RESEARCH COMMUNICATION

Retinoic acid regulates ornithine decarboxylase gene expression at the transcriptional level

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Retinoic acid (RA) is important for normal mammalian development and growth. Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the biosynthesis of the polyamines, and we have previously shown that ODC mRNA levels are suppressed by RA in human skin cells. Using HeLa cells, we now show that treatment with 0.5 μ M RA for 24 h suppresses endogenous ODC mRNA levels and the expression of a transfected ODC/chloramphenicol acetyltransferase plasmid (Kpn-ODCCAT), containing sequences from –1450 to +810 of the human ODC gene. Co-transfection with either the α -RA receptor (α -RAR) or a chimeric α -RA/oestrogen receptor (α -RAER) followed by treatment with the cognate hormone suppresses expression of Kpn-ODCCAT and Not-ODCCAT, which con-

tains sequences from –250 to +514. Liganded α -RAR suppresses the activity of Kpn-ODCCAT more markedly than does liganded α -RAER (98 % and 80 % suppression, respectively), whereas both receptors have very similar effects on Not-ODCCAT expression (73 % and 67 % suppression, respectively). The unliganded α -RAR suppresses Kpn-ODCCAT by 76 %, whereas unliganded α -RAER has no significant effect. These data show that RA regulates ODC-gene expression at the transcriptional level, and that α -RAR, but not α -RAER, can confer full hormonal responsiveness. This suggests that the activating function present in the α -RAR ligand-binding domain is required for full transcriptional regulation.

INTRODUCTION

Retinoids exert profound effects on mammalian development and cell growth; depending on the tissue, they can be either proliferative or anti-proliferative (for review, see [1]). Their actions are predominantly mediated by the various nuclear retinoic acid (RA) receptor (RAR) and retinoid X receptor (RXR) subtypes. Expression of receptor subtype isoforms is tissue- and cell-specific [2], and both RAR and RXR homo- and hetero-dimers can modulate gene transcription [3].

In skin cells, RA is anti-proliferative and decreases the activity of ornithine decarboxylase (ODC; EC 4.1.1.17), whose levels closely correlate with the growth state of the cell [4], and which has recently been identified as a proto-oncogene [5]. ODC is the first and rate-limiting enzyme in the biosynthesis of the polyamines, which are essential for cell growth [6]. We have shown that in human skin cells, retinoids decrease expression of ODC mRNA [7]. To examine one of the pathways used by RA, we have studied its effect on human ODC-gene expression in HeLa cells. We have found that RA suppresses endogenous ODC mRNA levels, and the activity of two chimeric ODC-gene/chloramphenicol acetyltransferase (ODCCAT) reporter plasmids, thus showing that RA acts at the transcriptional level. Full suppression is dependent on sequences present in the α -RAR, but not in a chimeric α -RA/oestrogen receptor (α -RAER). These data demonstrate that one important component of the action of RA is to suppress ODC-gene transcription, and that the *trans*-activation function in the ligand-binding domain of the α -RAR is necessary for full suppression.

METHODS

Reagents

All-*trans*-retinoic acid (RA), oestradiol (E_2) and all components of the medium were purchased from Sigma Biochemical Co. (St. Louis, MO, U.S.A.).

Plasmid construction

The *KpnI*–*HindIII* or the *NorI* fragment of the human ODC gene (–1450 to +810 or –250 to +514, respectively) [8] was inserted by using linkers into the *HindIII* site of SV0CAT (Figure 1a). The α -RAER was constructed by introducing a *XhoI* site at the junction of the C/D domain into the human α -RAR [9], and ligating the 5'-fragment of the α -RAR with the 3'-*XhoI* fragment of the human oestrogen receptor hER28 [10] (Figure 1b), followed by subcloning into the *EcoRI* site of pSG5 [11], and was kindly supplied by Dr. P. Chambon (Strasbourg, France). All plasmids were purified through two CsCl gradients.

Cell culture and transfection

HeLa and COS-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % newborn-calf serum, and were transfected by the calcium phosphate procedure, by using a total of 30 μ g of supercoiled DNA. Generally, 25 μ g of reporter plasmid/60 cm² plate was transfected with 0.3 μ g of α -RAER or α -RAR and 6 μ g of Rous Sarcoma Virus promoter/ β -galactosidase (RSV- β gal) to normalize the transfection efficiencies. Cells were treated with 0.5 μ M RA, 10 nM E_2 , or ethanol

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's Modified Eagle's Medium; E_2 , oestradiol; ODC, ornithine decarboxylase; RA, all-*trans*-retinoic acid; α -RAER, α -retinoic acid/oestrogen receptor; RAR, retinoic acid receptor; RSV- β gal, Rous Sarcoma Virus promoter/ β -galactosidase plasmid; RXR, retinoid X receptor.

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Figure 1 Maps of ODCCAT and receptor constructs

(a) The human ODC gene [8] and the human ODCCAT gene constructs are shown. Kpn-ODCCAT extends from the *KpnI* (K) site at -1450 to the *HindIII* (H) site at $+810$; Not-ODCCAT contains the *NotI* (N) fragment spanning -250 to $+514$, ligated into the *HindIII* site of SVOCAT. (b) Construction of the α -RAER from the human α -RAR [9] and the human oestrogen receptor hER28 [10]. A *XhoI* site was created at the junction of the C/D domain in the α -RAR cDNA, and the *EcoRI/XhoI* 5'-fragment ligated to the *XhoI/EcoRI* fragment of hER28 and subcloned into pSG5 [11].

alone ($< 0.1\%$) in Methyl Red-free DMEM containing 10% newborn-calf serum (charcoal-stripped) after transfection; fresh agent in fresh medium was again added at 12 h. At 24 h, cells were harvested, lysed by three freeze/thaw cycles, and β -galactosidase activity was determined spectrophotometrically [12]. CAT activity was measured with [3 H]chloramphenicol (New England Nuclear) and butyryl-CoA (Boehringer Mannheim Biochemicals) as described by Seed and Sheen [13].

Statistics

Significance of differences between treated and untreated cultures was determined by Student's *t* test.

RNA Isolation and Northern blotting

RNA was isolated by the guanidinium isothiocyanate/CsCl method, and fractionated on formaldehyde gels [7]. Blots were hybridized to the human ODC cDNA, pODC10/2H [14], or to the rat cyclophilin cDNA IB15 [15], to correct for variability in RNA between lanes. Hybridizations, washings, and quantification by laser-scanning densitometry were performed as described previously [7].

RESULTS AND DISCUSSION

We have previously shown that RA decreases ODC mRNA levels in cultured normal human epidermal cells [7]. RA is also anti-proliferative in HeLa cells, which, like human skin [16],

express predominantly the α - and γ -RARs [17], and we have therefore used HeLa cells to dissect the mechanisms governing the response of ODC-gene expression to RA.

We first assessed the effect of RA on endogenous ODC gene expression in HeLa cells. Cultures were treated with $0.5\ \mu\text{M}$ RA for 24 h, and ODC mRNA levels were determined by Northern blotting (Figure 2). We found that ODC mRNA levels were decreased by 50% by RA, and slightly increased (1.5–2-fold) by E_2 ($10\ \text{nM}$), the ligand for the chimeric RA/ E_2 receptor (α -RAER). Therefore, RA decreased ODC mRNA levels in HeLa cells to the same extent as in normal human skin cells [7].

To determine if the effects of RA on ODC mRNA levels were exerted at the transcriptional level, two ODCCAT (Figure 1) constructs were transfected into HeLa cells and tested for RA responsiveness. Treatment of HeLa cells transfected with Kpn-ODCCAT or with Not-ODCCAT with $0.5\ \mu\text{M}$ RA for 24 h decreased CAT expression by 58% and 27% , respectively (Table 1), demonstrating that RA suppressed ODCCAT gene transcription through multiple ODC DNA-sequence elements. Kpn-ODCCAT contained all sequences necessary for RA responsiveness. E_2 treatment ($10\ \text{nM}$, 24 h) suppressed both Kpn- and Not-ODCCAT expression to a similar extent (by 33% and 26% respectively). HeLa cells have been reported to show undetectable amounts of E_2 receptor, as determined by E_2 -binding assays [18]. Therefore, the origin of the small E_2 effects is unknown.

HeLa cells express multiple RAR subtypes [17]. To simplify our study by examining regulation by a single subtype, we co-transfected plasmids expressing the α -RAER or α -RAR with ODCCAT plasmids. The α -RAER contains the variable and the DNA-binding domains of the α -RAR, and the hinge and

Figure 2 Effect of RA on ODC mRNA levels

HeLa cells were treated with $0.5\ \mu\text{M}$ RA or $10\ \text{nM}$ E_2 for 24 h, RNA was isolated, and ODC and cyclophilin mRNA levels were determined by Northern blotting. C, control.

Table 1 Effect of RA and E_2 on CAT activity in HeLa cells

HeLa cells were transfected with Kpn- or Not-ODCCAT and treated for 24 h before harvesting. CAT activity was determined and, after normalization to β -galactosidase activity, was expressed relative to CAT activity in cultures treated with vehicle alone. Results are means $\pm$ S.D. ($n = 8$ –15). Significance: * $0.05 > P > 0.01$; ** $0.005 > P > 0.001$.

CAT construct	CAT activity (%)		
	Vehicle	$0.5\ \mu\text{M}$ RA	$10\ \text{nM}$ E_2
Kpn-ODCCAT	100.0 ± 30.2	$41.9 \pm 9.3^{**}$	$66.4 \pm 17.9^*$
Not-ODCCAT	100.0 ± 5.4	$72.8 \pm 10.8^{**}$	$74.5 \pm 15.9^{**}$

Table 2 Suppression of ODCCAT activity in HeLa cells by co-transfected α -RAR or α -RAER

HeLa cells were transfected with the indicated plasmids and treated for 24 h before harvesting. CAT activity was determined, normalized to β -galactosidase activity, and expressed as a percentage of control CAT activity. Results are means $\pm$ S.D. ($n = 9-12$). Significance: **0.005 > P > 0.001.

Treatment	CAT activity (%)	
	Kpn-ODCCAT	Not-ODCCAT
Vehicle	100.0 $\pm$ 8.2	100.0 $\pm$ 5.4
10 nM E ₂	77.1 $\pm$ 24.5	74.5 $\pm$ 15.9
α -RAER	74.4 $\pm$ 23.1	96.4 $\pm$ 18.2
α -RAER + 10 nM E ₂	20.7 $\pm$ 12.9**	33.5 $\pm$ 12.8**
Vehicle	100.0 $\pm$ 16.6	100.0 $\pm$ 2.9
0.5 μ M RA	50.1 $\pm$ 23.3**	72.8 $\pm$ 10.8**
α -RAR	23.6 $\pm$ 16.2**	67.0 $\pm$ 16.8**
α -RAR + 0.5 μ M RA	2.2 $\pm$ 2.5**	27.6 $\pm$ 10.2**

hormone-binding domains of the E₂ receptor (Figure 1). Therefore, the α -RAER binds DNA at α -RAR response elements, but is activated by E₂. Although receptor over-expression can suppress gene transcription [19], we found that Kpn-ODCCAT was not significantly affected by expression of the unliganded α -RAER at the level of 0.5 μ g of α -RAER/100 mm² plate.

We first asked whether α -RAER + E₂ suppressed ODCCAT-gene expression to the same extent as did RA. HeLa cells were co-transfected with Kpn- or Not-ODCCAT and α -RAER, and treated with 10 nM E₂ or with vehicle alone (Table 2). E₂ treatment resulted in suppression of Kpn-ODCCAT and Not-ODCCAT expression to 21% and 33% of control levels respectively, suggesting that the *NotI* fragment of the human ODC gene contained all DNA sequences necessary for suppression by α -RAER + E₂, but that additional sequences present only in the *Kpn* fragment were required for a full response to RA (compare Table 1 with Table 2).

Because the response of the ODCCAT constructs to α -RAER + E₂ was different from the endogenous response to RA, we tested both ODCCAT constructs with transfected α -RAR with or without RA. HeLa cells were co-transfected with either Kpn- or Not-ODCCAT and α -RAR, and cells were treated with 0.5 μ M RA or vehicle for 24 h (Table 2). Surprisingly, the unliganded α -RAR, at concentrations at which no effect was observed with the unliganded α -RAER, suppressed Kpn-ODCCAT activity by 76%, and Not-ODCCAT activity by 33%. RA treatment further suppressed the activity of Kpn-ODCCAT and Not-ODCCAT to 2% and 28% of control levels respectively. Thus Kpn-ODCCAT and Not-ODCCAT respond differently to α -RAR and α -RAER, in both their liganded and unliganded states. Furthermore, Kpn-ODCCAT appears to contain a DNA sequence that responds to α -RAR, but not to α -RAER.

Suppression by the unliganded α -RAR could be caused by dimerization with an RXR [3]. However, since the E₂ receptor is not known to heterodimerize with other members of the steroid-hormone receptor superfamily, we would not expect the α -RAER to form heterodimers. To test if the α -RAR or α -RAER reproduced these effects in cells with very low endogenous retinoid receptor amounts [20], we co-transfected COS-1 cells with Kpn-ODCCAT and either α -RAER or α -RAR. A small but significant suppression was seen with the α -RAER, but no suppression was seen with the α -RAR (Table 3). Therefore,

Table 3 Suppression of ODCCAT activity by unliganded α -RAER and α -RAR in HeLa and in COS-1 cells

Cells were co-transfected with Kpn-ODCCAT and the indicated receptor plasmid, and treated for 24 h. CAT activity was determined, normalized to β -galactosidase activity, and expressed as percentage of control CAT activity. Results are means $\pm$ S.D. ($n = 9-21$); N.D., not determined in these experiments. Significance: **0.01 > P > 0.005.

Transfected plasmid/treatment	CAT activity (%)	
	HeLa	COS-1
Kpn-ODCCAT		
+ vehicle	100.0 $\pm$ 16.0	100.0 $\pm$ 4.2
+ 0.5 μ M RA	N.D.	105.0 $\pm$ 12.2
+ 0.3 μ g of α -RAER	93.0 $\pm$ 14.3	80.9 $\pm$ 9.1**
+ 0.3 μ g of α -RAR	54.9 $\pm$ 14.3**	104.8 $\pm$ 13.3
+ 0.3 μ g of α -RAR + 0.5 μ M RA	N.D.	106.6 $\pm$ 18.2

additional proteins present in HeLa cells are necessary for ODC regulation by the α -RAR.

Taken together, these data show complex regulation of ODC-gene expression by retinoids at the transcription level. In HeLa cells, the α -RAR and α -RAER both cause ligand-dependent suppression of ODCCAT-gene expression, which parallels the response of endogenous ODC-gene expression to RA. We postulate a strongly RA-responsive site in Kpn-ODCCAT which is not found in Not-ODCCAT, with a more weakly responsive site, which can only fully respond in the presence of transfected receptors, in Not-ODCCAT.

Interestingly, suppression by unliganded α -RAR is not reproduced with the α -RAER, and is not reproduced in COS-1 cells. In the RARs [3], E₂ receptor and glucocorticoid receptor, a *trans*-activation function is localized to the same region as is important for receptor dimerization, and can influence target gene sensitivity (for review, see [21]). Our data suggest that the specific activating function in the α -RAR is important for full regulation of ODC-gene transcription; only partial regulation can be achieved by replacing this with the activating function of the E₂ receptor present in the α -RAER. This regulation is dependent on proteins which are present in HeLa cells, but not present in COS-1 cells. Determination of the specific receptor isoforms that are involved in ODC regulation and the sequences of their response elements in the ODC gene will begin to delineate critical components of the pathways which are activated in the control of cell growth by RA.

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