Biochimica et Biophysica Acta 1788 (2009) 1861–1868

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

The ATRA-dependent overexpression of the glutamate transporter EAAC1 requires RARβ induction

Massimiliano G. Bianchi [a], Gian C. Gazzola [a], Silvia Cagnin [a], Hiroyuki Kagechika [b], Ovidio Bussolati [a,*]

[a] Department of Experimental Medicine, Unit of General and Clinical Pathology, University of Parma, via Volturno 39, 43100 Parma, Italy
[b] School of Biomedical Science, Tokyo Medical and Dental University, 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

ARTICLE INFO

Article history:
Received 16 February 2009
Received in revised form 29 April 2009
Accepted 8 May 2009
Available online 18 May 2009

Keywords:
EAAT3
C6 cells
Glioma
Retinoids
RXR
Slc1a1
Transport

ABSTRACT

The mechanisms underlying trafficking and membrane targeting of EAAC1, the rodent counterpart of the human EAAT3 carrier for anionic amino acids, are well characterized. In contrast, much less is known on the regulation of Slc1a1, the gene that encodes for the transporter. We have recently found that all-*trans* retinoic acid (ATRA) stimulates EAAC1 expression and anionic amino acid transport in C6 rat glioma cells. We report here that the ATRA effect on EAAC1 activity was inhibited by the specific RAR antagonist LE540 and mimicked by Am80, a RAR agonist, but not by the RXR agonist HX630. Moreover, the ATRA-dependent induction of Slc1a1 mRNA required the synthesis of a protein intermediate and was not associated with changes in the messenger half-life. ATRA treatment induced the expression of both Rarb mRNA and RARβ protein several hours before the induction of Slc1a1, while the mRNA for RFX1, a transcription factor recently involved in Slc1a1 transcription, was unchanged. In addition, Rarb silencing markedly inhibited the ATRA-dependent increase of both Rarb and Slc1a1 mRNAs. We conclude that in C6 glioma cells the induction of Slc1a1 by ATRA requires the synthesis of RARβ, suggesting that the receptor is involved in the regulation of the transporter gene.

1. Introduction

The glutamate transporter EAAT3 and its rodent counterpart EAAC1, although present in several cell types, are mainly expressed in central nervous system (CNS) neurons [1]. As other members of the EAAT family, EAAC1, which is encoded by the *Slc1a1* gene, is involved in the regulation of the extracellular glutamate concentration and in synaptic plasticity [2]. Alterations in the activity and/or expression of the transporter have been implied in several neurological and psychiatric conditions [3]. For these reasons, the regulation of EAAC1/EAAT3 activity has been investigated in a number of studies (see [3] for a recent review). Most of these involve rapid mechanisms that modify transporter trafficking between the plasma membrane and intracellular compartments, where most of the carrier proteins are located [4].

Much less is known about EAAC1/EAAT3 regulation at the gene level. Ma et al. [5] have shown that the transfection of rat glioma C6 cells with the human transcription factor RFX1 induces EAAC1 expression. More recently, we have found that in the same cell model the chronic exposure to all-*trans* retinoic acid (ATRA) causes a marked induction of Slc1a1, causing the increased abundance of EAAC1 carrier proteins and the proportional stimulation of transport activity [6]. Under the same conditions, no induction of Slc1a2 (for the GLT-1 transporter) or Slc1a3 (for the GLAST transporter) is observed [6], suggesting that inducibility by ATRA is a peculiarity of Slc1a1, at least in C6 cells. In this cell model ATRA treatment also causes the increased expression of Plp, a marker of the oligodendrocytic differentiation pathway and a well known ATRA target gene [7]. The induction of Plp mRNA by ATRA in C6 cells is a slow phenomenon, requires a protein intermediate and has been attributed to an increase in the half-life of the messenger [8].

A variety of genes are direct or indirect targets of retinoids and their expression is modulated by ATRA at both transcriptional and post-transcriptional levels. In particular, the transcription of direct ATRA targets is promoted by a complex formed by ATRA, a heterodimer of RAR–RXR receptors and several other co-stimulator molecules. Indirect regulation of gene expression by ATRA comprises various mechanisms such as (a) the induction of an intermediary (usually a transcription factor) that is directly responsible for the effect, (b) the modulation of mRNA stability, and (c) the interaction

Abbreviations: ATRA, all-*trans* retinoic acid; CNS, Central Nervous System; DMEM, Dulbecco's modified Eagle Medium; EAAC1, Excitatory Amino Acid Carrier 1; EAAT, Excitatory Amino Acid Transporter; EBSS, Earle's Balanced Salt Solution; FBS, Fetal Bovine Serum; Rarb, retinoic acid receptor, beta, gene; RAR, Retinoic Acid Receptors; RXR, Retinoid X Receptors

* Corresponding author. Sezione di Patologia Generale e Clinica, Dipartimento di Medicina Sperimentale, Università di Parma, Via Volturno, 39, 43100 Parma, Italy. Tel.: +39 0521 033783; fax: +39 0521 033742.
E-mail address: ovidio.bussolati@unipr.it (O. Bussolati).

0005-2736/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2009.05.005

with nuclear receptors other than the classical RAR–RXR dimers [9]. Moreover, retinoids can affect transcription through non genomic effects, such as the modulation of MAPK cascade [10] or of Akt signaling pathway [11].

The essential step required for the direct transcriptional effects of ATRA is represented by the interaction of the retinoid with the heterodimer formed by a member of RAR receptor family and a RXR receptor. For each of the three RAR subtypes (α, β, and γ), several isoforms exist that differ for their N-terminal regions [12]. There are two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2) and four major isoforms for RARβ (β1 and β3, initiated at the P1 promoter, and β2 and β4, initiated at the P2 promoter). Two isoforms also exist for each of the three RXR receptors thus far characterized (α, β, and γ) [13]. Upon the interaction with the ligand, the RAR–RXR heterodimer exchanges co-repressor partners with co-activators and the transcription of the target gene can start. While RARα is expressed in most tissues [12], the expression of RARβ and RARγ is tissue specific and highly regulated [14]. Interestingly, two of the RARβ isoforms, RARβ2 and 4, are transcribed from a promoter that contains a DR5 sequence and, hence, are inducible by retinoids [15,16]. Consistently, ATRA treatment markedly affects the expression of RARβ receptors in many cell types. In particular, in C6 cells ATRA stimulates the expression of one of the "heavy", 55 kDa isoform of RARβ, while RARα is not affected by the retinoid [17].

In this report we have studied the mechanisms underlying the effect of the retinoid on EAAC1, obtaining evidence that involves the synthesis and the activation of a RARβ receptor as one of the steps in the regulatory process.

2. Methods and materials

2.1. Cells and treatments

The rat CNS-derived C6 glioma cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C and 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were used for less than 10 passages from thawing with no apparent change in morphology or sensitivity to treatments.

For ATRA treatment, unless otherwise specified, culture medium was substituted 12 h after the passage with fresh medium supplemented with 0.1% FBS in the absence or in the presence of ATRA. ATRA, obtained from Sigma, was used at the concentrations detailed for each experiment starting from a 10 mM stock solution in DMSO. The RAR and RXR agonists were synthesized by one of us (HK) according to the methods reported previously [18–20]. The identification and purity of the compounds were determined by melting point, NMR, and Mass Spectroscopy. The compounds are very stable to heat, light, acids and bases and do not decompose under the experimental conditions adopted. RAR and RXR agonists and inhibitors were used at the concentrations detailed in the experiments starting from stock solutions in DMSO (10 mM, 10 mM, 1 mM, and 1 mM for, respectively, Am80, HX630, LE540 and HX531). In the controls, the vehicle was used at the maximal concentration adopted in the single experiment.

2.2. Transport activity

The initial influx of D-[^{3}H]aspartate, a high-affinity substrate of EAATs [1], was measured in 96-well multidish plates (Falcon, Becton, Dickinson Biosciences, Franklin Lakes, NJ, USA), where C6 cells had been seeded at a density of $10 \cdot 10^3$ cells/well, or in Falcon 24-well multidish plates, where cells had been seeded at a density of $50 \cdot 10^3$ cells/well. For the experiments, cells were washed twice in a modified EBSS (Earle's Balanced Salt Solution, containing (in mM) 117 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 NaH_2PO_4, 0.8 $MgSO_4$, 5.5 glucose, buffered with 20 mM HEPES-NaOH at pH 7.4) and incubated in the same saline solution supplemented with D-[^{3}H]aspartate (1 μM, 2 μCi/ml) for 1 min.

At the end of the assay, multiwell dishes were washed twice with ice-cold urea (300 mM) and cell monolayers were extracted with absolute ethanol. The extracts were added to scintillation fluid and counted with a Wallac Trilux^2 liquid scintillation spectrometer (Perkin-Elmer, Boston, MA, USA). Cell proteins were determined directly in the well with a modified Lowry procedure and measured with a Wallac Victor^2 Multilabel Counter (Perkin-Elmer). Amino acid influx is expressed as pmoles mg of protein^{-1}·min^{-1}.

2.3. RT-qPolymerase Chain Reaction

Total RNA was isolated with RNeasy Mini Kit® (Qiagen S.p.a., Milan, Italy). After reverse transcription, 25 ng of cDNA from each sample was amplified in a total volume of 25 μl with 2× SYBRGreen qPCR SuperMix-ROX (Rovalab, Taltow, Germany), along with the following forward and reverse primers (5 pmol each) for the genes: 5′ CGA CTT GCC GTA CCT GGA CT 3′ and 5′ GCC CAC GGG ACT CAA CAC GA 3′ for Slc1a1; 5′ ATA CCC CAG AGC AAG ACA CC 3′ and 5′ AGC AGA TGG CAC TGA GAA GA 3′ for Rarb; 5′ ACT GCC TCT TTC TTC TTC CT 3′ and 5′ ATT TTC CCA AAC AAT GAC AC 3′ for Plp; 5′ AAC CAG AGA GCC GAT TTT AGA G 3′ and 5′ AAC TGT TGC TAC CCA CCC TAC T 3′ for Rfx1; 5′ AGC CTC AAG ATC ATC AGC AAT G 3′ and 5′ CAC GAT ACC AAA GTT GTC ATGGA 3′ for Gapdh. All the primers were designed with the help of Primer 3 program [21] according to the known or predicted (in the case of Rfx1) rat sequences reported in GenBank. Quantitative PCR was performed in a 36 well Rotor-Gene 3000 (Corbett Research, Rotor-Gene™ 3000, version 5.0.60, Mortlake, Australia). For all the probands each cycle consisted of a denaturation step at 95 °C for 30 s, followed by separate annealing (30 s) and extension (30 s) steps at a temperature characteristic for each proband. Fluorescence was monitored at the end of each extension step. A no template, no-reverse transcriptase control was included in each experiment. At the end of the amplification cycles a melting curve analysis was added. The analysis of the data was made according to the Relative Standard Curve Method [22]. qRT-PCR data were expressed as the ratio between proband mRNA and Gapdh mRNA.

2.4. Western analysis

For RARβ expression, protein extraction and quantification were performed with the method of Farinha et al. [23] with minor modifications. C6 cells, grown to subconfluence on 10-cm tissue culture plates, were rinsed twice in PBS and lysed in 300 μl of Sample Buffer 1× (31.25 mM Tris–HCl pH 6.8, 3% SDS, 10% glycerol, 100 mM DTT, 0.02% bromophenol blue). After solubilization, cell lysates were collected in Eppendorf tubes and passed 10 times into 25 G and then 27 G needles. The quantification of proteins was performed with a modified Lowry assay [23]. Briefly, the protein sample was added with 1 ml of H_2O and 100 μl of sodium deoxycholate (0.15%) and the mixture incubated for 10 min. At the end of this period, 100 μl of 72% trichloroacetic acid was added to the sample that was vortexed and span at 14,000 rpm for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in 400 μl of H_2O, supplemented with 400 μl of reagent A (1 volume of CTC; 1 volume of 10% sodium dodecyl sulphate; 1 volume of 0.8 M NaOH; 1 volume of H_2O, where CTC is a mixture of 25 ml of 0.4% $CuSO_4 \cdot 5H_2O$, 25 ml of 0.8% potassium tartrate, and 200 ml of 5% Na_2CO_3), incubated for 10 min, and added with 200 μl of Reagent B (1 volume of Folin–Ciocalteau reagent + 4 volumes of H_2O). Absorbance at 750 nm was read after 30 min and protein content was calculated from bovine serum albumin standards. Aliquots of 60 μg were loaded on a 10% gel for SDS-PAGE. After the electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience, Dassel,

Germany). Non-specific binding sites were blocked with an incubation in PBS-Tween 0.1%, containing 5% of non fat dried milk (Amersham Pharmacia Biotech Italia, Milan, Italy), for 2 h at room temperature. The blots were then exposed overnight at 4 °C to anti-RARβ1-2 (1:250) or anti RARα polyclonal antisera (1:200), obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), diluted in blocking solution. After washing, the blots were exposed for 1 h to horseradish peroxidase-conjugated anti-rabbit IgG (ExactaCruz, Santa Cruz Biotechnology) diluted 1:10000 in blocking solution. For the standardization of the total cell lysate and the intracellular fraction, stripped membranes were exposed to monoclonal antiserum against GAPDH (Chemicon International Inc., Temecula, CA, USA, 1:1000). The membranes were washed and the immunoreactivity visualized with enhanced chemiluminescence (Millipore, Billerica, MA).

The Western Blot analysis of EAAC1 expression was performed as described by Bianchi et al. [6].

2.5. Silencing experiments

Silencing of Rarb mRNA was carried out in C6 cells using the N-TER™ Nanoparticle siRNA Transfection System from Sigma-Aldrich. The same manufacturer provided also the oligonucleotides against rat Rarb1-2 (oligos #3834795 and #3834796 (siRNA1); oligos #3834797 and #3834798 (siRNA2); oligos #3834799 and #3834800 (siRNA3)) and the scrambled siRNAs (oligos #3834801 and #383802). The preparation of oligonucleotide/peptide mixtures was performed according to the manufacturer's instructions.

The protocols used for Rarb silencing are detailed in the legends of the single experiments.

2.6. Statistical analysis

Statistical analysis of transport data was performed with a one-way ANOVA with the Bonferroni post hoc test. qRT-PCR data were analyzed with a *t*-test for unpaired data (Fig. 5) or with a one-way ANOVA with the Bonferroni test for selected pairs of conditions (Figs. 3 and 8). In all cases, *P* values>0.05 were considered not significant.

2.7. Materials

FBS and culture medium were purchased from Celbio, (Pero, MI, Italy). D-[2,3-^{3}H]aspartic acid (12 Ci/mmol) was obtained from Perkin-Elmer (Monza, Italy). Sigma was the source of the other chemicals, including ATRA, whenever not stated otherwise.

3. Results

3.1. ATRA-dependent EAAC1 stimulation is mimicked by RAR agonists and inhibited by RAR but not by RXR inhibitors

To investigate the mechanism underlying Slc1a1 induction in ATRA-treated cells we examined the effects of agonists or antagonists of RAR and RXR receptors on the ATRA-dependent stimulation of EAAC1 transport activity. The results of this analysis, recounted in Fig. 1, indicate that the specific Retinoic Acid Receptor (RAR) agonist Am80 mimicked the maximal ATRA effect, while the RXR agonist HX630 was ineffective (Panel A). However, cells treated simultaneously with Am80 and HX630 exhibited a transport activity for aspartate significantly higher than cells treated with 1 μM ATRA. These data closely reflect the behaviour of EAAC1 protein, as shown in Fig. 2. Indeed, besides ATRA, also Am80 caused an evident EAAC1 over-expression while treatment with HX630 did not substantially increase transporter expression. The highest level of EAAC1 expression was detected upon the simultaneous exposure to Am80 and HX630.

Fig. 1, Panels B and C, reports the results obtained with RAR and RXR antagonists. These experiments, in which ATRA was used at low,

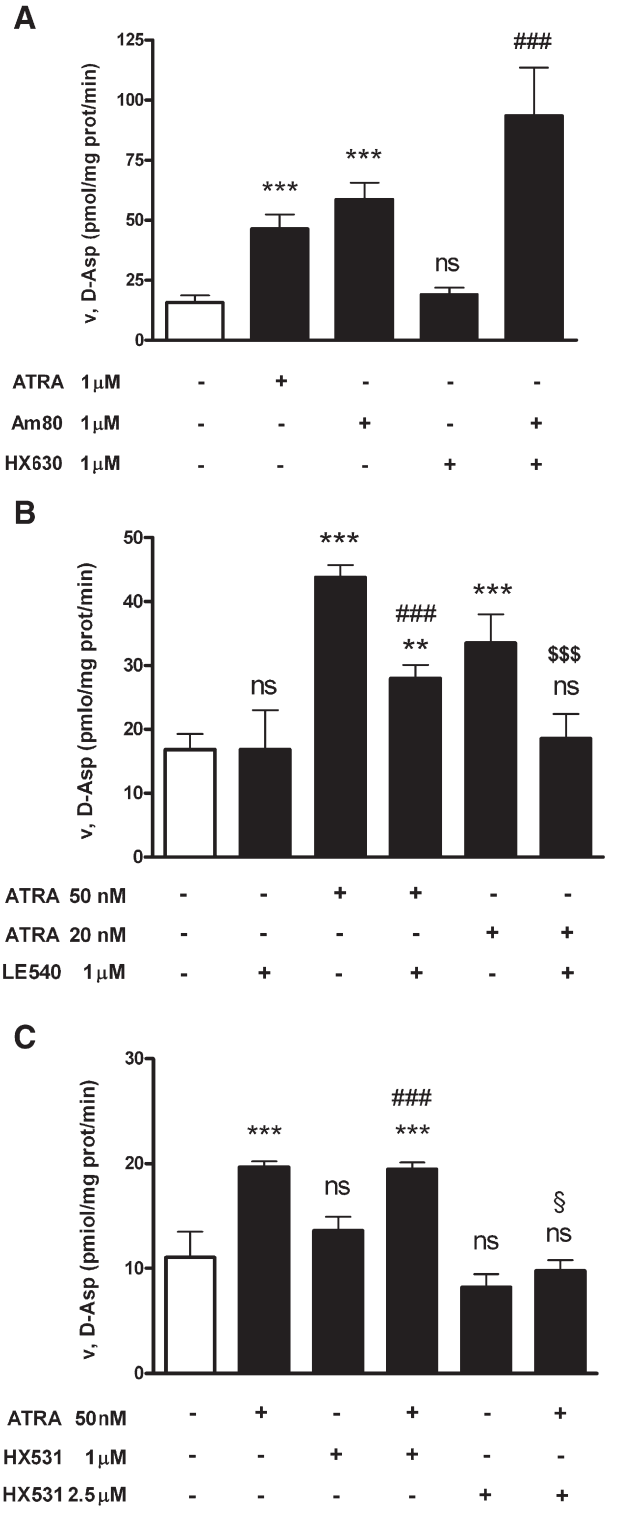

Fig. 1. Effect of RAR and RXR agonists and antagonists on ATRA-dependent increase of EAAC1 activity. C6 cells were seeded in 96-well (Panel A) or in 24-well multiwell dishes (Panels B and C). After 24 h, cells were incubated in the absence (control, empty bars) or in the presence of the indicated agonists or antagonists (solid bars), as detailed in each panel. Transport of D-Asp was measured after 3 days. Bars are means ± SD of five (Panel A) or three (Panels B and C) independent determinations in representative experiments, repeated twice with comparable results. For all panels, $^{**}P<0.01$, $^{***}P<0.001$ vs. control cells; ns, not significant vs. control cells; for panel A, $^{###}P<0.001$ vs. cells treated with ATRA and vs. cells treated with 1 μM Am80; for panel B, $^{###}P<0.001$ vs. cells treated with ATRA and vs. cells treated with 50 nM ATRA, $^{$$$}P<0.001$ vs. cells treated with 20 nM ATRA; for panel C, $^{###}P<0.001$ vs. cells treated with 1 μM HX531, $^{§}P<0.05$ vs. cells treated with 50 nM ATRA.

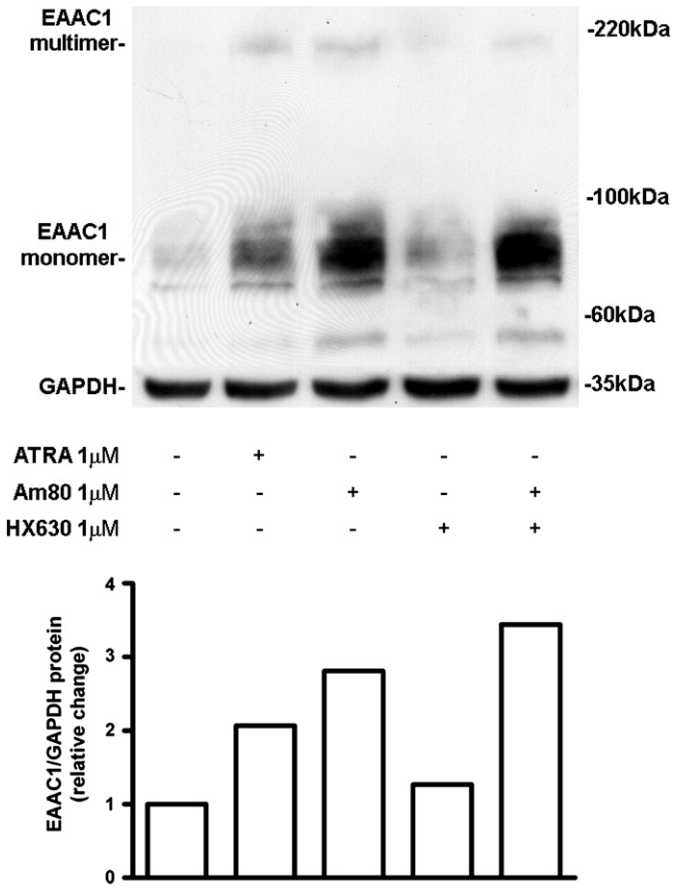

Fig. 2. Effects of RAR or RXR agonists on EAAC1 expression in C6 cells. C6 cells were incubated for 72 h in the absence or in the presence of ATRA (1 μM), the RAR agonist Am80 (1 μM), the RXR agonist HX630 (1 μM), or both agonists. Cells were then lysed and lysates examined for the expression of EAAC1. GAPDH was used as a loading control. The lower part of the figure reports the densitometric analysis of the blot. The experiment was repeated twice with comparable results.

sub-maximal concentrations, indicated that the specific RAR antagonist LE540 significantly inhibited the effect of 50 nM ATRA and completely suppressed transport stimulation at 20 nM ATRA (Panel B). On the contrary, ATRA effect was not inhibited by 1 μM HX531, an RXR antagonist that also inhibits RARs at high concentrations, but was significantly lowered when the inhibitor was used at 2.5 μM (Panel C). In summary, these results indicate that the ATRA-dependent stimulation of EAAC1 transport activity requires the interaction of the retinoid with a RAR receptor.

3.2. Slc1a1 induction requires protein synthesis and does not involve changes in the messenger half-life

ATRA-dependent stimulation of EAAC1 activity is associated with the increase in the level of Slc1a1 mRNA [6]. To investigate the mechanism underlying this effect we have performed ATRA treatment in the presence of the protein synthesis inhibitor cycloheximide and compared the behaviour of the transporter messenger with that of other two ATRA target mRNAs, Rarb, encoding for the RARβ receptor, and Plp, which encodes for the proteolipid protein (Fig. 3). The results obtained indicate that the inhibitor completely suppressed the increase in Slc1a1 mRNA levels caused by ATRA, demonstrating that the ATRA-dependent stimulation of Slc1a1 expression requires the new synthesis of a protein. The requirement for active protein synthesis is not shared by all the other ATRA target genes. Indeed, the mRNA for RARβ was still significantly increased by ATRA even in the presence of cycloheximide while the ATRA-dependent induction of Plp mRNA was not detectable under the same conditions (see also [8]). However, both Rarb and Plp mRNAs were significantly more abundant in cells treated with cycloheximide, even in the absence of ATRA (Panels B and C). Moreover, the effects of ATRA and cycloheximide on Rarb expression were clearly additive (panel B). These results, consistent with previous observations [24,25], indicated that in C6 cells Rarb is superinduced by the protein synthesis inhibitor. Although the mechanisms underlying gene superinduction by cycloheximide are not fully understood, they are usually attributed to decreased mRNA degradation or increased stabilization of nuclear receptors [26–28].

ATRA-dependent increase of Plp mRNA is particularly interesting since it is an example of ATRA effect at post-transcriptional level, being mediated through changes in the half-life of the messenger [8]. The possibility that the same mechanism operates in ATRA-dependent stimulation of Slc1a1 expression has been investigated

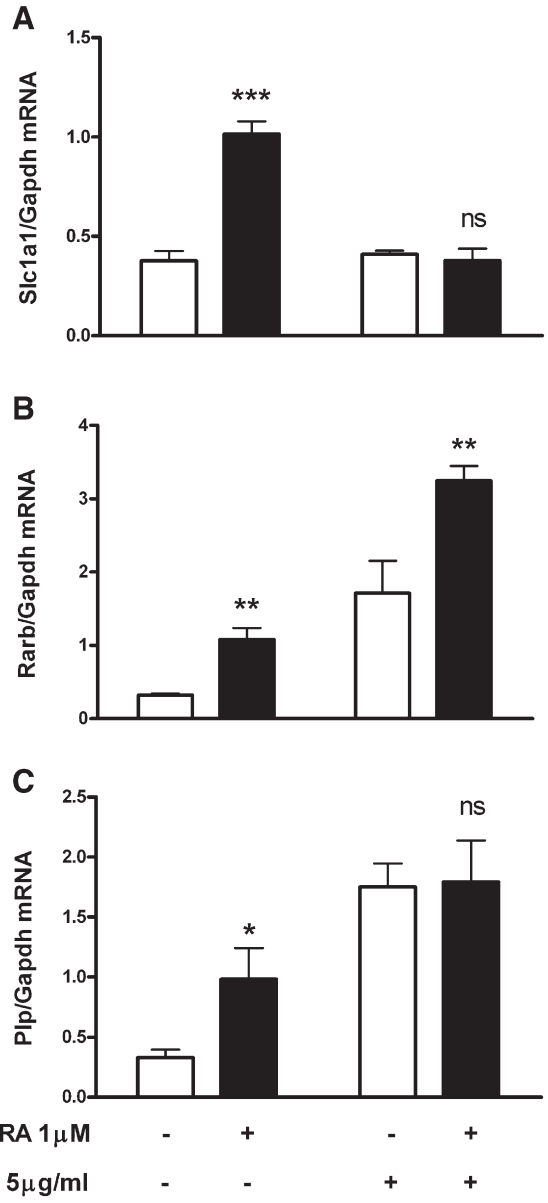

Fig. 3. Dependence of Slc1a1 induction upon protein synthesis. C6 cells were incubated for 3 days in the absence or in the presence of 1 μM ATRA and of 5 μg/ml cycloheximide. At the end of this period, the relative abundance of Slc1a1, Plp, and Rarb mRNA was determined with RT-PCR. Data are means ± SD of four determinations obtained in two different experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. respective control, ATRA-untreated cells; ns, not significant vs. respective, ATRA-untreated controls.

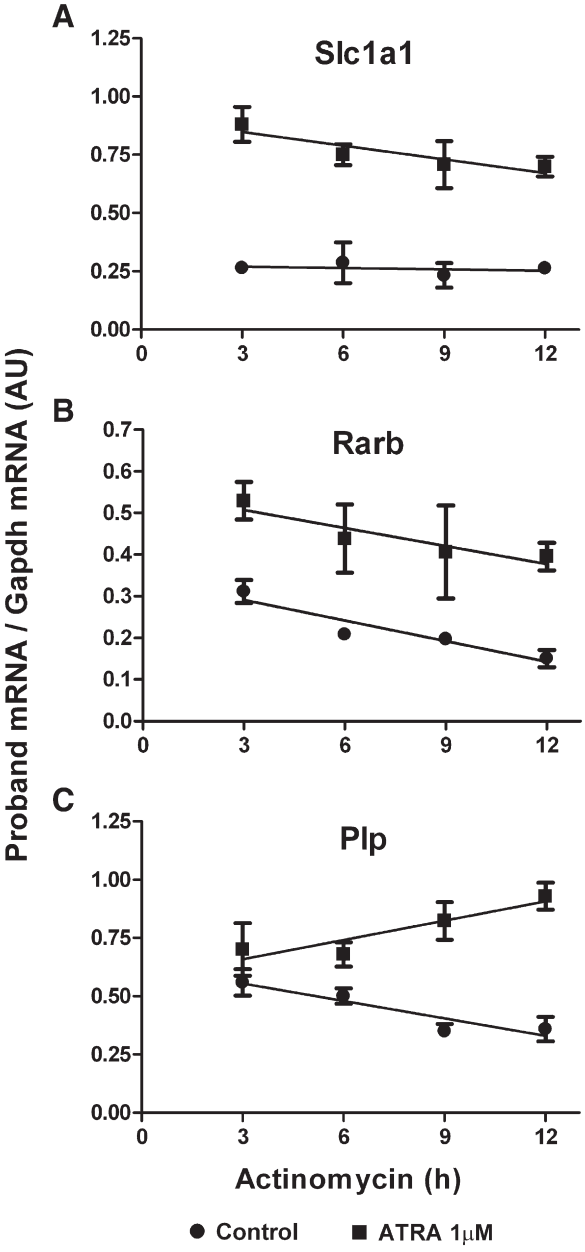

Fig. 4. ATRA effects on the half-life of Slc1a1, Rarb and Plp mRNAs. C6 cells were incubated for 24 h in the absence or in the presence of 1 μM ATRA. After this period, 2.4 μM actinomycin D was added to the extracellular medium and incubation prolonged, with or without ATRA, for further 12 h. At the indicated times, RNA was extracted and the expression of the indicated genes (Panel A, Slc1a1; Panel B, Rarb; Panel C, Plp) was determined through RT-PCR. Points are means of four determinations obtained in two distinct experiments. Lines represent the best fit linear regressions. The slopes of the regression lines obtained in the absence and in the presence of ATRA were significantly different ($P<0.01$) only for Plp (Panel C).

incubating control or ATRA-treated cells with actinomycin D, so as to block transcription, and determining mRNA levels at different times of incubation. The results (Fig. 4A) indicate that during the incubation with actinomycin D the time course of the ratio between Slc1a1 and Gapdh mRNAs was not statistically different in control and ATRA-treated cells. The behaviour of Rarb mRNA (Fig. 4B) is comparable, while, as expected [8], the ratio of Plp and Gapdh mRNAs increased progressively if the incubation in the presence of actinomycin was performed in ATRA-treated cells, indicating that the half-life of Plp messenger is longer in the presence of the retinoid than in its absence.

3.3. Slc1a1 induction is preceded by Rarb but not by Rfx1 induction

The transfection of C6 cells with the human transcription factor RFX1 has recently been reported to activate Slc1a1 expression [5]. Interestingly, RFX1 protein is induced by ATRA in human myeloid models [29]. Thus, although no direct evidence is available about ATRA sensitivity of the rat *Rfx1* gene, the possibility exists that the protein intermediate required for Slc1a1 induction is RFX1. In order to verify this hypothesis, we have performed a short-term time course of Slc1a1 and Rfx1 mRNAs during ATRA treatment (Fig. 5). The results indicate that, while a significant Slc1a1 induction is detected at 16 h of treatment as well as at longer times (Panel A), Rfx1 is not induced at any time during ATRA treatment (Panel B). Rather, a moderate decrease in the expression levels of the transcription factor is detected

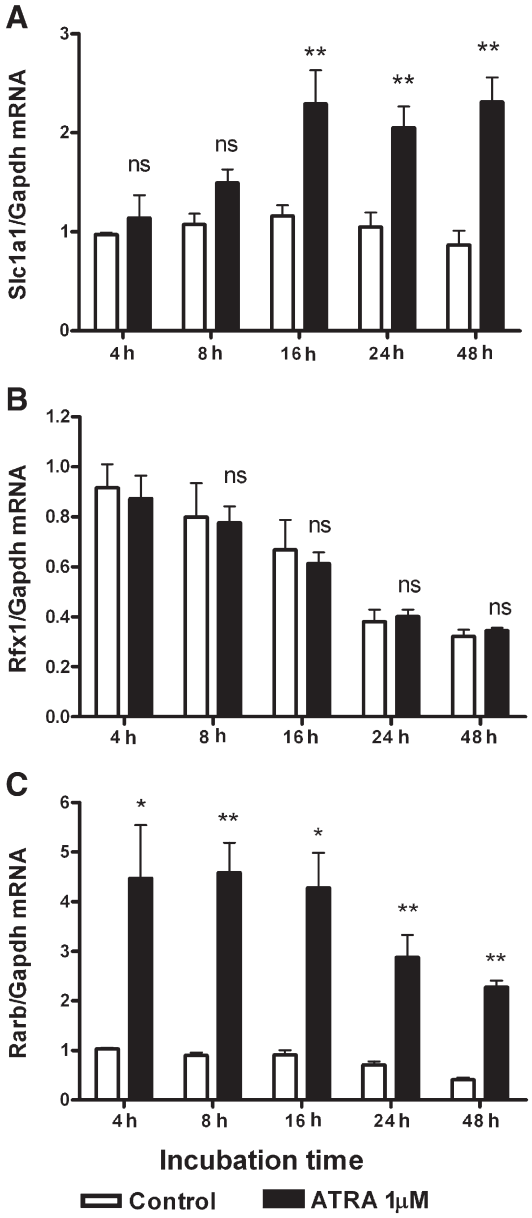

Fig. 5. ATRA effects on gene expression in C6 cells. C6 cells were incubated for the indicated times in the absence or in the presence of 1 μM ATRA. At the indicated times, RNA was extracted and the expression of the indicated genes (Panel A, Slc1a1; Panel B, Rfx1; Panel C, Rarb) was determined through RT-PCR. Data are expressed relatively to Gapdh mRNA abundance and are means of four determinations obtained in two distinct experiments. ns, not significant, *$P<0.05$, **$P<0.01$ vs. control, ATRA-untreated cells extracted at the same experimental time.

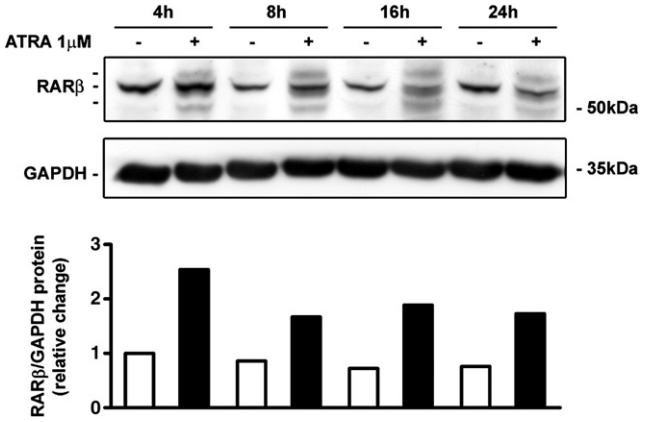

Fig. 6. Induction of RARβ by ATRA in C6 cells, C6 cells were incubated for the indicated times in the absence or in the presence of 1 μM ATRA. Cells were then lysed and the lysates were examined for the expression of RARβ. GAPDH expression was used as a loading control. The lower part of the figure reports the densitometric analysis of the results. The experiment was repeated twice with comparable results.

during the incubation at low serum, both in the absence and in the presence of the retinoid. Consistently, no increase in RFX1 protein is detected during ATRA treatment up to 72 h of treatment (data not shown).

As expected from literature [8], a rapid, ATRA-dependent induction of Rarb is instead detectable from the earliest time of treatment (4 h) and still observed, although at lower levels, after 48 h (Panel C). The fast induction of RARβ under our experimental conditions has been confirmed at protein level, through a Western Blot that, moreover, indicated that the induction of the receptor was an early change induced by ATRA. The primary antibody used in this experiment recognizes several RARβ isoforms and, consistently, at least two bands are induced during the treatment while a third band, already present under control conditions, is enhanced (Fig. 6). The densitometric analysis indicates that the maximal expression of the receptor is detected at 4 h to decrease at later times, although it is still clearly detectable at 24 h.

Under the same conditions no changes in RARα expression were detected either at mRNA or protein level (not shown).

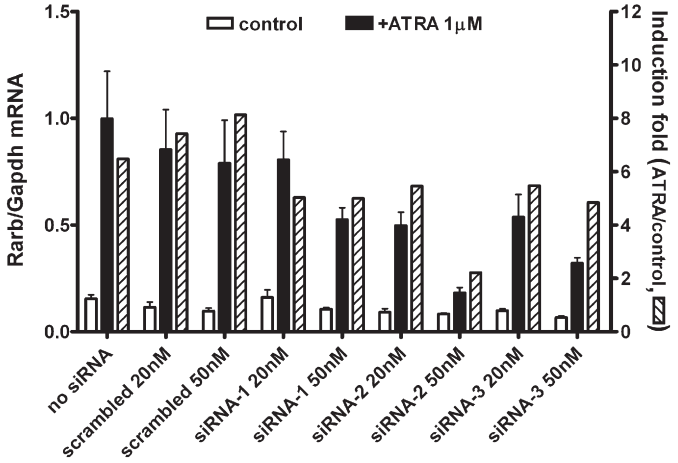

Fig. 7. Effects of anti-Rarb-siRNA on the ATRA-induced expression of Rarb. The culture medium of C6 cells was replaced with medium containing 10% FBS and the anti-Rarb siRNA/peptide or scrambled siRNA/peptide complexes (as indicated, see Methods). After 24 h, medium was renewed again with a fresh, siRNA-free medium, supplemented with 0.1% FBS, in the absence or in the presence of ATRA 1 μM. mRNA was extracted after 8 h and the abundance of Rarb and Gapdh mRNAs was determined with RT-PCR. Data of Rarb mRNA abundance are expressed relatively to Gapdh mRNA and are means of two determinations with SD in a representative experiment. The experiment was repeated twice with comparable results.

3.4. Slc1a1 induction is inhibited by Rarb silencing

To directly evaluate the role of RARβ in ATRA effects on Slc1a1, we used a silencing approach aimed to interfere with Rarb induction by ATRA. We preliminarily tested three siRNAs (1, 2, 3), targeted to Rarb1-2 mRNAs, at two different concentrations and found that scrambled siRNA did not affect the induction of Rarb caused by a 8h-incubation with ATRA. Rarb induction was instead markedly affected by one of the three anti-Rarb siRNAs used (siRNA-2). Rarb silencing by siRNA-2 was dose-dependent, with a 70% inhibition of ATRA-dependent induction observed with a concentration of 50 nM (Fig. 7). The inhibitions observed with the other two siRNAs were smaller and poorly dose-dependent. Attempts to use combinations of low concentrations of two siRNAs or prolongation of ATRA treatment over 24 h yielded smaller inhibitions than those observed with siRNA-2 alone after a 24h-ATRA treatment (not shown).

We then tested the effects of siRNA-2 on Slc1a1 induction by ATRA. In these experiments we had to perform ATRA treatment for 24 h so as to achieve a significant Slc1a1 induction. To maintain an effective silencing for this relatively long time, we decided to prolong the exposure to siRNA throughout ATRA treatment, raising the FBS concentration to 10% and ATRA concentration to 10 μM (see the legend to Fig. 8 for experimental details). Under these conditions, Rarb-silenced cells exhibited an effective inhibition of both Rarb and Slc1a1 ATRA-dependent induction (Fig. 8A and B). In cells treated with the scrambled siRNA, ATRA increased Slc1a1 expression by 140%

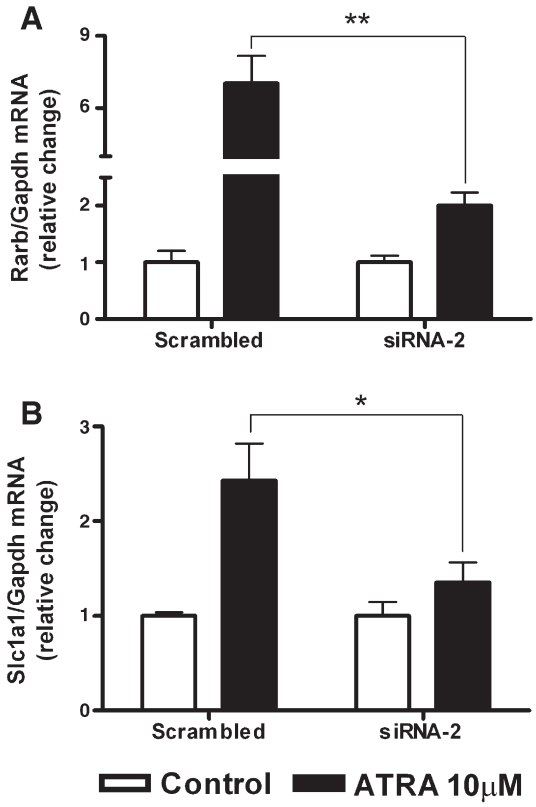

Fig. 8. Effect of RARβ silencing on the ATRA-induced expression of Slc1a1. The culture medium of C6 cells was replaced with serum free medium containing the anti-Rarb siRNA/peptide or scrambled siRNA, both at a concentration of 100 nM, and the incubation was prolonged for 4 h. An equal volume of medium supplemented with 20% FBS was then added to each culture so as to lower the siRNA concentration at 50 nM and to raise the FBS concentration to 10%. After 20 h, ATRA (10 μM) or vehicle was added to the incubation medium. mRNA was extracted after further 24 h of incubation and the abundance of Rarb (Panel A) and Slc1a1 (Panel B) was determined with RT-PCR. Data represent means of three determinations in a representative experiment repeated twice with comparable results.

(Fig. 8B), while the modest induction observed in Rarb-silenced cells (35%) was not significant.

4. Discussion

ATRA is a major regulator of gene expression in eukaryotes and the expression of several hundreds of genes is affected by this retinoid. However, only a portion of these genes are directly activated by the ATRA according to the classical mechanism of binding and activation of a heterodimeric complex of RAR–RXR receptors. In many other cases, ATRA works indirectly through several mechanisms, the best known of which involve the synthesis of factors that, conversely, target gene transcription or cause changes in messenger half-life. The results recounted in this contribution imply RARβ as the mediator of ATRA effects on EAAC1 transporter. This conclusion is based on several lines of evidence.

First, the pharmacological characterization of ATRA-dependent stimulation of EAAC1 activity (Fig. 1) or expression (Fig. 2) strongly suggests that one or more receptors of the RAR family are involved since the RAR-specific agonist Am80 fully mimicked ATRA effect. Interestingly, under the same conditions, the synthetic rexinoid HX630 does not increase aspartate transport and EAAC1 expression, but, in its presence, Am80 produces a greater stimulation than in its absence. This synergistic effect may be explained given that RXRs are unable to respond to rexinoids (RXR-selective agonists) in the absence of RAR ligands, since, under this condition, the dissociation of corepressors from the heterodimer does not occur [30]. However, the rexinoid receptor is not transcriptionally silent since RAR activation produces larger biological effects in the presence of a RXR ligand than in its absence, as originally demonstrated by Lotan et al. for the inhibition of cancer cell proliferation [31] and, more recently, by Idres et al. for the induction of CYP26A1 [32]. The prevalent involvement of a RAR in ATRA effects was also confirmed by data obtained with RAR or RXR antagonists (Fig. 1B and C).

Second, Rarb is induced before the stimulation of Slc1a1 expression, a finding consistent with its role as a transcription factor needed for the synthesis of the transporter. Indeed, cycloheximide suppresses Slc1a1 mRNA induction, indicating that the effect requires a protein intermediate, which may work either at transcriptional or at post-transcriptional level. However, ATRA treatment does not change the half-life of Slc1a1 mRNA compared with that of the housekeeping, ATRA-independent Gapdh (Fig. 3A). On the contrary, under the same conditions, the post-transcriptional effect of ATRA on another target gene, such as Plp [8], is readily observed as an increase in the messenger half-life (Fig. 3C). Similar effects have been detected in C6 cells for other ATRA target genes, such as β1-adrenergic receptor [33] and connexin 43 [34]. It is, therefore, likely that the protein intermediate required for Slc1a1 induction is a factor acting at the transcriptional level. A possible candidate is RFX1, since the expression of human RFX1 was found to stimulate Slc1a1 expression in C6 cells [5]. However, endogenous Rfx1 is not induced in C6 cells during ATRA treatment at both mRNA and protein level, thus rendering unlikely its involvement in ATRA-dependent Slc1a1 regulation. On the contrary, Rarb is a typical, direct ATRA target gene [9] and its induction is readily detected at early times of ATRA treatment of C6 cells. Moreover, also RARβ proteins are greatly induced at early ATRA treatment times so that high levels of these receptors are present when Slc1a1 expression is stimulated.

Third, and more importantly, Rarb silencing markedly inhibits the induction of the transporter. The silencing approach adopted here is quite effective, producing a marked fall in Rarb mRNA levels both in ATRA-treated and in control cells. In particular, Rarb induction is suppressed by almost 70%. The inhibitory effect on Slc1a1 is comparable, indicating a strong relationship between RARβ expression and Slc1a1 induction. The specificity of the inhibition is demonstrated by the smaller effect of the same protocol on the induction of Plp mRNA (not shown).

These results point to Slc1a1 as a RARβ-dependent ATRA target gene, although they do not allow to identify the precise mechanism underlying the effect. Indeed, it is possible that RARβ acts at genomic level, as a classical transcription factor, but the data presented would be also compatible with a non genomic effect of RARβ, as most recently demonstrated for ATRA stimulation of iodide transport in breast cancer cells [11]. Interestingly, two non repeating RARβ binding sites are present at −191 bp and at −2696 bp of the rat *Slc1a1* gene (as identified through TESS: Transcription Element Search Software on the WWW, URL: http://www.cbil.upenn.edu/tess). Similar sequences are present also in the mouse *Slc1a1* gene but not in the human counterpart. Further experiments are needed to characterize the role of these sequences, as well as to identify the RARβ isoform involved in Slc1a1 induction.

Acknowledgement

This study was supported by University of Parma, FIL.

References

[1] Y. Kanai, M.A. Hediger, The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects, Pflugers. Arch. 447 (2004) 469–479.
[2] A.V. Tzingounis, J.I. Wadiche, Glutamate transporters: confining runaway excitation by shaping synaptic transmission, Nat. Rev. Neurosci. 8 (2007) 935–947.
[3] P.M. Beart, R.D. O'Shea, Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement, Br. J. Pharmacol. 150 (2007) 5–17.
[4] W. Yang, M.S. Kilberg, Biosynthesis, intracellular targeting, and degradation of the EAAC1 glutamate/aspartate transporter in C6 glioma cells, J. Biol. Chem. 277 (2002) 38350–38357.
[5] K. Ma, S. Zheng, Z. Zuo, The transcription factor regulatory factor X1 increases the expression of neuronal glutamate transporter type 3, J. Biol. Chem. 281 (2006) 21250–21255.
[6] M.G. Bianchi, G.C. Gazzola, L. Tognazzi, O. Bussolati, C6 glioma cells differentiated by retinoic acid overexpress the glutamate transporter excitatory amino acid carrier 1 (EAAC1), Neuroscience 151 (2008) 1042–1052.
[7] W. Zhu, M. Kanoh, P. Ye, I. Laszkiewicz, J.E. Royland, R.C. Wiggins, G. Konat, Retinoic acid-regulated expression of proteolipid protein and myelin-associated glycoprotein genes in C6 glioma cells, J. Neurosci. Res. 31 (1992) 745–750.
[8] M. Lopez-Barahona, M. Minano, E. Mira, T. Iglesias, H.G. Stunnenberg, A. Rodriguez-Pena, J. Bernal, A. Munoz, Retinoic acid posttranscriptionally up-regulates proteolipid protein gene expression in C6 glioma cells, J. Biol. Chem. 268 (1993) 25617–25623.
[9] J.E. Balmer, R. Blomhoff, Gene expression regulation by retinoic acid, J. Lipid Res. 43 (2002) 1773–1808.
[10] P. Lefebvre, P.J. Martin, S. Flajollet, S. Dedieu, X. Billaut, B. Lefebvre, Transcriptional activities of retinoic acid receptors, Vitam. Horm. 70 (2005) 199–264.
[11] E. Ohashi, T. Kogai, H. Kagechika, G.A. Brent, Activation of the PI3 kinase pathway by retinoic acid mediates sodium/iodide symporter induction and iodide transport in MCF-7 breast cancer cells, Cancer Res. 69 (2009) 3927–3936.
[12] P. Germain, P. Chambon, G. Eichele, R.M. Evans, M.A. Lazar, M. Leid, A.R. De Lera, R. Lotan, D.J. Mangelsdorf, H. Gronemeyer, International Union of Pharmacology. LX. Retinoic acid receptors, Pharmacol. Rev. 58 (2006) 712–725.
[13] P. Germain, P. Chambon, G. Eichele, R.M. Evans, M.A. Lazar, M. Leid, A.R. De Lera, R. Lotan, D.J. Mangelsdorf, H. Gronemeyer, International Union of Pharmacology. LXIII. Retinoid X receptors, Pharmacol. Rev. 58 (2006) 760–772.
[14] M. Mark, N.B. Ghyselinck, P. Chambon, Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis, Annu. Rev. Pharmacol. Toxicol. 46 (2006) 451–480.
[15] P. Dolle, E. Ruberte, P. Leroy, G. Morriss-Kay, P. Chambon, Retinoic acid receptors and cellular retinoid binding proteins. I. A systematic study of their differential pattern of transcription during mouse organogenesis, Development 110 (1990) 1133–1151.
[16] A. Zelent, C. Mendelsohn, P. Kastner, A. Krust, J.M. Garnier, F. Ruffenach, P. Leroy, P. Chambon, Differentially expressed isoforms of the mouse retinoic acid receptor beta generated by usage of two promoters and alternative splicing, EMBO J. 10 (1991) 71–81.
[17] P. Reboul, P. George, P. Louisot, P. Broquet, Study of retinoic acid effect upon retinoic acid receptors beta (RAR-beta) in C6 cultured glioma cells, Biochem. Mol. Biol. Int. 36 (1995) 1097–1105.
[18] H. Kagechika, E. Kawachi, Y. Hashimoto, T. Himi, K. Shudo, Retinobenzoic acids. 1. Structure–activity relationships of aromatic amides with retinoidal activity, J. Med. Chem. 31 (1988) 2182–2192.

[19] H. Umemiya, H. Fukasawa, M. Ebisawa, L. Eyrolles, E. Kawachi, G. Eisenmann, H. Gronemeyer, Y. Hashimoto, K. Shudo, H. Kagechika, Regulation of retinoidal actions by diazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterodimers, J. Med. Chem. 40 (1997) 4222–4234.
[20] M. Ebisawa, H. Umemiya, K. Ohta, H. Fukasawa, E. Kawachi, G. Christoffel, H. Gronemeyer, M. Tsuji, Y. Hashimoto, K. Shudo, H. Kagechika, Retinoid X receptor-antagonistic diazepinylbenzoic acids, Chem. Pharm. Bull. (Tokyo) 47 (1999) 1778–1786.
[21] S. Rozen, H. Skaletsky, Primer3 on the WWW for general users and for biologist programmers, Methods Mol. Biol. 132 (2000) 365–386.
[22] S.A. Bustin, Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays, J. Mol. Endocrinol. 25 (2000) 169–193.
[23] C.M. Farinha, F. Mendes, M. Roxo-Rosa, D. Penque, M.D. Amaral, A comparison of 14 antibodies for the biochemical detection of the cystic fibrosis transmembrane conductance regulator protein, Mol. Cell. Probes 18 (2004) 235–242.
[24] C. Nervi, T.M. Vollberg, J.F. Grippo, D.A. Lucas, M.D. George, M.I. Sherman, K. Shudo, A.M. Jetten, Expression of nuclear retinoic acid receptors in wild-type and mutant embryonal carcinoma PCC4.aza1R cells, Cell. Growth Differ. 1 (1990) 535–542.
[25] M. Lansink, T. Kooistra, Stimulation of tissue-type plasminogen activator expression by retinoic acid in human endothelial cells requires retinoic acid receptor beta 2 induction, Blood 88 (1996) 531–541.
[26] Q. Ma, Induction and superinduction of 2,3,7,8-tetrachlorodibenzo-rho-dioxin-inducible poly(ADP-ribose) polymerase: role of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator transcription activation domains and a labile transcription repressor, Arch. Biochem. Biophys. 404 (2002) 309–316.
[27] D.D. Hershko, B.W. Robb, C.J. Wray, G.J. Luo, P.O. Hasselgren, Superinduction of IL-6 by cycloheximide is associated with mRNA stabilization and sustained activation of p38 map kinase and NF-kappaB in cultured caco-2 cells, J. Cell. Biochem. 91 (2004) 951–961.
[28] A. Joiakim, P.A. Mathieu, A.A. Elliott, J.J. Reiners Jr., Superinduction of CYP1A1 in MCF10A cultures by cycloheximide, anisomycin, and puromycin: a process independent of effects on protein translation and unrelated to suppression of aryl hydrocarbon receptor proteolysis by the proteasome, Mol. Pharmacol. 66 (2004) 936–947.
[29] M. Zajac-Kaye, N. Ben-Baruch, E. Kastanos, F.J. Kaye, C. Allegra, Induction of Myc-intron-binding polypeptides MIBP1 and RFX1 during retinoic acid-mediated differentiation of haemopoietic cells, Biochem. J. 345 (Pt. 3) (2000) 535–541.
[30] S. Westin, R. Kurokawa, R.T. Nolte, G.B. Wisely, E.M. McInerney, D.W. Rose, M.V. Milburn, M.G. Rosenfeld, C.K. Glass, Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators, Nature 395 (1998) 199–202.
[31] R. Lotan, M.I. Dawson, C.C. Zou, L. Jong, D. Lotan, C.P. Zou, Enhanced efficacy of combinations of retinoic acid- and retinoid X receptor-selective retinoids and alpha-interferon in inhibition of cervical carcinoma cell proliferation, Cancer Res. 55 (1995) 232–236.
[32] N. Idres, J. Marill, G.G. Chabot, Regulation of CYP26A1 expression by selective RAR and RXR agonists in human NB4 promyelocytic leukemia cells, Biochem. Pharmacol. 69 (2005) 1595–1601.
[33] M. Lopez-Barahona, T. Iglesias, I. Garcia-Higuera, F. Mayor Jr., A. Zaballos, J. Bernal, A. Munoz, Post-transcriptional induction of beta 1-adrenergic receptor by retinoic acid, but not triiodothyronine, in C6 glioma cells expressing thyroid hormone receptors, Eur. J. Endocrinol. 135 (1996) 709–715.
[34] X. Zhang, Z. Ren, J. Zuo, C. Su, R. Wang, Y. Chang, F. Fang, The effect of all-trans retinoic acid on gap junctional intercellular communication and connexin 43 gene expression in glioma cells, Chin. Med. Sci. J. 17 (2002) 22–26.