Post-transcriptional induction of \( \beta_1 \)-adrenergic receptor by retinoic acid, but not triiodothyronine, in C6 glioma cells expressing thyroid hormone receptors

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Thyroid hormone (triiodothyronine: T\(_3\)) has been shown to control the expression of \( \beta_1 \)-adrenergic receptors (\( \beta_1 \)-AR) in cardiac myocytes, but not in C6 glioma cells. This cell specificity has been attributed to low expression of \( \beta_3 \) receptors and high expression of the c-erbA\(_{\alpha2}\) splice variant that interferes with the action of \( \beta_3 \). To check this hypothesis we have expressed the c-erbA/thyroid hormone receptor (TR)\(_{\alpha2}\) gene in C6 glioma cells and investigated their response to thyroid hormone. Cells expressing TR\(_{\alpha2}\), but not wild-type cells, were responsive to \( T_3 \) as shown by increased expression of mitochondrial hydroxymethylglutaryl CoA synthase after \( T_3 \) exposure. However, \( T_3 \) had no effect on \( \beta_1 \)-AR gene expression in either set of cells. The \( \beta_1 \)-AR mRNA concentrations were, however, altered by retinoic acid (RA) treatment. Retinoic acid caused a rapid up-regulation of \( \beta_1 \)-AR mRNA levels that was blocked by cycloheximide. Retinoic acid did not increase the \( \beta_1 \)-AR gene transcription rate in run-on experiments. These results indicate an indirect post-transcriptional effect of RA. Control of \( \beta_1 \)-AR expression in C6 cells is also exerted at the translational level, because there was no correlation between mRNA and protein induction, as determined by radioligand binding studies. We conclude that lack of responsiveness of the \( \beta_1 \)-AR gene in C6 cells to \( T_3 \) is not due to high expression of c-erbA\(_{\alpha2}\) but to undefined cell-specific factors.

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The \( \beta_1 \)-adrenergic receptor (\( \beta_1 \)-AR) is a member of the family of G-protein-coupled catecholamine (adrenaline and noradrenaline) receptors. Upon hormone binding, \( \beta_1 \)-AR stimulates the enzyme adenylate cyclase leading to the generation of cAMP. Both the \( \beta_1 \) and \( \beta_2 \) adrenergic receptors are expressed in the brain, where their density seems to be regulated by thyroid hormone (1–3). Thyroid hormone also increases \( \beta \)-adrenergic receptors in the heart muscle and isolated cardiac myocytes (4, 5), in which it specifically activates \( \beta_1 \)-AR gene transcription (6). In contrast to cardiac myocytes, glial C6 cells have been shown to be unresponsive to thyroid hormone in terms of \( \beta_1 \)-AR induction (6). This fact has been attributed to the high expression of the c-erbA\(_{\alpha2}\) form, which results from differential splicing of the TR/c-erbA\(_{\alpha1}\) gene and encodes a protein that is unable to bind thyroid hormone but inhibits transcriptional activation by \( T_3 \) (7, 8). In addition, C6 cells express low levels of \( T_3 \) receptors: levels of the RNAs coding for the active receptors TR/c-erbA\(_{\alpha1}\) and TR/c-erbA\(_{\beta1}\) are undetectable or much lower than those of c-erbA\(_{\alpha2}\) RNA in Northern analyses. For these reasons, we decided to express in C6 cells an exogenous TR/c-erbA\(_{\alpha3}\) gene to investigate whether the lack of \( \beta_1 \)-AR inducibility by \( T_3 \) in these cells could be due to the pattern of expression of the different erbA forms or, alternatively, to an intrinsic inability of \( \beta_1 \)-AR to respond to \( T_3 \) treatment.

On the other hand, the vitamin A metabolite all-trans-retinoic acid (RA), considered as a modulator of differentiation of a number of cell types in higher organisms, causes a threefold increase in the number of \( \beta \)-adrenergic receptors in mouse teratocarcinoma F9 cells (9). Recently, RA has also been reported to induce the expression in C6 glioma cells of proteolipid protein (PLP), the major protein constituent of myelin (10, 11). These data prompted us also to investigate possible actions of RA on \( \beta_1 \)-AR expression in these cells, which are a commonly used system to study hormonal effects on glial cells. C6 glioma cells express high and low levels, respectively, of \( \beta_1 \)-adrenergic and \( \beta_2 \)-adrenergic receptor RNA and protein (6, 12). The RNA levels for
both receptors are down-regulated by their agonist isoproterenol and also by contact inhibition and by protein kinase C activators such as phorbol esters (12, 13). We report here the effects of RA and T3 on the expressions of the β1-AR gene in C6 glioma cells, and discuss them in relation to their reported regulatory actions in different cell types and on other genes.

Materials and methods

Culture techniques

The C6 glioma cell line was grown in RPMI medium containing 10% horse serum and 5% fetal calf serum (Gibco, UK). Cells were maintained at 37°C in a 5% CO2 atmosphere. The TR/c-erbAα1 and v-erbA-expressing cells were generated by using recombinant retroviruses encoding the chicken TR/c-erbAα1 or v-erbA genes described previously (14). Expression of active TR proteins was assayed by T3-binding assays (not shown). These analyses showed that the concentration of TRs increased around tenfold in TR/c-erbAα1 cells. The concentrations of T3 (25 × 10−5 mol/l) and RA (10−6 mol/l) used gave maximal biological responses in these cells.

Isolation and analysis by RNA

Poly(A)+ RNA was purified as described elsewhere (15), electrophoresed on a 1.2% agarose gel containing 2.2 mol/l formaldehyde and blotted onto Gene Screen membranes according to standard protocols (16). The cDNA probes were labeled by the random priming method (17). Hybridizations were carried out overnight at 65°C in 7% SDS, 500 mmol/l sodium phosphate buffer (pH 7.2) and 1 mmol/l EDTA (18). Filters were washed twice for 30 min each in 1% SDS and 40 mmol/l sodium phosphate buffer (pH 7.2) at 65°C. Sizes of respective mRNAs were calculated using an RNA ladder as a marker (BRL, Bethesda). Membranes were exposed to Kodak X-OMAT AR films. Autoradiograms were analyzed using a La Clé scanner connected to a Macintosh Ilci computer using Adobe Photoshop™ and NIH Image programs. All the experiments were done in triplicate unless stated otherwise. Statistical comparison between means was done by Student’s t-test.

Run-on assay

Nuclear run-on reactions were performed using [α-32P]UTP (Amersham) and 2 × 107 nuclei (9). Labeled RNA was purified by phenolchloroform extraction after the addition of 3 volumes of guanidine isothiocyanate solution (20) and isopropanol precipitation. Unincorporated label was removed by centrifugation through Sephadex G-50 spin columns. The probes were 5 μg of linearized plasmid DNA immobilized on nylon filters after denaturation in NaOH. Hybridizations were performed in 0.2 mol/l sodium phosphate buffer (pH 7.2), 1 mmol/l EDTA, 7% SDS and 45% formamide containing 250 μg/ml E. coli tRNA as a carrier, at 42°C for 3 days. Following hybridization, the filters were washed in 40 mmol/l sodium phosphate buffer (pH 7.2) containing 1% SDS at 37°C.

Binding assays

Radioligand binding studies were conducted using intact cells. Control or RA-treated cells were detached and washed three times with RPMI + 20 mmol/l HEPES (pH 7.4) medium. Resuspended cells were incubated (0.5 × 105 cells/assay) for 1 h at 37°C in the same medium in the presence of 2 nmol/l [3H]dihydroalprenolol (Amersham), and binding reactions were terminated by rapid filtration over GF/C glass-fiber filters (Whatman). Total cellular β-adrenergic receptor was defined as the amount of radioligand binding inhibited by 50 μmol/l propranolol; similar results were obtained by displacing with the specific β1-adrenergic receptor antagonist ICI 89,406 (a generous gift from ICI Pharmaceuticals). All experiments were carried out in quadruplicate (SEM < 10%).

Results

Lack of effect of thyroid hormone on β1-AR mRNA levels in C6 cells

As shown in Fig. 1, C6 cells were found to express the β1-AR gene. Addition of RA but not T3 induced β1-AR mRNA by about a factor of 3, as measured by densitometric analysis of autoradiographs after correction by expression of the cyclophilin gene used as control. The combination of RA plus T3 did not change the response to RA. The presence of cycloheximide in the culture medium blocked the effect of RA, suggesting an indirect effect through the synthesis of other RA-induced proteins. In order to clarify conclusively whether the unresponsiveness to thyroid hormone of the β1-AR gene in C6 cells is the consequence of the very low expression of T3 receptors and high expression of the c-erbAα2 isofrom, as suggested (6), we analyzed the pattern of expression of endogenous TR/c-erbA by Northern blotting. Results obtained were analogous to those reported previously: higher expression of c-erbAα2 RNA (2.6 kb) than of TR/c-erbAα1 RNA (5.0 kb) (Fig. 2). We then decided to express in these cells an exogenous (chicken) TR/c-erbAα1 cDNA and study the effect of T3 administration. For comparison, the mutant viral v-erbA gene, whose product is unable to bind hormone, was also expressed in parallel. Expression of both genes in C6 cells was carried out by using retroviral vectors available in our laboratory (see Materials and methods).

First, their respective expression in infected cultures after G418 selection was analyzed. Figure 2 clearly shows the high levels of the respective retroviral mRNAs detected using the hormone-binding domain of the
Fig. 1. Retinoic acid, but not T₃, increases β₁-adrenergic receptor mRNA levels in glioma C6 cells. (A) Poly(A)⁺ RNA (10 µg) from C6 cells treated for 6 h with 10⁻⁶ mol/l retinoic acid (RA). 25 × 10⁻⁹ mol/l T₃, or both together in the presence or absence of 8 µg/ml cycloheximide (CHX) were sequentially hybridized with β₁-adrenergic receptor (β₁-AR) and cyclophilin (Cy) probes. The autoradiograms were exposed 18 h (β₁-AR) and 6 h (Cy), respectively. The size of the β₁-AR mRNA is indicated. (B) Densitometric determinations of the ratio of expression of β₁-AR mRNA versus Cy mRNA obtained as described in the text. Results are the mean ± s of four sets of data.

Fig. 2. Northern analysis of exogenous (chicken) TR/c-erbAα₁ or v-erbA gene expression in C6 cells. Normal C6 cells were infected with recombinant retroviruses encoding the (chicken) TR/c-erbAα₁ or the v-erbA genes (see text): 10 µg of poly(A)⁺ RNA from C6 cells infected or uninfected, and treated or not with 25 × 10⁻⁹ mol/l T₃ for 2 days, were hybridized first with a specific probe corresponding to the hormone binding domain of the chicken TR/c-erbAα₁ cDNA (A) and then with a rat-specific probe corresponding to the same domain (B). The autoradiograms were exposed for 5 days.

v-erbA-expressing cells. This result indicates that the low level of endogenous TR is not the reason for the T₃ unresponsiveness of C6 cells. Figure 3 also shows that RA induced a similar increase (around threefold, as measured by densitometric scanning and correcting by cyclophilin expression) in β₁-AR mRNA levels in both TR/c-erbAα₁- and v-erbA-expressing cells than that observed in control C6 cells.

To prove that the exogenously expressed TRc-erbAα₁ gene in C6 cells was biologically active and to discard any possible aberrant character or uncharacterized mutation of the C6 cells that might make them totally T₃-resistant, we tested the hormonal inducibility of the mitochondrial hydroxymethylglutaryl CoA synthase (HMGOA synthase) gene, which is T₃-inducible in rat liver (21). As seen in Fig. 4, TR/c-erbAα₁ C6 cells but not uninfected or v-erbA cells responded to thyroid hormone, increasing the HMGOA synthase RNA levels in a statistically significant fashion (p < 0.05).

Retinoic acid increases β₁-AR mRNA levels by a post-transcriptional mechanism

In view of the upregulation of β₁-AR mRNA levels following RA addition, we investigated whether this
could be the result of an effect on the transcription rate of the gene. To test this possibility, run-on assays were performed using nuclei from untreated cells or cells treated with RA for 30 or 120 min. Retinoic acid β receptor (RAR-β) gene was used as a positive control of RA regulation, and cyclophilin as an unchanged gene. As shown in Fig. 5, the effect of RA on RAR-β transcription was significant, with p < 0.01 (asterisks) both at 30 min and at 120 min after addition to the culture medium. In contrast, no changes in transcription were observed with the β1-AR probe. Together with the cycloheximide sensitivity data, this finding suggests that the observed effect of RA can be ascribed to a post-transcriptional stabilization of β1-AR mRNAs.

β1-Adrenergic receptors in RA-treated C6 cells

To investigate whether the observed increase in β1-AR mRNA induced by RA correlates with a higher number of β1-ARs, radioligand binding studies were performed. β1-Adrenergic receptor protein expression was assessed by using the lipophylic antagonist...
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Fig. 5. Transcription rate of the β1-adrenergic receptor (β1-AR) gene in C6 cells. (A) Twenty million nuclei from C6 cells treated or not with 10^{-5} mol/l retinoic acid (RA) for 30 or 120 min were used to perform nuclear run-on assays. The probes were 5 µg of linearized plasmal DNA of β1-AR gene, retinoic acid β receptor (RAR-β) gene as an RA-induced gene and cyclophilin (Cy) as control gene. The autoradiograms were exposed for 12 days. (B) Densitometric scanning was performed as described in the text for Northern analysis. Determinations of the ratio of transcription of β1-AR versus Cy and RAR-β versus Cy are shown. Results are the mean ± SD of three sets of data. **Significant differences versus untreated samples, p < 0.01.

[3H]dihydroalprenolol: no statistically significant increase in the total number of cellular receptors was found in RA-treated cells, and even a slight decrease was observed at short times of treatment (Fig. 6). These results indicate that the regulation of β1-AR expression is a complex process that takes place at both mRNA and protein levels.

Discussion

The expression of relatively high levels of β1-ARs in neurons and glia is well documented. Despite the importance of adrenergic function, very little is known about the agents controlling the expression of these receptors. An influence of the thyroid status on the number of different receptors, including β-adrenergic (1–3), α-adrenergic (22) and other receptors (23), in the central nervous system (CNS) has been reported. Hypothyroidism induced by propylthiouracil (PTU) treatment causes a reduced expression of β-ARs in the developing rat brain (24). Correspondingly, primary cultures of astrocytes from hypothyroid animals display a similar reduction in receptors (24). Another recent study suggests a permissive effect of thyroid hormone because PTU treatment resulted in a decrease in the number of β-receptor binding sites but, however, no increase was observed following T3 administration (25).

The results described in this paper show that thyroid hormone has no effect on the expression of β-ARs in C6 cells. This discrepancy may be due to an intrinsic T3 unresponsiveness of the C6 cells used, or to the requirement of the concerted action of other factors. Supporting the hypothesis of a cell-type specific effect, thyroid hormone has been shown to upregulate the β1-AR gene at the transcriptional level in cultured cardiac myocytes. In contrast, in the same study thyroid hormone had no effect on C6 cells (6). It was suggested that the high expression of the c-erbAα2 isoform could interfere with the action of low levels of active TRs in these cells. However, we show here that over-expression of TR/c-erbAα1 by retrovirally mediated gene transfer, which makes these cells sensitive to T3, has no effect on the response of β-ARs. On the other hand, the use of a long-term cultured transformed cell line such as C6 may contribute to the differences observed. In line with this, several groups have described differences in the response of C6 cells to β-adrenergic activation, depending on the passage (26).
Together, these results indicate that much care should be taken when extrapolating the data obtained in C6, and probably other cell lines, to the in vivo situation, and that C6 cells may not be an appropriate system in which to study thyroid hormone effects in the brain.

On the other hand, our results indirectly demonstrate that RA has a probable post-transcriptional effect on the β1-AR gene, leading to an increased accumulation of its mRNA. In agreement with the possibility of post-transcriptional regulation by RA, we do not detect changes in the transcriptional rate of the β1-AR gene in run-on assays, and the presence of neither high levels of TR/c-erbAα1 nor of v-erbA protein are able to block the increase in β1-AR mRNA. These proteins have been described to inhibit transcriptional effects of RA in other cell systems (27, 28). As shown in Fig. 3, cycloheximide does not block RA induction of β1-AR mRNA in TR/c-erbAα1-expressing cells. Expression of TR/c-erbAα1 might regulate, recruit or stabilize a protein(s) required for RA action, which could be a co-activator or perhaps the RAR protein. Alternatively, it may substitute in the nucleus for a short-lived, cycloheximide sensitivity protein required for RA action. The lack of a transcriptional effect by RA is further supported by run-on experiments. As in the case of the PLP gene in the same C6 cells (9) and of other genes in different cell lines (29–31), RA may affect β1-AR mRNA stability, affecting its half-life. Recent data suggest that the stability and turn-over of the β1-AR mRNA is a highly regulated process, and that different agents, including protein kinase A, insulin or dexamethasone, may modulate it (32). It is tempting to speculate that RA may interact at this level with other extracellular messengers in order to modulate β1-AR expression. Interestingly, a novel action of RA has been described recently (33). Retinoic acid can regulate gene expression by a mechanism independent of the transcriptional rate or the stabilization of cytoplasmic mRNAs. Thus, RA regulates alkaline phosphatase expression by stabilizing the nascent RNA chains in the nucleus (33). The regulation of RNA processing at the level of this very early post-transcriptional step is another possible mechanism of RA action on the β1-AR gene in C6 cells.

The lack of correlation between the increase in mRNA levels and the number of total active receptors indicates a complex regulation. Translational or post-translational regulation may contribute to receptor function. Muscle nicotinic acetylcholine receptor number (nAChR) is regulated at both levels (34, 35). Similarly to our observations, recent data show that alterations in nAChR mRNA levels induced by NGF in PC12 cells are not paralleled by changes at the protein level (36). The elevated receptor protein could also be kept in a non-functional state by modulation of its phosphorylation or stability, or by redistribution of internal pools in the case of an excess of pre-existing receptors. Examples of these types of control have been described elsewhere (37, 38). Testing these possibilities will require additional studies, including the search for other regulatory factors probably not present in C6 cells. An additional example of this phenomenon is the up-regulation by thyroid hormone of the expression of its own TR/c-erbAβ1 receptor gene in rat cerebral hemisphere astrocyte cultures (39). Here, again, thyroid hormone specifically increases TR/c-erbAβ1 mRNA levels without changing hormone binding capacity.

Based on a series of data and considerations, including its endogenous presence and the expression of different RA binding proteins and receptors. RA has also been proposed to play a crucial role in CNS development and regulation (40, 41). Emphasizing their importance, β-adrenergic receptors have been implicated in the modulation of the synthesis and release of growth factors in glial cells (42, 43). In line with our findings, RA has been reported to increase β1-AR mRNA and protein in mouse teratocarcinoma F9 cells (9). Whether this represents a real discrepancy with our data on the glial C6 cells due to cell-specific RA effects or simply differences due to culture conditions as discussed above remains to be clarified. Examples of cell-specific gene regulatory action of RA are known: RA induced RAR-β2 expression in lung and liver and in F9 cells but not in several pituitary cell lines (44, 45).

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References


32. Port JD, Huang LY, Malbon CC. β-Adrenergic agonists that down-regulate receptor mRNA up regulate a Mr 35,000 protein(s) that selectively binds to β-adrenergic receptor mRNAs. J Biol Chem 1992;267:24103–8


34. Salpeter MM, Loring RH. Nictinic acetylcholine receptors in vertebrate muscle; properties, distribution, and control. Prog Neurobiol 1985;25:291–325


44. Hu L, Gudas LJ. Cyclic AMP analogs and retinoic acid influence the expression of retinoic acid receptor α, β and γ mRNAs in F9 teratocarcinoma cells. Mol Cell Biol 1990;10:391–6

45. Davis KD, Lazar MA. Induction of retinoic acid receptor-β by retinoic acid is cell specific. Endocrinology 1993;132:1469–74