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Retinoic acid decreases retinoic acid and triiodothyronine nuclear receptor expression in the liver of hyperthyroidic rats

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Retinoic acid (RA) and triiodothyronine (T_3) exert many of their actions by binding to specific nuclear receptors (respectively, RA receptor (RAR) and T_3) receptor (TR) belonging to a 'superfamily' of receptors. Some heterologous regulation of these receptors has been shown, and in particular regulation of the maximum binding capacity of TR by either retinol or RA. Now, using hyperthyroidic rats as a model, the effect of RA on binding capacity and on the mRNA levels of TR and RAR was investigated. The results show that the benefit of vitamin A treatment for the hyperthyroidic state, which has been described for a long time, could be the result of a down-heteroregulation of TR by RA, the active metabolite of retinol.

Hyperthyroidism; Retinoic acid; Triiodothyronine nuclear receptor; Retinoic acid nuclear receptor; mRNA

1. INTRODUCTION

Retinoic acid (RA) and triiodothyronine (T_3) have been known for a long time to exert profound effects on development and differentiation. Many of the diverse cellular responses that these agents elicit are initiated by their binding to specific nuclear receptors, and result from the induction of the transcription of several genes [1]. Structural homologies among these receptors have led to considering them as belonging to a 'superfamily' of DNA binding proteins [2]. Moreover, it was shown that these receptors can be regulated by their own ligands (homologous regulation) [3] and in some cases by ligands that are not their own (heterologous regulation) [4–6]. With respect to RA and T_3, in euthyroidic rats or control cells it was predicted that retinol or retinoic acid could down-modulate the nuclear T_3 receptor (TR) and its messenger (c-erb A mRNA) [7,8]. These findings prompted us to examine, in rats made hyperthyroidic, the effects of RA on the properties of both its own receptor (RAR) as well as TR, and also on the level of their respective mRNA (RAR mRNA and c-erb A mRNA).

2. MATERIALS AND METHODS

2.1. *Experimental animals*

Pathogen-free male Wistar rats (weight range 180–200 g) were obtained from IFFA-CREDO (L'Arbresle, France). The animals were segregated into three groups of eight animals.

T_3 and RA treated rats. Animals were injected (at 18.00 h) intraperitoneally with 50 μg of T_3 (Sigma, no. 2752) in 0.01 N NaOH/100 g b.wt. per day for 3 days, and the third day they also received 500 μg of all-*trans*-RA (Sigma, no. 2625) in arachis oil/100 g b.wt. by gastric intubation. They were killed 12 h after RA was administered and samples of liver tissue were collected for analysis.

T_3 treatment. Animals were administered with T_3 as described above and then received arachis oil only.

Control animals. Rats received the vehicles only.

2.2. *Properties of nuclear receptors*

2.2.1. Receptors preparation

Nuclei were obtained as described by De Groot et al. [9]. TRs were obtained as described by Torresani et al. [10]. To obtain RAR, the nuclei were washed three times with binding buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.9) and then submitted to a DNAse I (Sigma no. D 4527) digestion for 30 min at room temperature, followed by a high salt extraction (0.5 NaCl). The nuclear extract was then obtained by centrifugation [12]. Protein content of the nuclear extracts was determined using the Bradford assay [11].

2.2.2 Binding studies

TR binding was performed according to Torresani et al. [10]. RAR binding was modified from Daly et al. [12]. Aliquots of nuclear extract (96 μl) were mixed with 4 μl of increasing concentrations (0.15–1.5 μM in dimethyl-sulfoxide) of [^{3}H]retinoic acid (52.5 Ci/mmol; NEN Dupont de Nemours, Paris, France). After 1 h incubation at 4°C, 50 μl of the incubation mixture was submitted to high-performance size-exclusion chromatography separation on a TSK gel G3000SW column (300 × 7.5 mm TosoHaas), and eluted with 0.3 M KH_2PO_4, pH 7.8, at a flow rate of 0.5 ml/min. The column was standardized with a mixture of human albumin (67 kDa), egg albumin (45 kDa), and horse myoglobin (16.8 kDa). Fractions of 0.2 ml were collected and counted in a liquid scintillation counter using Ready Value Cocktail (Beckman) as the scintillation liquid. Radioactive counts obtained in fractions containing the RAR-[^{3}H]RA complex were added and expressed as picomoles of bound ligand per mg of proteins. Non specific binding was determined by incubation in the presence of 1000-fold excess of unlabelled RA (Fig. 1). Scatchard analysis was then performed.

2.3. *Quantification of mRNAs*

The absolute values of c-erb-A and RAR mRNAs cannot be deter-

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mined directly, but a proportion can be deduced by comparing them with the β-actin internal standard simultaneously reverse transcribed and amplified in the same test tube.

Extraction of RNA was performed according to Sambrook et al. [13] modified by Pailler-Rodde et al. [14].

Oligonucleotide primers used for PCR were synthesized using an Applied Biosystem Model 381A DNA synthesizer. The position and sequence of the different primers are summarized in Table I. The position of the primers for c-erb-A and β-actin mRNAs was chosen according to Mitsuhashi et al. [15], Nudel et al. [16], and Murray et al. [17]. The primers for RAR mRNA were chosen by comparing the murine RAR-β sequence with those of human and chicken, and of murine RAR-α and -γ. These positions were prepared according to the sequence published [18–20].

Preparation of cDNA was carried out as described by Pailler-Rodde et al. [14].

Synthesized cDNA (15 μl) was amplified by the polymerase chain reaction (PCR) using *Taq* polymerase [21]. The amplification was performed according to Pailler-Rodde et al. [14]. The reaction was carried out for a total of 34 cycles.

For quantitative analysis of PCR products, 10 μl of the PCR reaction were sampled after each (from the 11th to 27th) amplification cycle and then at the last cycle [22], and the co-amplified fragments were separated by electrophoresis on a 10% acrylamide gel. The incorporated radioactivity was visualized by autoradiography, and the bands were excised from the gels and quantified by scintillation counting.

3. RESULTS

Results are summarized in Table II.

3.1. *Hormone binding*

After T_3 treatment (3 injections of 50 μg/100 g b.wt. in 60 h) the capacity (C_{max}) of TR in liver was increased by 68% relative to the control value. The administration of a single dose of RA (500 μg/100 g) by gastric intubation simultaneously with the last T_3 injection induced, 12 h after administration, a decrease in C_{max} of TR by 22% relative to the T_3 treatment value.

There was no significant effect on liver C_{max} of RAR in rats treated with T_3 alone compared to control animals. In contrast, the combination of T_3 and RA induced a decrease in C_{max} of RAR of 24% relative to the control value.

3.2. *mRNA abundance*

The abundance of c-erb-A mRNAs in the liver decreased by 51% relative to the control value in rats treated with T_3 alone, and by 77% in rats treated with T_3 and RA. In contrast the abundance of RAR mRNA was strongly increased by both treatments: the levels were increased approximately fourfold after T_3 treatment alone, and by twofold after T_3 and RA in combination.

4. DISCUSSION

4.1. *Effects of T_3 on c-erb-A mRNA and nuclear T_3 receptors*

In the present investigation only the mRNAs coding for proteins which bind T_3, i.e. c-erb-A α mRNA and c-erb-A β mRNA, have been quantified. These measurements have facilitated a comparison of the content of c-erb-A mRNAs with nuclear binding capacity as determined by isotopic displacement analysis.

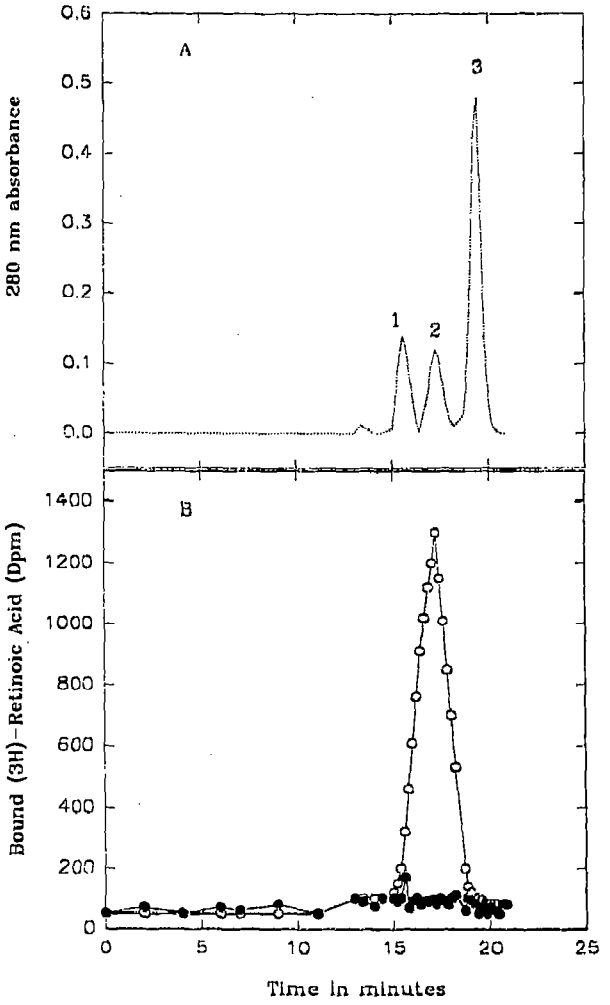

Fig. 1. Size-exclusion chromatography analysis of a nuclear extract incubated with a saturated concentration of [^{3}H]retinoic acid. (A) Ultraviolet (280 nm) standard profile: (1) human albumin, (2) egg albumin, (3) horse myoglobin. (B) [^{3}H]Retinoic acid bound to proteins in the absence (○, total binding) or in the presence of 100 μM unlabeled retinoic acid (●, non-specific binding).

It was observed that the administration of T_3 induced an increase in the C_{max} of TR associated with an increase in cytosolic malate dehydrogenase activity, an enzyme that is T_3 dependent (data not shown). Such a result is consistent with previous studies showing an increase in C_{max} of TR correlating with an increase in mitochondrial α-glycerophosphate dehydrogenase activity (another T_3 dependent enzyme) in T_3-treated rats [23,24]. These results differ from those obtained with various cell lines showing a time- and dose-dependent down-regulation of TR by T_3 [25–27]. To explain such a discrepancy between results obtained with either cell lines

Table I

Synthesized primers used for PCR

Primers		Sequence (5'3')	Complementary sites
β-Actin[a]	A1	AGGATGCAGAAGGAGATTACTGCC	2,814–2,837
	A2	GTAAAACGCAGCTCAGTAACAGTCC	3,159–3,135
c-erb-A[b]	E1	TCCTGATGAAGGTGACGGACCTGC	1,247–1,270
	E2	TCAAAGACTTCCAAGAAGAGAGGC	1,364–1,341
RAR[c]	R1	CTCACTGAGAAGATCCGGAAAGCCCACC	538–565
	R2	TTGGTGGCCAGCTCACTGAATTTGTCCC	680–653

[a]From rat cytoplasmic β-actin according to the sequence of Nudel et al. [16].
[b]From rat c-erb-A cDNA according to the sequence of Murray et al. [17].
[c]From murine RAR cDNA according to the sequence of Zelent et al. [18].

or intact animals it is suggested that T_3 administration in intact animals induces various biochemical events occurring at a post-transcriptional level, which are then able to modify the properties of the nuclear T_3 receptor.

Under the conditions used in our experiments, i.e. when measurements were performed 60 h after T_3 administration, the c-erb-A mRNA levels were decreased. Previous studies designed to assess the effect of the thyroidal state on the c-erb-A mRNA levels have yielded conflicting results. Our results are in agreement with those obtained on cell lines [28,29], but only partially consistent with those obtained in intact rats [30]. Indeed in this study T_3 reduces the amount of c-erb-A α mRNA by approximately 50% but has no effect on the c-erb-A β mRNA levels, while in control rats the level of c-erb-A β mRNA is approximately fourfold that of the c-erb-A α mRNA. The discrepancy observed between the level of TR and the level of c-erb-A mRNAs could be related to the time shift observed between a change in the level of the c-erb-A mRNAs and the change in the T_3 binding activity [31].

4.2. *Effects of RA on c-erb-A mRNAs and nuclear T_3 receptors in T_3-treated rats*

The effect of the RA administration on C_{max} of TR was related to the T_3 status of the rats: the C_{max} was increased relative to euthyroid rats but decreased relative to hyperthyroidic rats. So RA down-modulated the effect of T_3 on TR.

The decrease of c-erb-A mRNAs levels induced by RA and T_3-treated rats was more extensive than the decrease shown in only T_3-treated rats. So RA and T_3 act synergistically to down-modulate the c-erb-A mRNA level.

In hyperthyroidic rats the effect of RA on the C_{max} of TR is consistent with its effect on mRNA, and induces a decrease in T_3 binding. Such an effect has been shown in a mouse pre-adipocyte cell line in which RA decreases c-erb-A expression [29]. Moreover, in intact rats it was known that a retinol-overloaded diet induces decreases in c-erb-A mRNA levels and in the C_{max} of TR [14].

4.3. *Effects of T_3 on RAR mRNA and RAR*

There is some evidence for heteroregulation of superfamily receptors by ligands that are not their own, but results concerning the effect of T_3 are scarce [32] and none have been concerned with the effect on RAR. Our study showing elevated RAR mRNA levels after T_3 administration indicated the modulation of the messenger by T_3. It does not, however, allow the determination of whether this change is caused by a direct action on the RAR gene, mRNA processing, or mRNA stabilization. This increase of mRNAs is not consistent with an

Table II

Capacity of T_3 and RA nuclear receptor and proportion of c-erb-A and RAR mRNA to β-actin mRNA

	c-erb-A		RAR	
	Capacity[a] (pmol/mg prot)	mRNA[b] ($A_{erb\text{-}a}/A_{act}$)	Capacity (pmol/mg prot)	mRNA (A_{RAR}/A_{act})
Control	0.316 ± 0.022	0.35	4.46 ± 0.048	0.04
Hyper T_3	0.531 ± 0.018	0.17	4.36 ± 0.053	0.15
Hyper T_3 + AR	0.416 ± 0.022	0.06	2.81 ± 0.013	0.09

(C_{max}) values were obtained by Scatchard analysis.
[a]Each value is the mean ± S.E.M. of 8 animals; $P<0.05$ compared with controls (Student's t-test).
[b]Each value represents the mean of 4 separate measurements; $A_{erb\text{-}A}$, A_{RAR} and A_{act} are absolute values of c-erb-A, RAR and β-actin mRNA, respectively.

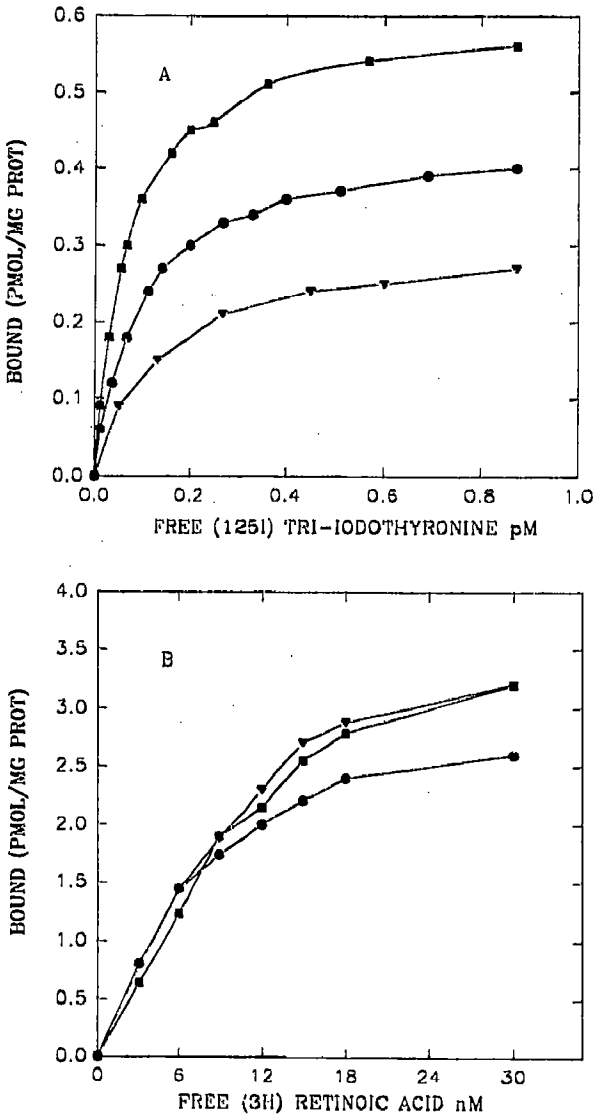

Fig. 2. Saturation binding curves of (A) [^{125}I]triiodothyronine obtained with c-erb-A and (B) [^{3}H]retinoic acid obtained with retinoic acid nuclear receptor. Hepatic nuclear fractions were obtained from control (▼), hyperthyroidic (■), and hyperthyroidic + retinoic acid (●) rats.

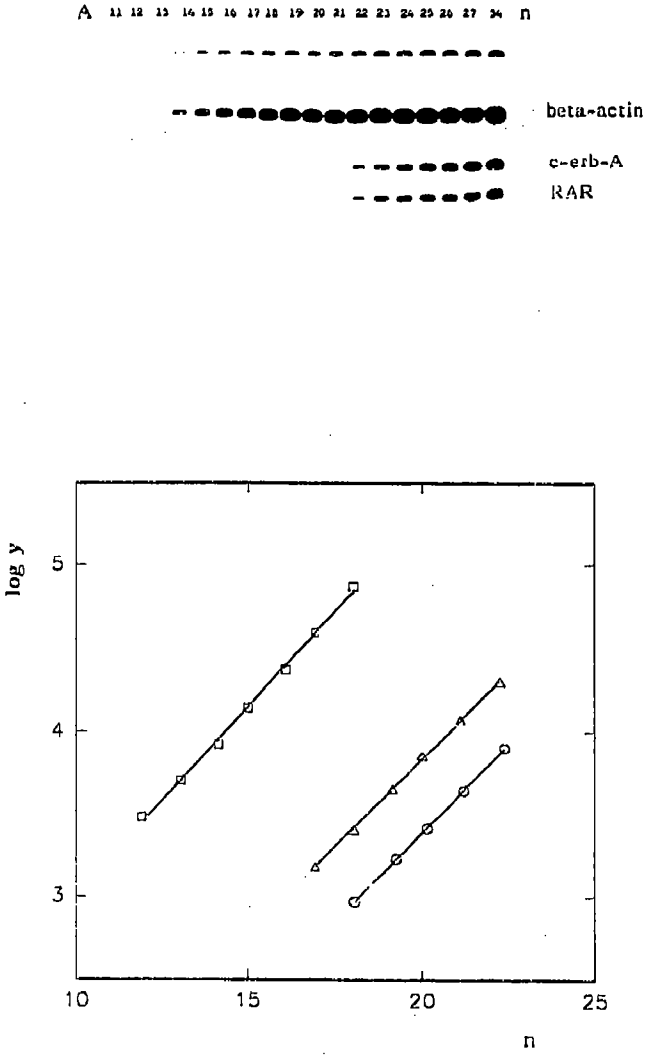

Fig. 3. Kinetic analysis of PCR products from β-actin, c-erb-A and RAR mRNAs. (A) Autoradiogram of acrylamide gel electrophoresis of PCR co-amplified products of rat transcripts of the β-actin (222 bp), c-erb-A (118 bp), and RAR (143 bp) genes. (B) Semi-logarithmic representation of the relative extent of amplification in control rats measured by counting the amount of ^{32}P incorporated into the fragments visualized in A; n is the number of PCR cycles; y is the incorporated radioactivity (cpm); (□) β-actin; (▽) retinoic acid receptor; (○) c-erb-A.

unchanged C_{max} of RAR. The mechanism involved in such a phenomenon is not known, but, as previously noted, a time shift between both phenomena has been implicated.

4.4. *Effects of RA on RAR mRNA and RAR in T_3-treated rats*

It has been shown that RA up-regulates the RAR-β, which is the major isoform of RAR in liver [33], and RAR-β mRNA [3], but the effect of RA in rats pretreated with T_3 was not known. This study provides evidence for an attenuation of the T_3-induced increase of RAR mRNA levels by RA. This attenuation of T_3 action concerned both RAR mRNA and RAR. So it is clear that when the RAR mRNAs level has been experimentally increased RA is able to down-regulate the mRNA levels.

In summary, these results have established that in hyperthyroidic rats the administration of RA induced an attenuation of C_{max} of TR and c-erb-A mRNA and of C_{max} of RAR and RAR mRNA. Future studies are needed to explore the mechanisms involved in the modulation of these receptors and in particular in the heterologous regulation of TR by RA. Such data are interesting because they can contribute to an understanding of how an excess of vitamin A has an inhibitory effect on thyroid function. It has been known for a long time that there is an amelioration of symptoms

in hyperthyroidic patients treated with large doses of retinol [34], and that vitamin A treatment decreased the severity of experimental thyroiditis in guinea pigs and rats [35]. Various mechanisms are suspected to account for these observations since vitamin A modifies thyroid hormone functions at multiple sites, including cellular uptake of T_3 [36] and peripheral metabolism of thyroid hormones [37]. Recently it was shown that retinol was an inhibitor of TSH-stimulated iodine metabolism [38]. Our results showed that it is also possible that the benefit of the vitamin A treatment is the result of a down-heteroregulation of TR by RA, the active metabolite of retinol.

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