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Didehydroretinoic Acid: Retinoid Receptor-Mediated Transcriptional Activation and Binding Properties

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ABSTRACT. All-*trans*-3,4-Didehydroretinoic acid (vitamin A_2 acid; DDRA) is one of the retinoids present in human skin, the most responsive tissue to retinoid treatment. To understand the mechanism of action of DDRA in the control of differentiation and tumorigenesis, we studied its interaction with cellular retinoic acid-binding proteins (CRABPs) and nuclear all-*trans*-retinoic acid (RA) receptors (RARs), and 9-*cis*-retinoic acid receptors (RXRs). The IC_{50} plots of DDRA for inhibition of [^{3}H]RA binding to CRABP I and II and to RARα, β and γ illustrate that this retinoid binds with the same affinity as RA to these proteins. DDRA, however, showed higher affinity than RA for RXRα. Evaluation of the transcriptional activation potential of DDRA in CV-1 cells showed that this retinoid induced RARα-mediated transcription to the same magnitude as RA in the 10^{-9} to 10^{-6} M concentration range. However, in comparison to RA, DDRA produced a 2- to 3-fold higher activation of the transcription mediated by RXRα homodimers, as well as RARβ–RXRα heterodimers. These results suggest that the biological activity of retinoids in the skin may be attained through the joint potential of both RA and DDRA. BIOCHEM PHARMACOL 53;7:1049–1053, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. DDRA; CRABPs; RARs; RXRs; IC_{50}; transcription

Certain retinoids, both naturally occurring and synthetic, function as important regulatory signaling molecules for cell growth and differentiation. Additionally, these compounds have been shown to inhibit or reverse the process of malignant transformation in certain cell types [1, 2]. In this group of retinoids, RA[§], the oxidized form of retinol, is generally considered as the compound with the highest activity in the above-mentioned biological processes. Its effectiveness as a clinical agent in the treatment of promyelocytic leukemia [3] and juvenile chronic myelogenous leukemia [4] has been demonstrated. Even though the mechanisms underlying the biological action(s) of RA are not well understood, the evidence to date indicates that two types of nuclear RA receptors, RARs and RXRs, may be involved in the RA-signaling pathway [1, 2, 5]. High-affinity CRABPs that are present predominantly in the cytosol may also function to modulate the effects of RA within the cells [6, 7]. The RARs, RXRs, and CRABPs have different patterns of expression during vertebrate embryogenesis [1, 8], suggesting that they each have particular functions. Another dimension at which the retinoid action could be fine-tuned is at the level of metabolism where vitamin A generates several active metabolites such as RA, all-*trans*-4-oxo-RA, 14-hydroxy-4,14-retroretinol [9], DDRA [10, 11] and 9-*cis*-RA [12]. 4-Oxo-RA, which was once considered to be a detoxification product of RA [13], is now thought to be a highly active compound that can modulate positional specification in early embryos [14].

Thus far, RA and 9-*cis*-RA have been recognized as the likely endogenous ligands for the nuclear retinoid receptors [1, 2, 5, 8]. Vitamin A-dependent epithelial cells have been shown to exhibit diverse cellular responses to retinoids, and are capable of utilizing the wide range of biologically active metabolic products of retinol. Quantitative studies of vitamin A in human tissues have revealed the presence of didehydroretinol (vitamin A_2), which constitutes 20–25% of the total retinoid content of human epidermis [10]. In addition, Thaller and Eichele [11] reported that DDRA is present at about a 5-fold higher concentration than RA in chick limb. The levels of 3,4-didehydroretinol, the precursor of DDRA [11, 15], are elevated markedly in multiple skin disorders, such as eczema, psoriasis, and basal cell carcinoma [16, 17]. Moreover, both *in vitro* and *in vivo* studies have shown that DDRA has biological potency similar to that of RA [18–20]. In our attempts to understand the mechanism of action of DDRA in the modulation of cellular differentiation, we have investigated in the present study its binding affinities to CRABPs, RARs, and RXRs as well as its role in the transcriptional activation mediated by RARs and RXRs.

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§ *Abbreviations*: RA, all-*trans*-retinoic acid; DDRA, all-*trans*-3,4-didehydroretinoic acid; 9-*cis*-RA, 9-*cis*-retinoic acid; RAR, retinoic acid receptor; RXR, 9-*cis*-retinoic acid receptor; and CRABP, cellular retinoic acid-binding protein.

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MATERIALS AND METHODS

RA and DDRA were gifts from Hoffmann–La Roche, Inc. (Nutley, NJ). [11,12-^{3}H]RA (40–60 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). CRABP I and II, RARs, and RXRs were prepared from COS-1 cells that were transfected by electroporation with pSG5-based expression plasmids carrying cDNA for mouse CRABP I or II, or human RARs or RXRs [21, 22]. The transfected cells were transferred to 150-mm plates containing Dulbecco's modified Eagle's medium (DMEM) and were harvested in 2–3 days. The cytosolic fractions from the cellular extracts contained recombinant CRABP I or II in large quantities. Nucleosols from the transfected cells were used in studies describing the ligand interactions with RARs and RXRs.

For determination of the competitive binding, portions of cytosol (100 μg of protein) or nucleosol (100 μg of protein) were incubated with [^{3}H]RA in the presence or absence of increasing concentrations of unlabeled competing ligand. For all binding assays, ligands bound to CRABP, RAR, or RXR were separated from free radioactivity by use of Sephadex PD-10 columns. Binding in the presence of 200-fold molar excess of unlabeled ligand was defined as nonspecific. The IC_{50} values were determined by plotting the data as percent specific binding versus the negative log of the concentration of the competing ligands [21, 23].

A modification of the calcium phosphate precipitation procedure was used for transient transfection, which is based on previously described methods [21–23]. Briefly, CV-1 cells (5×10^4 cells/mL/per well in 24-well plates), grown in DMEM containing 10% charcoal-treated fetal bovine serum, were cotransfected with plasmid DNAs consisting of: (i) 100 ng of TREpal-tk-CAT reporter vector; (ii) 100 ng of RAR cDNA (α, β or γ) and/or RXR cDNA (α, β, or γ) expression vector(s), and (iii) 150 ng of β-galactosidase cDNA (pCH110) vector. DNA–$CaPO_4$ precipitates were prepared by the successive addition of 25 μL of freshly prepared 2 × BBS [containing 50 mM *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 6.95] and 2.5 μL of 2.5 M $CaCl_2$ to DNA to a final volume of 50 μL. After 5–10 min at room temperature, to allow for the formation of precipitates, the cells were exposed to the DNA–$CaPO_4$ mixture overnight in a 4.0% CO_2 incubator.

The cells, after incubation for 24–48 hr in the presence of RA or DDRA (10^{-9}–10^{-6} M) with proper controls, were lysed in 0.25 mL of lysis buffer (0.5% Triton X-100 in 100 mM Tris–HCl, pH 7.8). After low-speed centrifugation of the plates, portions (50 μL) of the supernatants were mixed with [^{3}H]acetyl-CoA and chloramphenicol in Tris–HCl buffer, pH 7.8. Following a 2-hr incubation at 37°, the CAT reaction was terminated by the addition of 7 M urea. Following the addition of 4 mL of toluene-based scintillation fluid, radioactivity of the acetylated chloramphenicol (partitioned into the organic phase) was determined. For β-galactosidase activity, 50 μL of the supernatants was mixed with 200 μL of incubation buffer (100 mM sodium phosphate, pH 7.3, 100 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 4 mg/mL *O*-nitrophenyl-β-D-galactoside), and after a 1–2 hr incubation at 37°, the enzyme activity in the samples was measured from the absorbance at 405 nm. To account for the variation in the transfections, the values for β-galactosidase activity were used to normalize the CAT activity obtained from the same extracts [21–23]. The mean values of data obtained from two separate experiments with less than 10% variation are expressed as the experimental results in both the binding studies and the transfection assays.

RESULTS AND DISCUSSION

We evaluated the binding affinities of DDRA to the CRABPs and RARs/RXRs and compared them with those exhibited by RA for the same proteins. Addition of a 100-fold molar excess of DDRA, similar to that observed with RA, resulted in a 100% inhibition of [^{3}H]RA binding to both CRABPs (I and II) and RARα, β, and γ, indicating that both DDRA and RA possessed at least equal binding affinity for these proteins (data not shown). To make a more definitive assessment of the binding affinity of DDRA for CRABPs and RARs/RXRs, we determined the IC_{50} values of both RA and DDRA (Table 1). For CRABP I and II, both RA and DDRA showed IC_{50} values in the range of 300 nM. Similarly, as evidenced from the IC_{50} values, the two retinoids also expressed almost identical binding patterns with various RARs and RXRs (Table 1).

The IC_{50} plots in Fig. 1 illustrate the competition shown by DDRA for [^{3}H]RA binding to RARα, β, and γ. Both RA and DDRA expressed IC_{50} values between 5.0 and 7.2 nM for RARα, β, and γ (Table 1; Fig. 1). As with RA, DDRA did not show any significant receptor-specific differences in binding to these three RAR subtypes. Both RA and DDRA were, in general, poor binders of RXRs. However, DDRA showed a relatively higher affinity for RXRα than RA as reflected in the IC_{50} values of 6,250 and >10,000 nM, respectively. With RXRβ and γ, both retinoids expressed similar IC_{50} values of >10,000 nM.

TABLE 1. IC_{50} Concentrations for the binding of RA and DDRA to CRABP (I and II) and to RAR/RXR (α, β, and γ)

	IC_{50} (nM)	
Receptor protein	**RA**	**DDRA**
CRABP I	285	295
CRABP II	310	330
RARα	5.7	6.0
RARβ	5.0	5.7
RARγ	7.2	7.1
RXRα	>10,000	6,250
RXRβ	>10,000	>10,000
RXRγ	>10,000	>10,000

Data represent the mean values obtained from two independent experiments.

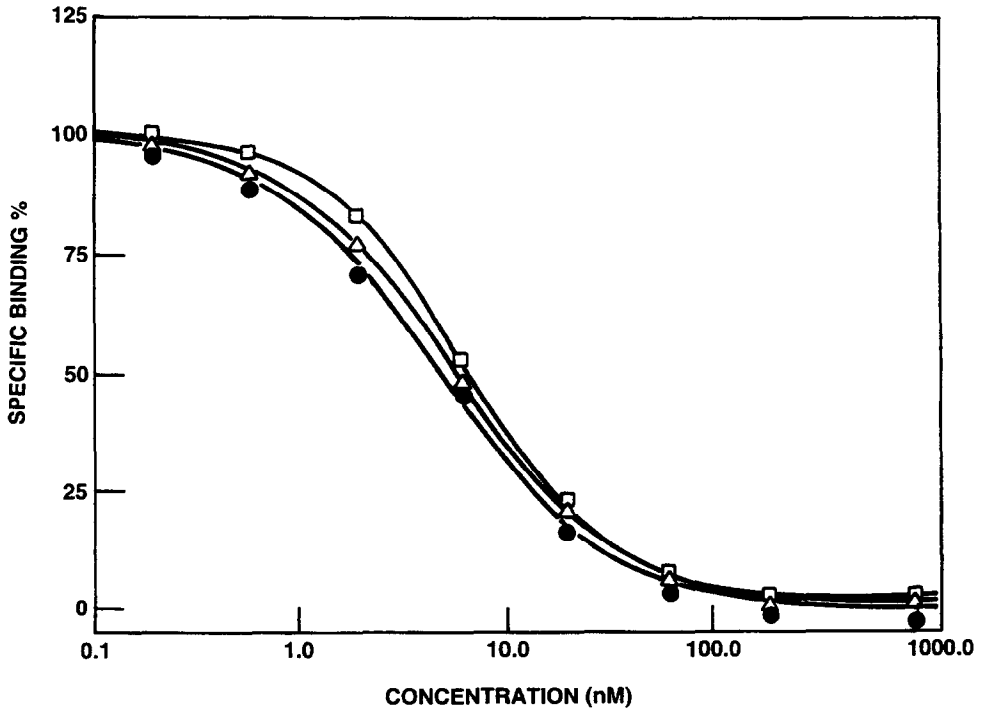

FIG. 1. Competition for [^{3}H]RA (5 nM) binding on RARα (△), β (●), and γ (□) by increasing concentrations of DDRA. Mean values of two independent experiments are plotted in the graph.

Almost all of the naturally occurring RAs, such as 4-oxo-RA, 4-hydroxy-RA, DDRA, and 13-*cis*-RA, have been shown to exhibit some degree of binding affinity for the CRABPs and/or RARs/RXRs [18, 20, 21, 24]. 9-*cis*-RA was found to possess the least binding affinity for CRABP [25]. None of the all-*trans*-RAs tested thus far showed any selectivity in binding towards either of the two CRABPs. This is also true with regard to the binding affinities of these RAs for RARs. Even though 4-oxo-RA and a few of the 3-substituted 4-oxo-RAs that were examined had shown significant binding affinity for CRABP, RA was proven to be the best binder [21]. Compared with RA, only DDRA, among the natural analogs of RA, showed a similar or a higher affinity for both CRABP and RARs (Table 1). Both RA and DDRA exhibited only weak binding to RXRs. Interestingly, however, DDRA, but not RA, showed a marked preference in binding to RXRα when compared with RXRβ and RXRγ (Table 1). These findings, in general, are in agreement with earlier published data on DDRA [18, 20, 24]. The present finding that DDRA had an IC_{50} value of 6,250 nM with RXRα, and its lack of binding to RXRβ and γ (>10,000 nM), is particularly interesting in the context of the RXR-mediated transcriptional activation potential of this retinoid as described below.

The RAR and RXR homo- and heterodimer-mediated transcriptional activation by both RA and DDRA was evaluated in CV-1 cells. In transfections where RARα, RARβ, and RARγ were expressed separately (Fig. 2), both RA and DDRA induced a similar magnitude of the reporter gene activation in the range of 10^{-9} to 10^{-6} M. Transcriptional activation mediated by RARβ and RARγ homodimers was slightly higher than that observed in the case of RARα expression for both retinoids. With RARβ, the basal activity (control) was always higher than that observed with the other two receptors. A striking difference between RA and DDRA was observed with regard to their RXRα homodimer-mediated transcriptional activation. DDRA produced a 2- to 3-fold higher activation of the reporter gene expression than RA at a concentration range of 10^{-8} to 10^{-6} M. Although transcriptional activity was higher at 10^{-9} M RA than DDRA at the same concentration, this difference was not significant. This conclusion is supported by the observation that, unlike in the case of DDRA, no concentration-dependent effect on the transcription was observed when RA was present in the concentration range of 10^{-9} to 10^{-7} M, indicating that the activity seen in the presence of 10^{-9} M RA was not ligand dependent. These findings are consistent with our current observation that DDRA possessed weak but relatively higher binding affinity than RA for the RXRα (Table 1). However, whether this weak binding by DDRA itself is responsible for the observed RXR-mediated high transcriptional activation remains to be established. Surprisingly, DDRA induced a 3-fold higher level of transcription when RXRα was expressed in the cells instead of RARs (Fig. 2), despite the observation that this retinoid binds with much less affinity to the former than to the latter (Table 1).

The pattern of transcriptional activation induced by RA and DDRA was similar when RARα–RXRα and RARγ–RXRα heterodimers were expressed in the cells (Fig. 3). However, with the RARβ-RXRα heterodimer, DDRA activated the transcription better than RA in a concentration-dependent manner. At the highest concentration level of 10^{-6} M RA or DDRA, however, a lack of correlation in concentration-dependence was observed. The average of the values derived from two experiments, as plotted

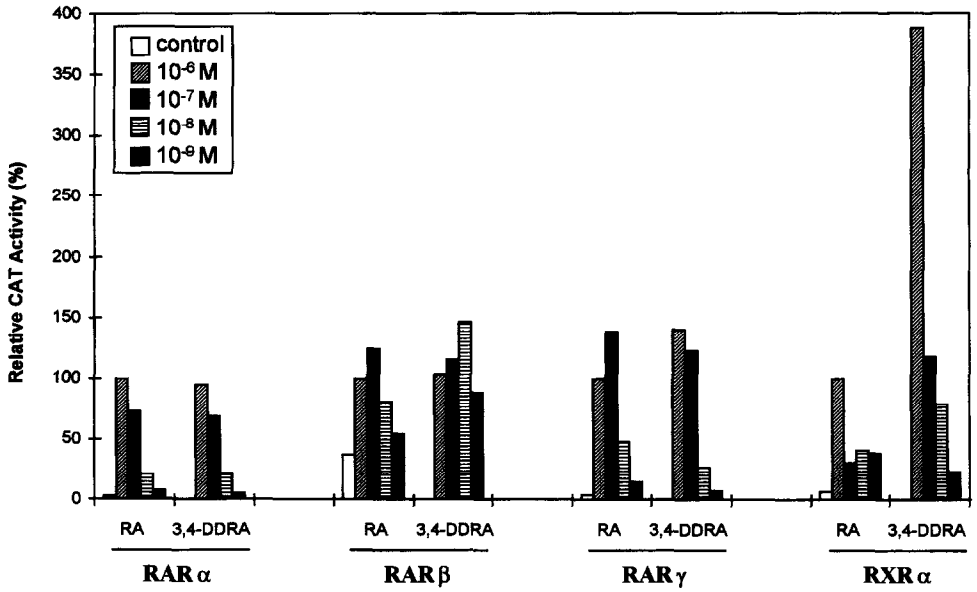

FIG. 2. Analysis of the transcription mediated by various retinoid receptors. CV-1 cells were transfected with the reporter plasmid TRE_2-tk-CAT and expression plasmid for RARα, RARβ, RARγ, or RXRα. CAT activity was determined after the cells were exposed to various concentrations (10^{-9} M to 10^{-6} M) of either RA or DDRA. CAT activity was normalized for transfection efficiency by the corresponding β-galactosidase activity expressed from the internal control plasmid pCH110. The mean values of data obtained from two separate experiments with less than 10% variation are shown in the graph.

in Fig. 3, indicates that the results shown here are significant. Together, these results suggest that compared with RA, DDRA is a better activator of RXR-mediated transcription, especially in combination with RARβ.

To have an insight into the cellular transport and the mode of action of retinoids, it is imperative that we understand the interactions of retinoids with their specific binding proteins and nuclear receptors. The general consensus is that retinoids that possess a terminal carboxyl group bind to CRABP to facilitate their cellular transport, and to nuclear receptors to activate the transcription of the target genes. Since DDRA is a major retinoid present in the skin and since this tissue is very responsive to retinoid treatment, analyses of its interactions with retinoid receptors and its role in the transcriptional regulation become important. We now understand that, consistent with its equipotent biological activity with RA in multiple systems, DDRA binds to CRABPs, RARs, and RXRs at least to the same extent as RA. Additionally, the retinoid expresses significant binding to RXRα as compared with RA. In transcriptional activation studies involving RXRs, this retinoid exhibited higher activity than RA. It is yet to be elucidated whether the two retinoids function within the cell by independent routes or in conjunction with each other.

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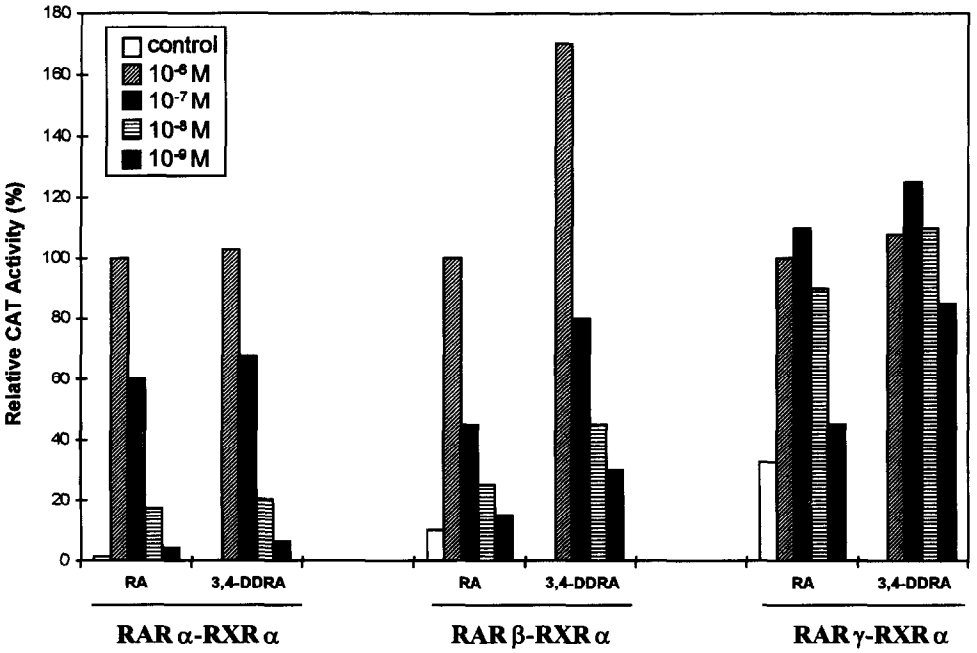

FIG. 3. Analysis of the retinoid-induced transcription mediated by RAR–RXR heterodimers. Expression plasmids for RARα and RXRα, or RARβ and RXRα, or RARγ and RXRα were used in addition to the TRE_2-tk-CAT reporter DNA in the transfection of CV-1 cells. CAT activity in the extracts was determined following the treatment of the transfected cells with RA or DDRA at concentrations ranging from 10^{-9} M to 10^{-6} M. The mean values of data obtained from two separate experiments with less than 10% variation are shown in the graph.

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