenesis through the mediation of protein(s).

Keywords. Thyroid hormone; mouse granulosa cells; steroidogenesis.

1. Introduction

Thyroid has long been implicated in the reproduction of vertebrates (Ball 1960; Sage 1973; Dodd 1975; Sen and Bhattacharya 1982; Gorbrnan *et al* 1983; Chakraborti and Bhattacharya 1984). Thyroid hormone has been found to influence both ovarian (Southren *et al* 1974; Akande 1975) and testicular (Kato *et al* 1970; Howland and Ibrahim 1973; Schreider *et al* 1979; Chadrashekar *et al* 1986) functions in mammals. Its involvement in both ovarian and testicular functions in humans is indicated by the fact that thyroprivic hypothyroidism from infancy, if untreated, leads to sexual immaturity in men, while hypothyroidism in women cause irregular menstrual bleeding due to failure of progesterone secretion (Ingbar and Woebar 1981). Alteration in steroid hormone metabolism due to hypothyroidism can be improved by restoring the euthyroid state (Gordon and Southren 1977). All these reports clearly suggest an influence of thyroid hormone on gonadal function, but precisely how it does so is still not clear. Recent reports from our laboratory showed high affinity and low capacity 3,5,3' -triiodothyronine (T_3) binding sites in the oocyte nuclei of perch (Chakraborti *et al* 1986; Maitra and Bhattacharya 1989) and in human corpus luteal cell nuclei (Bhattacharya *et al* 1988). Binding of T_3 has also been demonstrated in porcine granulosa cell nuclei (Wakim *et al* 1987). These information may imply a direct involvement of thyroid hormone in ovarian function. To clarify the situation further we describe here the *in vitro* binding of T_3

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to mouse granulosa cell nuclei and demonstrate that addition of T_3 to mouse granulosa cell *in vitro* causes a significant increase in progesterone release.

2. Materials and methods

2.1 *Isolation and incubation of mouse granulosa cells*

Mouse granulosa cells were obtained from immature female mice (18-23 days old) pre-treated with Humegon (post menopausal gonadotropin, Organon, Holland). Mice were sacrificed after 72 h of initial injection, the ovaries were removed and immediately transferred to chilled culture medium (medium 199 with Hank's salt, GIBCO, USA). Priming of immature mice by Humegon, which is follicle stimulating hormone (FSH), resulted in the proliferation of only one kind of ovarian cells, *i.e.* the granulosa cells. These cells were collected by gently tapping the ovary several times with a blunt instrument and then harvesting them by centrifugation (300 *g,* 0°C, 15 min). The cells were resuspended in 1 ml of the medium which was gassed with 95% O2/5% CO_2. Viability of the cells was 90% as estimated by using 0·1% trypan blue (BDH, England) dye exclusion method. Cells were counted in a haemocytometer and 1×10^6 cells were taken in each well of a microwell module (NUNC, Denmark, Model F 16). The cells were incubated at 37° C, with gentle shaking, under an atmosphere of oxygen, for 1 h and at the end of 1 h T_3 (50 ng or 100 ng or 150ng/incubation) was added. Incubations were carried out in a final volume of 400 μl. A 1 h preincubation time was necessary for recovery of the cells from initial shock. The incubation was continued for 3 h.

After completion of incubation the medium was separated from the cells by centrifugation at 300 *g* for 10 min at 0°C. Progesterone released into the medium was determined by radioimmunoassay (RIA).

2.2 *T_3 binding assay*

T_3 binding was performed using pure nuclear preparations. Granulosa cells were lysed by ultrasonication and nuclei isolated according to our earlier procedure (Chakraborti *et al* 1986; Maitra and Bhattacharya 1989) by following the description of Lawson *et al* (1987). For a Scatchard analysis (Scatchard 1949), Nuclear preparations containing 35 μg DNA were incubated in a final volume of 0·5 ml with varying concentrations of [^{125}I] T_3 (9·8-38 nM) in the absence (total binding) or presence of 500-fold excess of unlabelled T_3 (non-specific binding) at pH 7·5 and 37°C for 1 h, in a shaking water bath. After termination of the incubations, free and bound radioactivity were separated by addition of l·0 ml ice-cold 40% polyethylene glycol followed by centrifugation at 3000 *g* in a refrigerated centrifuge. The supernatant was aspirated and radioactivity in the pellet measured in a gamma counter (1282 Compugamma CS, LKB Wallac, Sweden). For competitive binding studies the above mentioned parameters were kept constant except the addition of different analogues.

2.3 *T_3 incubation and protein synthesis*

Granulosa cells (1×10^6/well) were incubated with [^{14}C] alanine in medium 199

supplemented with 1 mM of 18 different amino acids except alanine. The pH was adjusted to 7.0. After 40 min preincubation the cells were incubated for 2 h in the presence of T_3 (100 ng) alone or T_3 (100 ng) plus cycloheximide (100μg/ml) or in the absence of either chemical (control). The cells were then separated from the medium by centrifugation, resuspended in distilled water and subjected to ultrasonication. The sonicated material was precipitated with a final concentration of 10% TCA (w/v). The pellet obtained after centrifugation at 3000 *g* was washed first with 10% TCA and then with 5% TCA containing cold alanine (1 mM). TCA (7%) was next added to the pellet and the sample was heated for 30 min at 95°C, to hydrolyse aminoacyl tRNA. After further centrifugation and two washes with ethanol: ether (1:1), the final precipitate was dissolved in 500 μl of 1 (N) NaOH and the radio activity measured in a liquid scintillation counter (LSS 20, ECIL) in 10 ml of a toluene based cocktail containing PPO, POPOP and methylcellosolve.

2.4 *Progesterone (P_4) RIA*

Progesterone RIA was carried out by following the method of Korenman *et al* (1974), with minor modifications. Aliquots of the medium were incubated in phosphate buffered saline (10mM K-PO_4, pH 7·4, 0·15 M NaCl) containing 0·1% gelatin, progesterone antibody 1:500 dilution (donated by Professor Gordon Niswender, Colorado State University, USA) and [1, 2, 6, 7^{3}H] progesterone 15000cpm (Amersham, England). Incubations were carried out at 4°C for 16 h. Bound and free progesterone were separated by using dextran-coated charcoal (0·6% charcoal, 0·06% dextran). Bound [^{3}H] progesterone was counted in a liquid scintillation counter (LSS 20, ECIL) by using 10 ml of a toluene based cocktail containing PPO, POPOP and methylcellosolve.

2.5 *Effect of T_3 on steroidogenesis*

The effect of T_3 on steroidogenesis in mouse granulosa cells was monitored by measuring the conversion of [^{14}C] cholesterol to [^{14}C] progesterone. 1×10^6 cells/well were incubated in medium 199 in the presence of [^{14}C] cholesterol. After a preincubation of 30 min at 37°C incubations were carried out for 3 h in the presence of T_3 (100 ng) alone or T_3 (100 ng) plus cycloheximide or in the absence of either chemical (control). After completion of incubation the cells were seperated from the medium by centrifugation. The cells were then sonicated in 1 ml of 10 mM sodium-phosphate, pH 7·4 containing 10 µg/ml of Trypsin inhibitor and 0·05 M phenylmethylsulphonylfluoride (PMSF). Steroids were extracted from the homogenate with diethylether. The mixture of extracted steroids was chromatographed on a thin-layer plate and cholesterol, pregnenolone and progesterone were separated by using diisopropyl ether: petroleum spirit: acetic acid solvent system in the ratio of 70:30:1. Three different standards, one for each of the three different steroids, were chromatographed separately under similar conditions. The plates were developed in iodine and spots corresponding to the three steroids were excised and counted in a liquid scintillation counter in 10 ml of the scintillant mentioned previously in § 2·3.

2.6 *Statistical analysis of data*

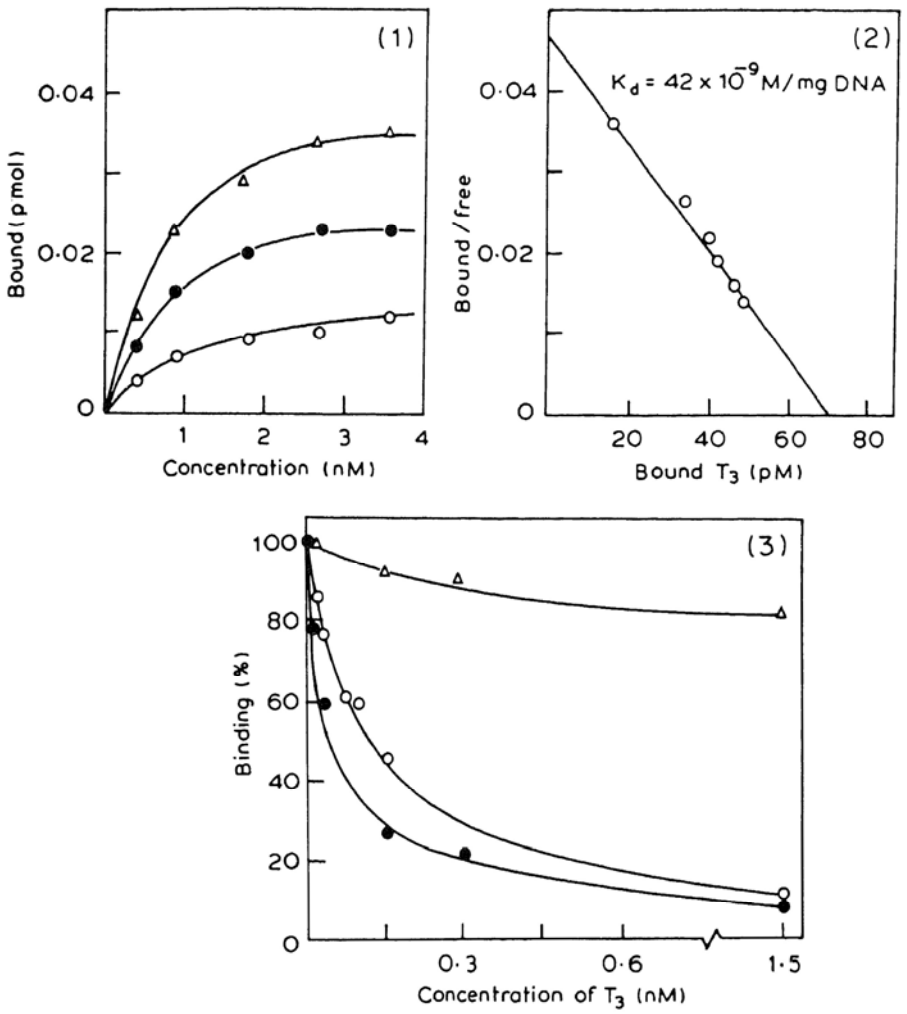
All data were analysed by Student's *'t'* test (Snedecor and Cochran 1968). The data presented are the mean ± SE of triplicate observations unless otherwise stated.

3. Results

Binding of T_3 to mouse granulosa cell nuclei were carried out at 37° C and pH 7·5 and maximal binding occurred at 60 min of incubation. Figure 1 shows the specific binding of T_3 to granulosa cell nuclei with increasing concentrations of radio-labelled T_3. Specific binding of [^{125}I] T_3 increased linearly from 0·5-2·0 nM and then levelled off indicating binding saturation. Scatchard analysis of the data were compatible with high affinity low capacity binding sites. The K_d was 42×10^{-9} M / m g DNA and maximum binding capacity (MBC) for granulosa cell nuclei was 1 pmol/mg DNA (figure 2). Results from competitive binding assays indicate analogue specificity of the T_3 receptors. While diiodotyrosine (DIT) was a poor competitor, T_4 could compete with T_3 in inhibiting [^{125}I] T_3 binding (figure 3), although less competitively. To observe the biological activity of T_3 on mouse granulosa cells *in vitro* three experiments were carried out: (i) Incubation of granulosa cells with T_3 in the presence of [^{14}C] alanine and other cold amino acids to have an idea about the increase in TCA precipitable protein synthesis; (ii) addition of varied concentrations of T_3 to mouse granulosa cell incubation and determination of the amount of progesterone released into the medium; (iii) to see the effect of T_3 on steroidogenesis, granulosa cells were incubated with a radiolabeled steroid precursor—[^{14}C] cholesterol. Addition of T_3 resulted in a three-fold increase of [^{14}C] alanine incorporation into TCA precipitable proteins. T_3 stimulation was significantly ($P<0·001$) inhibited by cycloheximide (figure 4). In the second experiment 50 and 100 ng of T_3 addition increased progesterone release in a dose dependent manner, while 150ng had no additional effect over 100 ng (figure 5) and, in the third experiment, T_3 stimulated steroidogenesis in granulosa cells significantly (*P* value for both pregnenolone and progesterone formation was $P< 0·001$) which was completely inhibited by cycloheximide (table 1).

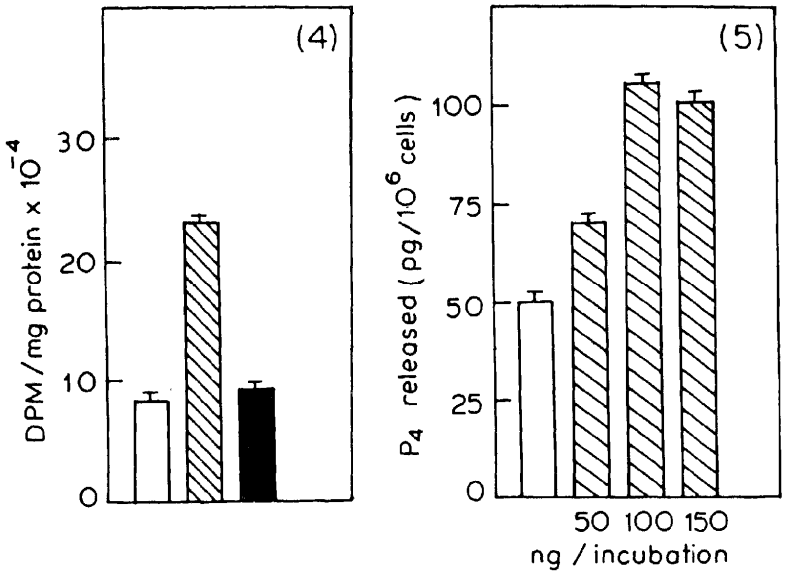
4. Discussion

The normal development of granulosa cells in mammals, so long attributed solely to the functions of pituitary gonadotropins, are now known to be modulated by non-gonadotropic hormones also (May and Schomberg 1981; Kirkland *et al* 1983). In uterus from hypothyroid rats, restoration of estrogen response was possible by thyroid hormone supplementation (Gardner *et al* 1978; Kirkland *et al* 1981). From a biological point of view it is important to understand the relationship between hormonal occupancy and specific biological response parameters. Our studies provide a support to the assumption that thyroid hormone is directly involved in ovarian cell function. The findings clearly show that T_3 can stimulate the release of progesterone from mouse granulosa cells. That this release is dose-dependent further indicates that T_3 induced steroid production is not an experimental artifact. The influence of gonadotropins, LH and FSH, does not arise as cells were



Figures 1-3. (1) Concentration curve of T_3 binding to granulosa cell DNA showing saturation of specific binding. 35 μg DNA from granulosa cell nuclei were incubated with 4·6-19 pmol of [^{125}I] T_3 for 60 min at 37°C. Non-specific binding (O) is in the presence of 500-fold excess cold T_3 . Specific binding (●) is the difference between total binding (Δ) and non-specific binding. **(2)** Scatchard analysis of the data obtained from figure 1. The concentration of bound hormone [^{125}I] T_3 is plotted against the ratio of the bound and free hormone. The intercept on the X-axis denotes the maximum binding capacity (MBC). K_d, dissociation constant. **(3)** Competitive inhibition of radiolabeled T_3 binding to mouse granulosa cell DNA by DIT (Δ), T_4 (O) and T_3 (●). 0·015—1·5 nM of cold hormones were used.

incubated *in vitro*. A previous report from this laboratory shows that T_3 could increase fish mitochondrial steroidogenesis several fold (Kaul and Bhattacharya 1988). Evaluation of a direct action of T_3 is possible by studying the binding of T_3 to granulosa cell nuclei in *in vitro* conditions. Specific binding of T_3 is believed to result in the initiation of atleast some of the effects of thyroid hormone (Oppenheimer 1979; Seelig *et al* 1981). T_3 is known to regulate gene expression by



Figures 4 and 5. (4) Incubation of mouse granulosa cells with T_3 and [^{14}C] alanine (with 18 other amino acids) to monitor protein synthesis. After a preincubation of 40 min the cells were incubated for 120 min with the indicated compounds. (□) Control, (▨) 100 ng T_3, (■) 100 ng T_3 and 100 μg/ml cycloheximide. **(5)** Effect of T_3 on release of progesterone by mouse granulosa cells. After a preincubation of 30 min the cells were incubated for 3 h in the absence of T_3 (□) or in the presence of the indicated amounts of T_3 (▨).

Table 1. Formation of pregnenolone and progesterone from radiolabelled cholesterol.

System	Steroid produced (pmol/1 × 10^6 cells)	
	Pregnenolone	Progesterone
Control (C)	1·30 ± 0	2·04 ± 0·5
C + T_3	2·38 ± 0	3·70 ± 0·35
C + T_3 + cycloheximide	1·50 ± 0·25	1·94 ± 0·68

100 ng T_3 and 100 μg/ml cycloheximide were used in the granulose cell incubations.

tightly binding to its receptors on DNA (Koerner *et al* 1975). These receptor sites have been located on the acidic chromatin proteins (Surks *et al* 1973; McLeod and Baxter 1976) and a good correlation exists between binding of T_3, T_4 and a number of thyroid hormone analogues to these sites and subsequent responses (Latham *et al* 1976). On entering the cell thyroid hormones are bound by the cytosol receptor proteins and subsequently by the nuclear and mitochondrial receptor proteins. Unlike steroid hormones, cytosol binding proteins here do not play a role in translocating the hormone to the nucleas but possibly serve as a reservoir site (Sterling and Milch 1975; Bernal and DeGroot 1980; Morgan, 1984). Binding of thyroid hormone to the nuclear receptor on the other hand, results in induction of

mRNA and protein synthesis which resemble steroid hormone receptor function (Tata 1974; Larsen and Ingbar 1992). In order to establish its true nature, a hormone receptor must satisfy certain general criteria. These are saturability, appropriate affinity and binding capacity. From our data it is clear that mouse granulosa cell nuclei contain a single species of high affinity (42×10^{-9} M/mg DNA) and low capacity (1 pmol/mg DNA) T_3 receptors which are saturable on addition of high doses of the hormone (T_3). Affinity of T_3 to the binding sites on mouse granulosa cells is comparable to the earlier findings in this laboratory on piscine ovarian nuclei (Chakraborti *et al* 1986) and nuclei from human corpus luteal cells (Bhattacharya *et al* 1988). In these previous studies it has been shown that thyroid hormone (T_4) and 3,3', 5-triidothyroacetic acid (Triac), a thyroid hormone analogue, competitively inhibit [^{125}I] T_3 binding to its nuclear receptor. The rates of binding, however, are not similar. Binding of T_3 to mouse granulosa cell nuclear receptor is also analogue specific, DIT is a poor competitor while T_4 competes very well. T_4 did not compete so well with T_3 in the case of perch ovarian or human corpus luteal nuclei (Chakraborti *et al* 1986; Bhattacharya *et al* 1988). This further strengthens the ground for belief that a single type of receptor binds T_3 as well as T_4 and their analogues. The difference in receptor occupancy indicates specificity for T_3. Comparable binding has been observed in other mammalian tissues as well (Arnott and Eastman 1983; Mukku *et al* 1983; Wakim *et al* 1987; Jannini *et al* 1990).

The valid question arising out of our observation is, what is the biological relevance of binding of T_3 to granulosa cells? It is known that protein synthesis is a pre-requisite of acute steroid secretion by steroidogenic cells. This has been amply demonstrated in Graafian follicles (Tsafriri *et al* 1973), rat Leydig cells (Cooke *et al* 1975) and hamster follicle cells (Greenwald and Limback 1984). Our findings show that addition of T_3 to granulosa cells incubated in medium containing [^{14}C] alanine increases the incorporation of this radiolabeled amino acid into TCA precipitable proteins. The increase is inhibited by a protein synthesis inhibitor, cycloheximide, which supports our previous observation on piscine ovary (Kaul and Bhattacharya 1988). Further evidence for the biological relevance of T_3 binding comes from our data where we show that progesterone is released in a dose-dependent manner from mouse granulosa cells. How T_3 increases progesterone release from these ovarian cells is a pertinent question which cannot be answered clearly at this moment. However, stimulation of steroidogenesis in granulosa cells by T_3 was monitored by observing the conversion of radiolabeled cholesterol to pregenenolone and progesterone. This conversion was remarkably stimulated by T_3 (pregnenolone 83% and progesterone 81 % as compared to control). T_3 stimulation of steroidogenesis in granulosa cells was completely inhibited by cycloheximide, indicating that T_3 possibly mediates its action *via* the synthesis of a protein or proteins.

In conclusion it may be remarked that high affinity specific binding of T_3 to mouse granulosa cell nuclei, increase in cellular protein synthesis, formation of pregnenolone and progesterone from cholesterol precursor and progesterone release from granulosa cells by T_3 clearly indicates its direct effect on the ovarian cells. Our observation will raise questions regarding the specific type (s) of protein (s) to be induced by T_3 in granulosa cells and the precise mechanism underlying T_3 stimulated progesterone release. This is a maiden report which shows that

reproductive abnormality in thyroid disorder may be linked directly to T_3 action and our finding put forward the need to investigate the details of this mechanism further.

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