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RETINOIC ACID DECREASES THYROID HORMONE RECEPTOR EXPRESSION IN PITUITARY GH1 CELLS

Paloma Pérez, Aurora Sánchez-Pacheco, Angel Pascual and Ana Aranda

Instituto de Investigaciones Biomédicas, CSIC, 28029 Madrid, Spain

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SUMMARY Retinoic acid produced a time and dose-dependent depletion of thyroid hormone receptors in GH1 cells without modifying their affinity for triiodothyronine (T3). A maximal decrease (50-70%) was obtained after 24-48 h incubation with 5-10 μM retinoic acid. Treatment with 0.8 nM T3 for 24 h caused a similar reduction and did not potentiate the decrease produced by these concentrations of retinoic acid. However, the combination of sub-maximally effective doses of both ligands had an additive effect on receptor levels. The reduction of receptor caused by retinoic acid is accompanied by a decreased expression of c-erbA α1 and α2 mRNAs, but the retinoid did not reduce the abundance of c-erbA β mRNA. In contrast, T3 decreased the levels of both α and β transcripts.

Both retinoic acid (RA) and thyroid hormones are important regulators of growth and developmental processes. The thyroid hormone receptors (the product of c-erbA proto-oncogenes) are highly homologous to the RA receptors (RAR) and are members of a superfamily of ligand-activated transcriptional regulators, which exert both positive and negative transcriptional control of gene expression through binding to short cis-acting DNA sequences named hormone-response elements (1).

Regulation of the expression of several members of the nuclear receptor superfamily by their own ligand is well documented and can result both in a decrease or an increase of receptor concentration, potentially leading to a desensititation or an amplification of the action of the ligands in responsive cells. The existence of different subtypes of RA receptors (2-6) and c-erbA proteins (8-12) which can be differently regulated (4,5,13-16) is well stablished. Treatment with RA produces a rapid increase of RAR β mRNA levels, whereas expression of other RAR forms is not affected (4,5). In contrast, T3 is known to down-regulate its own receptor levels as a consequence of a reduction of α and β c-erbA mRNAs in pituitary cell lines (12,13). Since regulation of these receptors can take place by ligands of other members of the superfamily we have examined the influence of RA on T3 receptor levels and c-erbA mRNAs in pituitary GH1 cells.

MATERIALS AND METHODS

Hormones and Chemicals - The synthetic retinoids etretinate (Ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate) and isotretinoin (13-cis-Retinoic acid) were kindly donated by Hoffman La-Roche. Triiodothyronine (T3), all-trans retinoic acid (RA), retinol and retinol acetate were obtained from Sigma Chemical Co.

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Cell culture. GH1 cells were grown as previously described (16-19) in RPMI medium containing 10% horse serum and 2.5% fetal calf serum. Twenty four hours before the experiments the medium was changed to RPMI medium supplemented with 10% newborn calf serum which had been stripped of thyroid hormone with charcoal and ion exchange resin (16) to ensure cellular depletion of thyroid hormones. Since RA, as does T3, binds to serum proteins which could reduce cell availability to the ligands, the cells were transferred to serum-free medium for the experiments.

Quantitation of T3 nuclear binding. Nuclear binding of [^{125}I]T3 was determined as previously described (16-19) in cells treated with RA for the times and with the concentrations indicated in the text. In some studies T3 binding was examined by incubation of intact GH1 cell monolayers or isolated nuclei for 3 hours with increasing concentrations of [^{125}I]T3 and the binding data analyzed according to the method of Scatchard. In other studies the level of receptors was determined by incubation of the cell monolayers in serum-free medium for 90 min with 0.8 nM [^{125}I]T3. This concentration of T3 gives an estimate of the total levels of receptor since it binds to greater than 90% of the thyroid hormone receptor population. To evaluate the effect of RA on the receptor down-regulation exerted by T3, the cell cultures were incubated for 24 h with different concentrations of radioactive hormone, and the total level of receptor was assesed by adjusting the [^{125}I]T3 concentration to 0.8 nM and further incubation for 90 min. The level of receptors in control cultures was examined by incubation for the same period (90 min) with 0.8 nM [^{125}I]T3. After the binding period the nuclei were isolated and the nuclear and extranuclear [^{125}I]T3 concentration analyzed.

RNA isolation and hybridizations. Total cellular RNA was extracted by the method of Chomzynski and Sachhi (20), and poly(A)$^+$ RNA was prepared by oligo dT-cellulose chromatography (21). The RNA was run on 1% formaldehyde-agarose gels, transferred to nylon membranes (Nytran), and probed with β and α c-erbA cDNAs. The ^{32}P-labeled cDNAs, the Pvu II fragment of rat α c-erbA (7) and the EcoR1 fragment of rat β1 c-erbA (8) were prepared using random oligonucleotide priming to a specific activity of approximately $9x10^7$ cpm x μg. Hybridizations were at 42ºC/50% formamide, and the membranes were washed at 42ºC with 1xSSC. After hybridization with c-erbAs the probe was stripped off and the blots re-hybridized with a control glycerol phosphate dehydrogenase (GAPDH) probe labeled by nick translation.

RESULTS

Figure 1 illustrates the effect of RA on thyroid hormone nuclear receptor levels. The effect of RA ranging from 1nM to 10 μM was examined after a 24 h incubation (left panel). Receptor levels were 140 fmol/100 μg DNA in control cells and RA caused a dose-dependent reduction of T3 nuclear binding. Binding decreased by 50-75% with 1-5 μM RA and a half-maximal decrease occurred at approximately 100 nM. The time-course of the effect on the receptor was examined using 1 μM RA (right panel). Cells incubated with RA showed a significant decrease of receptor levels after 6 h . The effect was maximal at 24 h and longer incubation times did not elicit further decreases (not illustrated). The extranuclear T3 levels did not decrease after treatment with RA,thus showing that a decrease in hormone availability is not the cause for the reduction of [^{125}I]T3 binding.

Fig.2A shows the influence of a 24-h incubation with 1 μM RA on the Scatchard plot of T3 nuclear binding assessed in intact cells. The estimated Kd was similar in control and RA-treated cells indicating that RA does not alter the apparent affinity of the receptor for T3, but rather reduces receptor number. To eliminate the possibility that the decrease in nuclear binding reflects a change in the amount of radiolabelled T3 reaching the nuclei rather than a decrease in receptor number, we performed binding experiments using isolated nuclei. Figure 2B shows a Scatchard plot of T3 binding to nuclei from control cells and cells incubated with RA. Again, the Kd values were similar and there was a decrease in the maximal binding capacity in conditions where there is no interference of extranuclear hormone binding.

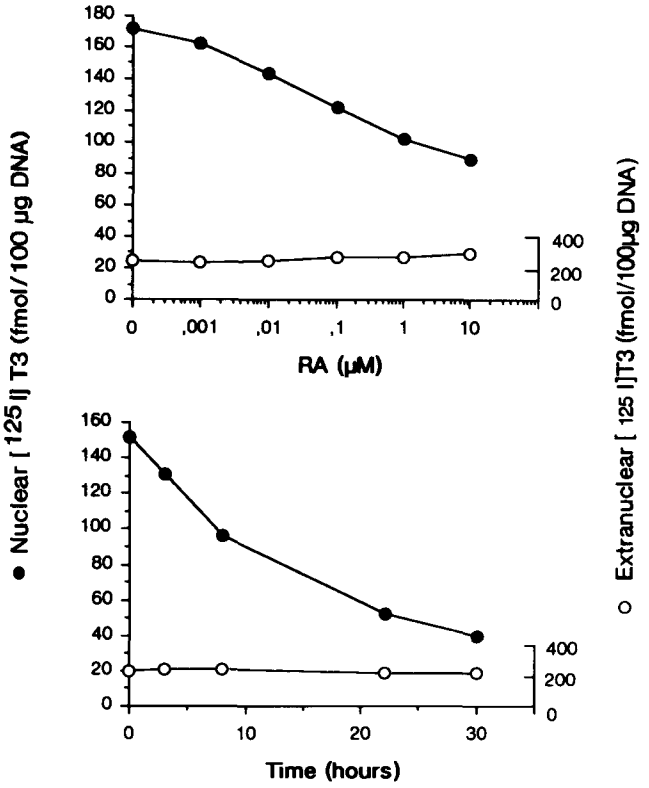

FIGURE 1. *Influence of retinoic acid on [125I]T3 binding.* Nuclear (●) and extranuclear (○) [125I]T3 binding was determined in GH1 cells incubated for 24 h with the concentrations of retinoic acid (RA) indicated (upper panel) or for different time-periods with 1 µM RA (lower panel).

Figure 3 compares the effect of RA with that caused by other retinoids. The level of receptors was studied after 24-h incubation with a 1 µM concentration. RA was the most active compound decreasing the receptor by 70% followed by isotretinoin (13-cis-retinoic acid) which produced a

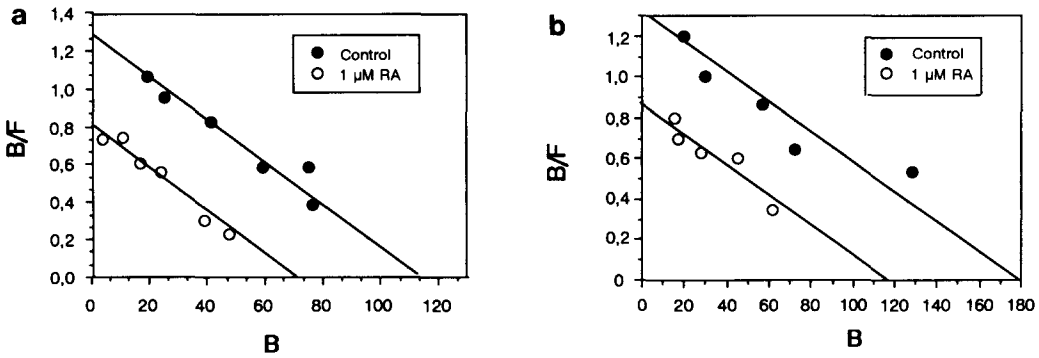

FIGURE 2. *Scatchard plot of [125I]T3 binding.* Nuclear binding was determined in control cultures and in cells incubated for 24 h with 1µM retinoic acid using intact cells (panel a) or isolated nuclei (panel b).

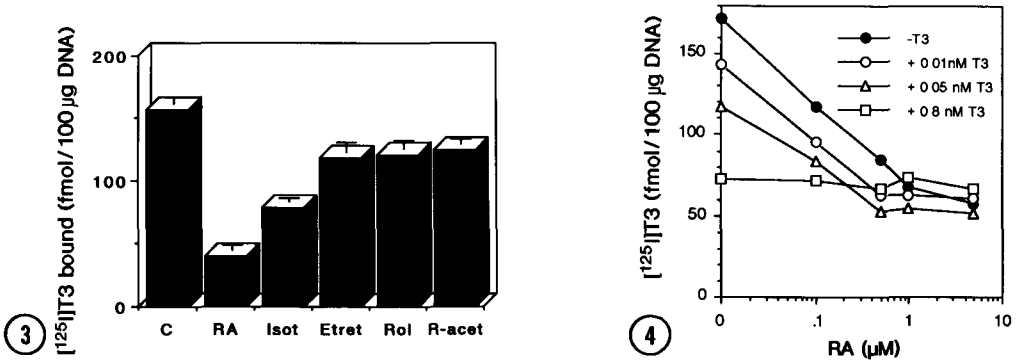

FIGURE 3. *Effect of different retinoids on the T3 receptor.* Receptor levels were determined in control cells (C) and in cells incubated for 24 h with 1 µM retinoic acid (RA), isotretinoin (Isot), etretinate (Etret), retinol (Rol), or retinyl acetate (R-acet).

FIGURE 4. *Combined effects of triiodothyronine and retinoic acid on thyroid hormone receptor levels.* GH1 cells were incubated with the concentrations of retinoic acid (RA) and radiolabelled T3 indicated for 24 h and the levels of receptors determined as described under "Materials and Methods".

50% reduction. Retinol, retinyl acetate and the synthetic retinoic etretinate were less effective causing only a 20-25% depletion.

It had been previously shown that T3 down-regulates its own nuclear receptor in GH1 cells (16-19). Figure 4 illustrates the receptor reduction resulting from a 24-h incubation with T3 (0-0.8nM) in combination with increasing concentrations of RA. T3 elicited the expected dose-dependent depletion of receptors and RA also lowered receptor levels. T3 0.01 and 0.05 nM potentiated the effect of low doses of RA, whereas the effect of RA above 0.5 µM or a receptor-saturating dose of T3 (0.8 nM) were already maximal and the combination of both did not produce a further decrease.

The molecular basis for the observed decrease in T3 receptors was addressed by Northern analysis of RNA prepared from RA and/or T3-treated cells. Figure 5 shows the results of a representative hybridization of total RNA (30 µg) with rat c-erbA cDNA probes (panel A). The c-erbAα cDNA probe hybridized to a major mRNA species 2.6 kb in size which appeared to decrease in the presence of both T3 and RA. Quantification from several experiments yielded a 52 % reduction in T3-treated cells, a 43 % reduction in RA-treated cells and a 60 % reduction in the presence of both. This band has previously been shown to correspond to c-erbAα2 mRNA which does not bind hormone (9-11), suggesting that this variant is also the major species of c-erbA in GH1 cells. Northern analysis using poly(A)$^+$ RNA (panel B) demonstrated the presence of an additional 5.0-6.0 kb mRNA which most likely represents the c-erbAα1 form (9-13) which encodes a functional T3 receptor. The changes found in the levels of c-erbAα1 mRNA paralleled those found for the receptor since T3 and RA and the combination of both decreased the levels of this mRNA by 47, 40 and 45 %, respectively. The β probe detected in all cases a single RNA species of molecular size around 6-6.5 kb whose abundance was reduced by in T3-treated cells with respect to control cells. Quantification of the ratio c-erbAβ mRNA/ GAPDH mRNA showed an average decrease of 52% after treatment with 0.8 nM T3 for 24 h. In contrast RA (1 µM for 24-

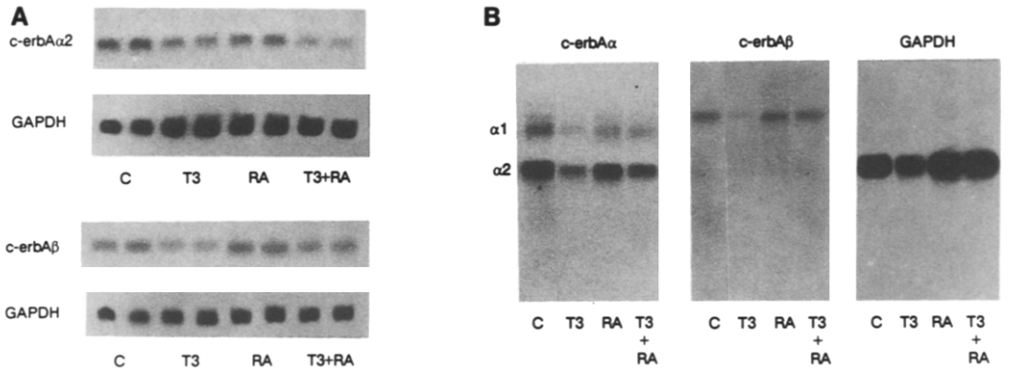

FIGURE 5. *Regulation of c-erbA mRNAs by retinoic acid.* 30 µg of total RNA (panel A) or 4 µg of poly(A)$^+$ RNA (panel B) were isolated from cells incubated for 24 h with 0.8 nM triiodothyronine (T3), 1 µM retinoic acid (RA) or the combination of both. The mRNAs for c-erbA α1 and α2, c-erbA β or glycerol phosphate dehydrogenase (GAPDH) were analyzed by Northern blot.

h) did not significantly decrease the levels of c-erbAβ mRNA which varied between 85 and 122% of control in 4 different experiments. The combination of T3 and RA did not cause a larger reduction than T3 alone (40-57% of control).

DISCUSSION

RA caused a time and dose-dependent decrease of nuclear [^{125}I]T3 binding in pituitary GH1 cells. This decrease reflects a reduction in the number of receptors, since their apparent affinity was not altered by the retinoid. Other retinoids had a similar effect although RA was the most active compound. The potency of the different retinoids in decreasing T3 binding agrees with their described trans-activating activity and relative affinity for the RARs (2-6). We have observed the presence of at least α and β RAR mRNAs in pituitary cell lines (unpublished results), but it is not known whether a specific RAR form mediates the down-regulation of thyroid hormone receptors demonstrated in this study.

As expected, T3 also decreased by 50-60% the level of thyroid hormone receptors. Samuels et al. (17) had suggested that this partial reduction could represent a heterogeneous population of receptors which are differently regulated.The existence of different receptor forms with a strongly conserved hormone binding domain which could explain that a unique class of binding sites is detected by Scatchard analysis has been recently confirmed. The proteins encoded by c-erbA mRNAs have been divided into α and β forms. The α1 and α2 forms are alternative splice products of a single α gene and whereas the c-erbAα1 mRNA encodes a functional receptor, the α2 protein product does not bind T3 (9-12).The β gene yields two T3-binding forms (β1 and β2), and unlike other members of the c-erbA family which have a wide tissue distribution, erbA β2 appears to be expressed only in the anterior pituitary gland (13). Using probes specific for either c-erbA β1 or β2, Hodin et al. (13) have found that T3 decreases the abundance of c-erbAβ mRNA levels and that this reduction is due to an almost total depletion of c-erbAβ2 form coupled with a

slight increase in c-erbAβ1 mRNA levels. Additionally, Lazar et al (12) have demonstrated that the α1 and α2 c-erbA mRNAs decrease by approximately 50% after T3 treatment of GH3 cells. We have found a similar reduction of c-erbA α and β mRNAs in GH1 cells treated with T3. In addition, we find that RA caused a decrease in the abundance of α1 and α2 c-erbA mRNAs but did not significantly alter the levels of c-erbAβ mRNA. Since there is a 1089-bp region of 100% identity between the r-erbA β1 and β2 coding sequences (13), the band that we detected by Northern analysis with a c-erbA β1 probe (8) probably consists of both β1 and β2 mRNAs that have the same size. Therefore, we cannot exclude the possibility that an opposite regulation of both forms leads to a normal level of total c-erbAβ levels. Our results indicate that RA causes a decrease in cellular thyroid hormone receptor concentration mainly by decreasing levels of α receptor mRNA.

The exposure to saturating concentrations of T3 and RA did not have larger effects than both ligands separately and the level of receptors did not fall by more than 60-70%. Concomitantly, the levels of α and β c-erbA mRNAs were not further reduced by T3 in the presence of RA, suggesting the existence of common mechanisms in the regulation of receptor expression.

The strong analogy between the DNA binding region of the c-erbAs and RARs suggest that they could bind similar elements and regulate some genes in common. In GH1 cells we have shown that both triiodothyronine (T3) and RA transcriptionally regulate rat growth hormone (GH) gene expression through sequences located in the 5'-flanking region of the gene (22). Furthermore, it has been reported that RA can activate gene expression through thyroid hormone response elements (TREs) derived from the TRE of the GH gene and that the RA and thyroid hormone receptors, irrespective of the presence of ligand, can form heterodimers and bind to the TREs with high affinity (23-26). Additionally, RARs recognize other response elements (RAREs) which fail to support transcriptional activation by thyroid hormones (27-28), thus showing that RA can regulate gene expression both in common and independently from these hormones. The regulation of the expression of c-erbAs by RA adds another level of complexity to the relationship between thyroid hormone and retinoids on the regulation of gene expression. It is attractive to especulate that T3 and RA could have a direct effect on receptor gene expression, but further studies will be needed to determine if they regulate the rate of transcription of the c-erbA genes, and which are the genic elements that mediate this effect.

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