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Thyroid Hormone Receptor Occupancy and Biological Effects of 3,5,3,-L-Triiodothyronine (T_3) in GH_4C_1 Rat Pituitary Tumour Cells

P. FILIPČÍK, V. ŠTRBÁK, J. BRTKO

Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovak Republic

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Summary

The GH_4C_1 pituitary cell line, an excellent model for a thyroid hormone action study, was used for determination of the relationship between thyroid hormone receptor occupancy and intensity of cell proliferation, prolactin (PRL) production, thyrotropin (TSH) inhibition and 3,5,3,-L-triiodothyronine (T_3) receptor down-regulation. Nuclear receptor population was progressively occupied by T_3 in concentrations ranging from 0.025 to 10.0 nM T_3. B_{max} ranged from 0.029 fmol/10^6 cells at the lowest T_3 concentration to B_{max} = 12.51 fmol/10^6 cells at the highest concentration. Each of the observed biological events is operative within distinct dose-response ranges in cultured GH_4C_1 cells. The maximal biological response (except the TSH inhibition and T_3 receptor down-regulation) does not require the occupation of the whole nuclear receptor population by T_3 and the intensity of none of the responses studied was directly proportional to thyroid hormone receptor occupancy.

Key words

GH_4C_1 rat pituitary cell line – 3,5,3,-L-triiodothyronine – Thyroid hormone receptors – Receptor occupancy – Biological response

Introduction

Thyroid hormones (TH) regulate a number of biological events in the pituitary. Their effects are mediated through intranuclear receptors (TR) that have been identified as transcriptional regulators. They are able to bind to specific DNA sequences and regulate complex networks of target genes, and thus control various aspects of growth and development. The existence of different isoforms of TR which are specific in their developmental and spatial distributions in the body and throughout, as well as their intermolecular interactions with other transcriptional factors or accessory proteins, makes the explanation of the mechanism of TR action rather complicated. Moreover, it is believed that various TR isoforms play different roles in biological events (Bradley *et al.* 1994).

Thyroid hormone receptor occupancy (THRO) is one of the critical factors involved in the process of regulation of TH specific events. Relationships between THRO and the intensity of the final biological effect had been investigated by using different cell lines, but controversial results were obtained (Shupnik *et al.* 1986, Halperin *et al.* 1990). Useful models for this type of study have been provided by pituitary tumour cell lines (GH_1, GH_3, GC, GH_4C_1) which possess unique characteristics of the original differentiated tissue such as production of hormones in combination with an immortalized growth potential. Moreover, these cell lines express intranuclear TH receptors in very high amounts and they respond to physiological concentrations of TH.

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E-mail physres@sun1.biomed.cas.cz
Fax +420 2 293 894
http://www.lf2.cuni.cz/physiolres/physiolres.htm

The aim of this study was to determine T_3 dose responses for nuclear receptor occupancy and several biological parameters including cell growth, prolactin (PRL) and thyrotropin (TSH) secretion and TR down-regulation in GH_4C_1 pituitary cells. We have also clarified the relationship between T_3 receptor occupancy and intensity of the biological effects which seem to be important for further understanding the mechanism of TH action. Thus, our data provide further insight into the mechanism of physiological regulations by thyroid hormones and can supplement other data obtained at the level of T_3-activated gene expression.

Material and Methods

Cell culture

GH_4C_1 cells were cultured in DMEM/F12 medium (Sigma, U.S.A.) supplemented with 2.5 % foetal calf serum and 12.5 % horse serum, which have been stripped of thyroid hormones prior to the experiments using Dowex 1x8 and charcoal – Norit A treatment (Serva, Germany) according to Samuels *et al.* (1979a,b). To avoid bacterial contamination, penicillin (100 μU/ml) (Slovakofarma, Slovakia), streptomycin (100 μg/ml) (Medika, Slovakia) or kanamycin (10 μg/ml) (Sigma, U.S.A.) were used. Cells were plated in 6-cm diameter tissue culture dishes or 24-well culture plates (Flow laboratories, U.K.) usually at the density of $5x10^4$ cells/cm^2. The cell number never exceeded $3x10^5$ cells/cm^2. Doubling time and cell viability were determined using a haemocytometer and the Trypan blue exclusion test.

Percentage of T_3 receptor occupancy and medium T_3 concentration

T_3 receptors were estimated using a modified method of Barlow and De Nayer (1988). Initially we determined both, the nuclear receptor occupancy in GH_4C_1 cells after 48 h incubation in the presence of different T_3 concentrations and T_3 nuclear receptor capacity at each T_3 dose. From these data, we calculated the relationship between the concentration of T_3 in the medium and the percentage of nuclear receptor occupancy. For the binding experiments, a six-point saturation analysis was performed in duplicates. Cells were plated in a normal growth medium that had been changed for the TH stripped one 12 h after plating. The cultures were supplemented with different concentrations of T_3 and the same dose (5.0 pM) of radioactive [^{125}I]T_3 (SA=44.4 TBq/g; New England Nuclear, U.S.A.). After 48 h, cell nuclei were isolated (Samuels *et al.* 1973) and specifically bound nuclear T_3 was determined. A similar experimental procedure was followed at each T_3 concentration for assessment of nuclear receptor capacity. In this case, we incubated the cells at different T_3 concentrations for 48 h and T_3-TR specific binding was determined during the last three hours of the period by incubation with radioactive T_3 (Halperin *et al.* 1990). Non-specific T_3 binding was determined in a thousandfold excess of non-radioactive T_3 and was always less than 8 % of the total nuclear radioactivity.

PRL and TSH determination

Prolactin and TSH in a conditioned medium of GH_4C_1 cells were determined by RIA using kits for the "Rat Pituitary Hormone Program" (NIADDK, NIH, U.S.A.) and normalized to the protein content.

Statistics

The differences between various experimental groups were compared by the analysis of variance. The figures give the standard error of the mean (±S.E.M.) and the results are the means of at least three independent experiments.

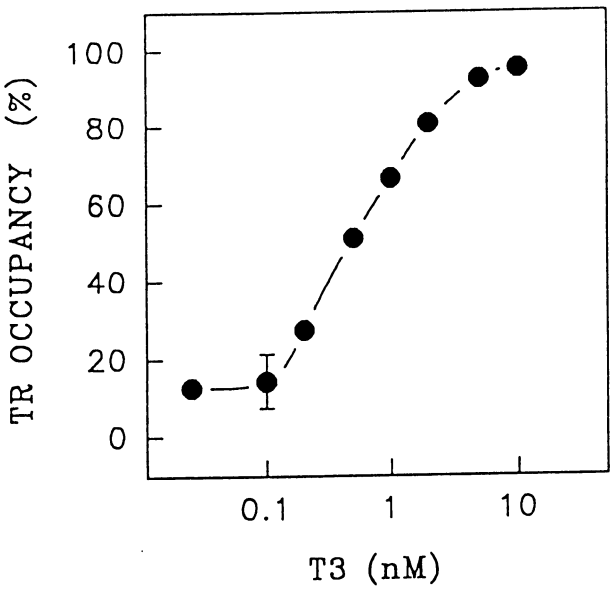

Fig. 1. *The dependence of nuclear TH receptor occupancy in GH_4C_1 cells in relation to different concentrations of T_3 in culture media. Non-specific binding was estimated in the presence of tenfold excess of non-labelled thyroid hormone and was usually less than 8 % of the total binding.*

Results and Discussion

Specific T_3 binding in nuclei of GH_4C_1 cells, TR occupancy and down-regulation of TR by T_3

The GH_4C_1 rat pituitary tumour cell line is a widely used experimental model. The cells produce PRL, TSH and represent an easily accessible source of

TR. Saturation analysis revealed the presence of specific T_3 binding sites characterized by an equilibrium association constant, K_a = 1.07 x 10^9 μmol and the maximal binding capacity, B_{max} = 12.19 fmol/10^6 cells, which indicates the presence of approximately 7300 T_3 binding sites per cell nucleus.

The maximal binding capacity and the receptor occupancy were determined for each of T_3 doses after 48 h incubation at 37 °C according to Halperin *et al.* (1990). Figure 1 shows the relationship between TH receptor occupancy and the concentration of T_3 in the culture medium. The TR population was occupied in the T_3 concentration range from 0.025 nM to 20.0 nM. At doses higher than 10 nM T_3, we observed down-regulation of nuclear receptors, their binding capacity being reduced by about 19.6 % ($p<0.02$) of the maximal value (Fig. 2). At this T_3 concentration, 92.0 % of TRs were occupied. In agreement with the data on receptor down-regulation, we also observed a significant reduction of the TR alpha mRNA accumulation as measured by Northern blot hybridization with c-erbA alpha cDNA probe (data not shown), which indicates that the inhibition of TR expression occurs at the pretranslational level. Based on these observations, most of the effects of thyroid hormone occurs within the concentration interval from 0.025 nM to 20.0 nM T_3, and resulting in 12.6 % TR occupancy to 10.0 nM T_3, at which almost all of the TR population might be expected to be already occupied

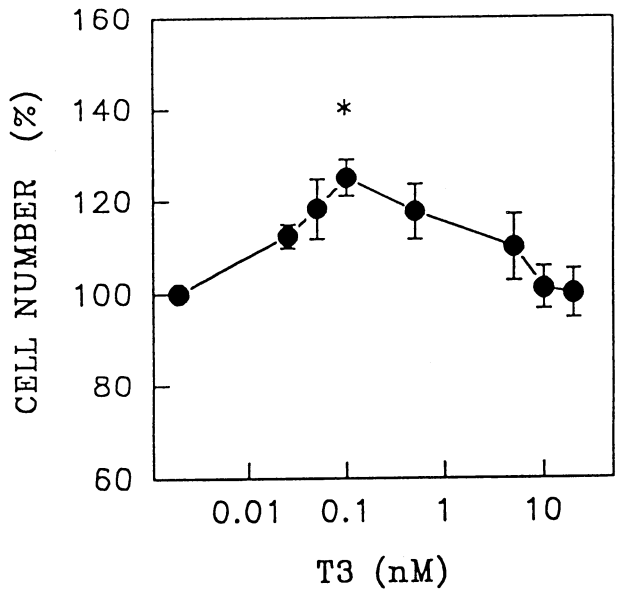

Fig. 3. *GH_4C_1 cell growth stimulation in the presence of T_3. (<20 % nuclear receptor occupancy). Doses higher than 0.1 nM T_3 inhibited cell division (* $p<0.05$; $n=10$, ANOVA).*

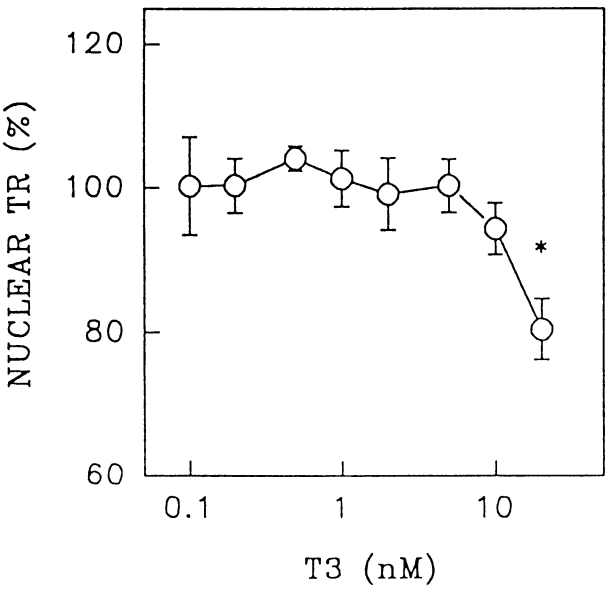

Fig. 2. *Down-regulation of TR by T_3. The TR binding capacity was estimated after 48 h cultivation in the presence of different amounts of unlabeled T_3 in short, 3 h incubations (at the end of cultivation) with radiolabeled T_3 (* $p<0.02$; $n=4$).*

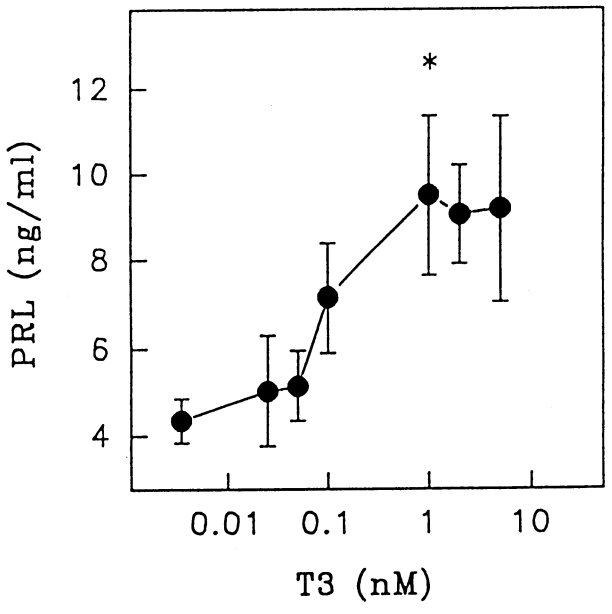

Fig. 4. *The effect of T_3 on PRL production in GH_4C_1 cells. Maximum of PRL production was observed at concentrations of 1.0 nM T_3 resulting in 66 % of TR occupancy. Higher doses of T_3 did not increase PRL production (* $p<0.05$; $n=3$).*

T_3 dose-dependent biological events: cell proliferation, regulation of PRL and TSH production

All the experiments were performed in a resin-treated serum containing medium, which was completely depleted of thyroid hormones and subsequently supplemented with a desired concentration of T_3. The doubling time of cell population (in a hormone-free medium) was extended from 38.8 h to 68.2 h which represents an increase of about 75.7±14.5 % in comparison to cells grown in a medium containing normal serum. T_3 increases significantly the cell division rate ($p<0.05$). Figure 3 shows the effect of T_3 dose on cell proliferation. The growth rate reaches its maximum at 0.025 nM T_3 which represents an occupancy of less than 20 % of the TR population. It is interesting to note that the cell growth rate was not maintained at higher T_3 concentrations but it was rather reduced to control level.

Figures 4 and 5 show the dose-dependent effects of T_3 on PRL and TSH production. The basal levels of PRL and TSH produced by GH_4C_1 cells were 85.0 ng PRL/ml media/10^6 cells and 76.0 μU TSH/ml media/10^6 cells as measured after 48 h in the cell culture media. We found that the GH_4C_1 cells respond to specific doses of 3,5,3,-L-triiodothyronine differently e.g. the PRL production increases whereas the production of TSH is decreased as measured by RIA in the cell culture medium. A significant increase of PRL production was observed at a concentration of 1.0 nM T_3 (118 % increase above the control values). This concentration caused a receptor occupancy by T_3 equal to 66 % and also represents the maximal stimulation of PRL production. Under the same conditions, we observed moderate inhibition of TSH production (Fig. 5). Ten times higher T_3 concentration resulted in almost complete TR occupation by T_3. At 20.0 nM T_3, we observed significant reduction of the T_3 nuclear receptor binding capacity, resulting in 52.5 % reduction (Fig. 2) when compared to the control values.

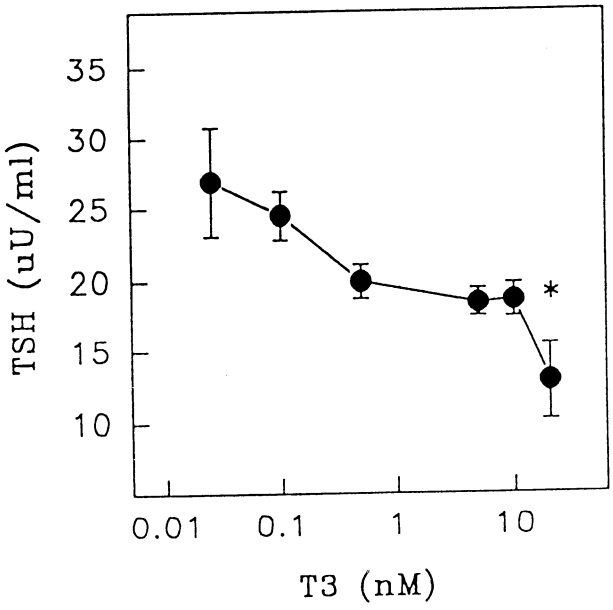

Fig. 5. *The effect of T_3 on TSH production in GH_4C_1 cells. Decreased TSH production was found at higher T_3 doses and significant differences occurred after saturation of the TR population (* $p<0.05$; $n=3$).*

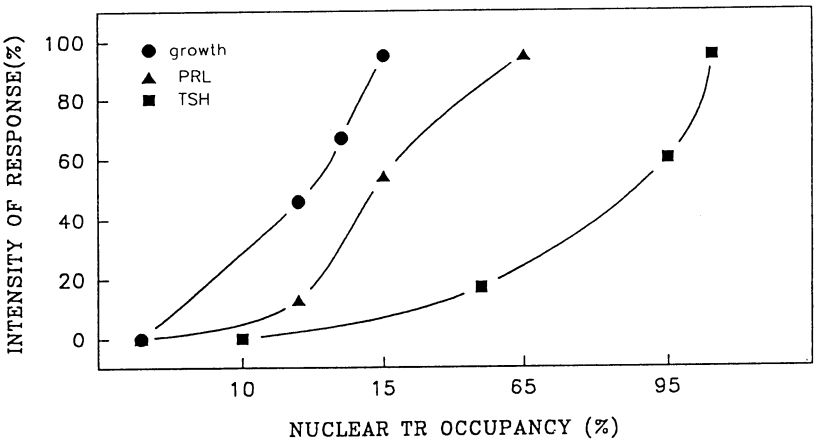

Fig. 6. *The relationship between intensity of selected biological responses and the percentage of nuclear thyroid hormone receptor occupancy in rat pituitary tumour cell line GH_4C_1. Full dots – cell growth, triangles – PRL production, full squares – TSH inhibition. Each of the responses reached its maximum at different T_3 concentrations and therefore at different receptor occupancy. The response ranges partly overlap and intensity of the processes are not directly proportional to receptor occupancy.*

Intensity of biological effects and the TH-receptor occupancy relationship

The experimental data were used to define the relationships between the intensity of biological effects and the percentage of thyroid hormone receptor occupancy in this particular cell line. Figure 6 represents a summary of the above experiments. Each of the responses is operative in different T_3 dose

ranges and different nuclear receptor occupancy intervals. It is obvious that the induction of a particular biological event requires the occupancy of a different minimal number of receptors by T_3 and no full receptor population occupancy by the thyroid hormone is needed for a maximal intensity of the above mentioned process (except for TR down-regulation). In certain cases (e.g. cell division), occupation of a higher than optimal percentage of TR population can inhibit the biological process. Cell growth seems to be a very sensitive response of the cells to T_3 stimulation. On the other hand, PRL production begins when more than 20 % of the TR population is being occupied by T_3, and a maximal response occurs in the middle of the occupancy range. A higher number of receptors (at least 80 % of the TR population) has to be occupied to inhibit TSH production as well as to down-regulate nuclear T_3 receptors. These effects are operative in distinct T_3 dose ranges which can partially overlap as is shown in Figure 6. The concentration of T_3 and percentage of the THRO corresponding to the maximal intensity of the above biological responses were as follows: a) the maximal growth rate was attained at 0.1 nM T_3 corresponding to T_3 receptor occupancy of less than 20 %; b) the maximal stimulation of PRL production was observed at 1.0 nM T_3 which corresponds to 66.0 % of THRO; c) inhibition of TSH production occurs at 20.0 nM of T_3 when more than 95.7 % T_3 receptors are occupied by T_3.

Several different pituitary cell lines (GC, GH_1, GH_3) have been employed in the studies on thyroid hormone effects. Because of their differentiated phenotype *in vitro*, they represent a valuable experimental model. Our data contribute to the long-lasting discussion whether or not the final effect of TH is directly proportional to THRO. Conflicting opinions can be illustrated by previous reports of other authors (Samuels 1983, Yaffe and Samuels 1984, Nyborg *et al.* 1984, Shupnik *et al.* 1985, Halperin *et al.* 1990). Our data are in principal agreement with those published by Halperin *et al.* (1990). The authors have established an experimental model using the GC pituitary cell line. They were able to determine unique dose-response regions for three different biological responses (cell division, growth hormone production and nuclear receptor down-regulation) to T_3 within the wide range (from 1 nM to 10 nM T_3) of nuclear receptor occupancy. In agreement with these data, we found cell growth to be the most sensitive response of the cells to TH. Down-regulation of c-erbA mRNA accumulation as well as the final protein product content at concentrations higher than 10 nM of T_3 were also observed in GH_4C_1 cells. Between these extreme effects, PRL production and TSH inhibition were observed and T_3 dose-response ranges were determined.

In conclusion, we found that the above biological events are operative in distinct T_3 dose-response ranges and that the intensity of any particular response is not directly proportional to thyroid hormone receptor occupancy. Moreover, full receptor population occupancy is not necessarily needed for achieving the maximal intensity of the biological response. In contrast, increased concentrations of the thyroid hormone (above the optimal level) might lead to inhibition of the biological response. These data also suggest that this cell line can be used as a suitable model for clarifying the mechanism leading to different sensitivity of pituitary cells towards T_3. It is very likely that one of the possible mechanisms is based on a different number and composition of thyroid hormone responsive elements in the regulating regions of target genes.

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Reprint requests

P. Filipčík, Institute of Neuroimmunology, Slovak Academy of Sciences, Dúbravská cesta 9, 842 46 Bratislava, Slovak Republic.