Acyclic retinoid activates retinoic acid receptor β and induces transcriptional activation of p21^{CIP1} in HepG2 human hepatoma cells

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Abstract

Acyclic retinoid (ACR), a novel synthetic retinoid, has recently been demonstrated by us to inhibit the *in vitro* growth of human hepatoma cells, and this effect was associated with decreased expression of cell cycle-related molecules. These results, taken together with previous *in vitro* and clinical studies with ACR, suggest that this agent may be useful in the chemoprevention and therapy of hepatoma and possibly other human malignancies. In the present study, we further examined the molecular effects of ACR on the HepG2 human hepatoma cell line, focusing on the expression of nuclear retinoid receptors and the cell cycle inhibitor protein p21^{CIP1}. Reverse transcription-PCR assays and Western blot analyses indicated that these cells express retinoic acid receptors (RARs) α, β, and γ, retinoid X receptors (RXRs) α and β, and peroxisome proliferator-activated receptors (PPAR) γ mRNA. Treatment with ACR caused a rapid induction within 3 h of RARβ mRNA and the related protein, but there was no significant change in the levels of the mRNA or proteins for RARs α and γ, RXRs α and β, and PPARγ. There was also a rapid increase in p21^{CIP1} mRNA and protein in HepG2 cells treated with ACR, and this induction occurred via a p53-independent mechanism. In transient transfection reporter assays, we cotransfected the retinoic acid response element-chloramphenicol acetyltransferase (CAT) reporter gene into HepG2 cells together with a RARβ expression vector. RARβ expression markedly stimulated CAT activity (up to about 4-fold) after the addition of ACR. However, CAT activity in the presence of ACR was only about 2-fold higher than that in the absence of ACR, when cells were cotransfected with RARs α and γ or RXRα. These findings suggest that the growth inhibitory effects of ACR are mediated at least in part through RARβ and that both RARβ and p21^{CIP1} play critical roles in the molecular mechanisms of growth inhibition induced by ACR. [Mol Cancer Ther. 2004;3(3):309–316]

Introduction

Retinoids are a group of structural and functional analogues of vitamin A that exhibit major effects on cell proliferation and differentiation and on pattern formation during development (1). During the past 10 years, there has been increasing evidence that both natural and synthetic retinoids can exert an inhibitory effect on the development of several types of human carcinomas (2, 3). The novel synthetic retinoid, acyclic retinoid (ACR), has been shown to prevent the recurrence of hepatoma after surgical resection of primary tumors (4, 5). In these studies, ACR did not cause the typical toxic effects seen with conventional retinoids (4, 5). This unique agent was also reported to decrease the incidence of carcinogen-induced skin tumors and spontaneous hepatomas in mice (6). ACR was also shown to induce apoptosis in the Huh7 human hepatoma cell line (7). In a recent study, we found that ACR inhibits the growth of the Huh7, Hep3B, and HepG2 human hepatoma cell lines (8). In HepG2 cells, this growth inhibition by ACR was associated with arrest of cells in the G_1 of the cell cycle, early induction of the p21^{CIP1} protein, and subsequent reduction in the level of both cyclin D1 protein and mRNA (8). We also demonstrated that ACR inhibits β-catenin-stimulated cyclin D1 promoter activity in HepG2 cells (8).

The activity of various retinoids, including all-*trans*-retinoic acid (ATRA) and 9-*cis*-retinoic acid (9-*cis*-RA), is thought to be mediated by interactions with two subfamilies of nuclear retinoic acid receptors—retinoic acid receptors (RARs) and retinoid X receptors (RXRs; Ref. 9). These subfamilies both include three distinct subtypes, designated α, β, and γ, which are encoded by distinct genes (10, 11). All of these receptors are ligand-dependent transcription factors and control the activity of target genes by binding to DNA response elements, including retinoic acid response elements (RAREs; DR5) and retinoid X response elements (DR1; Refs. 9, 12). The RARs bind both ATRA and 9-*cis*-RA, while the RXRs bind only 9-*cis*-RA (13). These receptors are also thought to bind a variety of synthetic retinoids, some of which preferentially bind to specific subtypes (3).

Normal oral mucosa epithelial cells express RARs α, β, and γ and RXRα (14–16), while preneoplastic lesions and head and neck squamous cell carcinomas (HNSCCs)

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often display selective suppression of RARβ expression (14, 17, 18). These findings suggest that loss of RARβ expression is associated with the development of HNSCCs. Treatment of the human hepatoma cell line with ATRA causes a marked increase in the level of RARβ mRNA but not in the level of RARα mRNA (19). However, *N*-(4-hydroxyphenyl)retinamide (fenretinide) selectively activates transcription of RARγ and, to a lesser extent, RARβ but not transcription of RARα and RXRα in human breast carcinoma cell lines (20). Novel synthetic retinoids that bind selectively to RXRs are termed rexinoids (2). The rexinoids LGD1069 (targretin or bexarotene) and LG100268 are highly effective in rat mammary carcinogenesis prevention model systems (2). LGD1069 also inhibits proliferation of human HNSCC cell lines (21).

These findings suggest that different retinoids can act through different retinoid nuclear receptors to alter the expression of genes that inhibit the growth of cancer cells. Therefore, in the present study, we investigated whether ACR acts through specific nuclear RARs to inhibit the growth of human hepatoma cells by examining cellular levels of the related mRNA and proteins before and after treating the cells with doses of ACR that inhibit the growth of these cells. In parallel studies, we also examined whether ACR acts via specific retinoid receptors in enhancing the transcriptional activity of a RARE-chloramphenicol acetyltransferase (CAT) reporter in transient transfection reporter assays.

The activities of the cell cycle control proteins are negatively regulated by a series of cyclin-dependent kinase inhibitors, including $p21^{CIP1}$ (22). A recent study demonstrates that the promoter region of the $p21^{CIP1}$ gene contains a functional RARE sequence and that this sequence confers retinoid induction of $p21^{CIP1}$ mRNA through RAR-RXR heterodimers (23). Therefore, the $p21^{CIP1}$ gene appears to be a retinoid target gene (23). In a recent study, we found that ACR causes rapid induction of the $p21^{CIP1}$ protein in HepG2 human hepatoma cell line (8). In addition, $p21^{CIP1}$ is a primary response gene in the induction of differentiation of HL-60 human leukemia cells by ATRA (24). Therefore, in the present study, we also examined the effects of ACR on the induction of $p21^{CIP1}$ mRNA by carrying out time course study using reverse transcription-PCR (RT-PCR) assays and Northern blot analysis.

Materials and Methods

Chemicals

ACR [(2*E*,4*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-2,4,6,10,14,-hexadecapentaenoic acid; Fig. 1; NIK333; Nikken Chemicals Co., Ltd., Tokyo, Japan] was provided by Dr. H. Moriwaki (Gifu University School of Medicine, Gifu, Japan; Ref. 7).

Cell Lines and Treatment with Chemicals

The human hepatoma cell line HepG2 (25) was maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies) in an incubator with humidified air with 5% CO_2 at 37°C. Cells were plated in 10 cm culture dishes (1×10^6 cells/dish) in DMEM plus 10% FBS, at concentrations determined to yield 60–70% confluent cultures, treated with the indicated concentrations of ACR, and harvested at the indicated times. As an untreated solvent control, cells were treated with DMSO (Sigma Chemical Co., St. Louis, MO) at a final concentration of <0.1%.

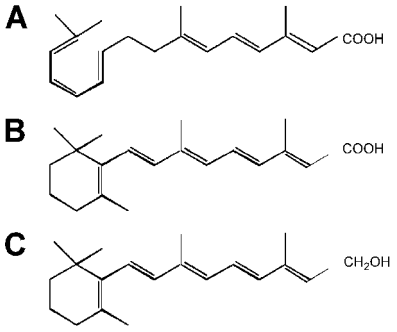

Figure 1. Chemical structures of ACR (**A**), ATRA (**B**), and vitamin A (**C**).

Western Blot Analysis

HepG2 cell lysates were prepared with a modified radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 0.5% deoxycholic acid, 1 mM EDTA, 2 mM EGTA, 1 mM DTT, 25% glycerol]. Proteins (50–100 mg) were separated by SDS-PAGE, transferred onto an Immobilon-P transfer membrane (Millipore, Bedford, MA), and incubated with the appropriate antibodies as described previously (8). The primary antibodies used in this study were RARβ (sc-552; Santa Cruz Biotechnology, Santa Cruz, CA), $p21^{CIP1}$ (Ab-1; Oncogene Research Products, Darmstadt, Germany), and p53 (sc-126, Santa Cruz Biotechnology). Anti-mouse IgG or anti-rabbit IgG antibodies (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) were used as the secondary antibodies. Each membrane was developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

Transient Transfection CAT Reporter Assay

Reporter assays were performed as described previously (8). HepG2 cells were plated into six-well, 35 mm diameter plates (0.5×10^5 cells/well) in DMEM plus 10% FBS and cultured overnight to allow for cell attachment. The RARE-CAT reporter construct (RARE-CAT) consisting of the RARE and the CAT gene and RARs α, β, and γ and RXRα expression vectors were generously provided by Dr. D. A. Talmage (Institute of Human Nutrition, Columbia University, New York, NY). The pcDNA3 plasmid (Invitrogen, San Diego, CA) was used as a control vector. RARE-CAT (2 μg) reporter was transfected into HepG2 cells. The expression vectors (2.0 μg/assay) were cotransfected as described in the legend of Fig. 3 using the Lipofectin reagent (Invitrogen). After transfection, the HepG2 cells were incubated with ACR (30 μM) or 0.1% DMSO in DMEM plus 10% FBS for 24 h. Cell extracts were then prepared, and each sample was assayed in triplicate using a CAT-ELISA system (Roche Diagnostics GmbH, Mannheim,

Germany) according to the manufacturer's instructions. The absorbance was measured using a spectrophotometric microtiter plate reader at 405 nm wavelength. A cytomegalovirus-driven β-gal expression plasmid was cotransfected with the above plasmids to normalize for transfection efficiency. Differences in reporter activities between the experimental groups were analyzed by Student's t test. All statements of significance are $P < 0.05$.

RT-PCR Analysis

These assays were performed as described earlier (8). Total RNA was isolated from frozen cells using a Trizol reagent as recommended by the manufacturer (Life Technologies). cDNA was amplified from total RNA (1 μg) using a SuperScript One-Step RT-PCR System with Platinum Taq (Life Technologies). PCR was conducted for 30–40 cycles in a thermal controller (Programmable Thermal Controller; MJ Research Inc., Watertown, MA). The primers used for amplification are shown in Table 1. β-actin-specific PCR products from the same RNA samples were amplified and served as internal controls. Each amplification cycle consisted of 0.5 min at 94°C for denaturation, 0.5 min at 55°C for primer annealing, and 1 min at 72°C for extension. After PCR amplification, the fragments were analyzed by agarose gel electrophoresis and stained with ethidium bromide.

Northern Blot Analysis

These assays were also performed by established procedures (8). Total RNA was isolated as mentioned above. An aliquot (20 μg/lane) of each RNA sample was separated on a formaldehyde-agarose gel and then blotted onto a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). rRNA was used as a loading control. A $p21^{CIP1}$ cDNA probe was synthesized using primers C1F and C2R as described in the RT-PCR assays and labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using a Prime-It II Random Primer Labeling Kit (Stratagene, Cedar Creek, TX). Total RNA obtained from WRL68 human embryonic liver cells (American Type Culture Collection, Manassas, VA) was used as template for RT-PCR. Membranes were hybridized with the labeled cDNA probe in Church buffer [7% SDS, 1% BSA, 1 mM EDTA, 0.25 M $Na_2HPO_4 \cdot 7H_2O$ (pH 7.2)] at 65°C for 24 h and washed with 2× SSC at 65°C. Each filter was exposed to Kodak XAR film (Kodak, Rochester, NY).

Results

ACR Causes a Rapid Increase in the Levels of Expression of RARβ mRNA and Protein

In a previous study, we found that ACR inhibits the growth of HepG2 human hepatoma cells, with an IC_{50} value of about 30 μM when the cells were grown in DMEM containing 10% FBS (8). Therefore, in the present study, we treated HepG2 cells with this concentration of ACR in DMEM plus 10% FBS. We then isolated RNA at 3, 6, 13, and 24 h after the addition of the drug and examined by RT-PCR assays the levels of expression of RARs α, β, and γ, RXRs α, β, and γ, and peroxisome proliferator-activated receptors (PPAR) γ mRNA (Fig. 2A). We included assays for PPARγ mRNA because PPARγ heterodimerizes with RXRα (9). To quantify the expression levels of mRNA, PCR products were generated during both plateau and log-phase reactions by conducting 32–40 cycles of PCR as described in our recent study (8). With this approach, we found that these products reflect corresponding levels of mRNA by Northern blot assays (8). In samples treated with ACR (30 μM), there was a marked increase in the RARβ band intensity within 3 h after the addition of the drug and

Table 1. Oligonucleotide primers used for PCR amplification

Gene	Name	Sequence	Size of the PCR product (bp)
RARα	AF2 (forward)	5′-CCC CGA GAC CCC CTC TGA AGG-3′	400
	AR2 (reverse)	5′-GGA GCT GCT CGC ACT CAA AGC-3′	
RARβ	BF2	5′-TGC AAT TGA AAC ACA GAG CAC-3′	435
	BR2	5′-TGG CAG AGT GAA GGG AAA GTT-3′	
RARγ	RARGF	5′-TTC GAG ATG CTG AGC CCT AGC TTC C-3′	428
	GR4	5′-CTG AGG GCT CAG CTC ATA GCT-3′	
RXRα	XAF1	5′-ACA GCT GCA TTC TCC CAT CAG-3′	460
	XAR2	5′-CAT GGC CAG GCA CTT CTG GTA-3′	
RXRβ	XBF1	5′-TCC CCA AAT CCC CTT TCC CAG-3′	462
	XBR2	5′-GTC CAC TGT GCA GTC TTT GTT-3′	
RXRγ	XGF2	5′-ACT GGC TCT ACA TCC ATG AGC-3′	440
	XGR2	5′-CTT ATC GTC CTC TTG AAG AAC-3′	
PPARγ	PPF3	5′-ATG ACC ATG GTT GAC ACA GAG-3′	427
	PPR2	5′-GCT TCA ATC TGA TTG TTC TCC-3′	
$p21^{CIP1}$	C1F	5′-CTC AGA GGA GGC GCC ATG TCA-3′	443
	C2R	5′-GCC GTT TTC GAC CCT GAG AGT-3′	
p53	P1F	5′-TCA GAT CCT AGC GTC GAG CCC-3′	438
	P2R	5′-GGG TGT GGA ATC AAC CCA CAG-3′	
β-actin	FBA	5′-CCA GGC ACC AGG GCG TGA TG-3′	436
	RBA	5′-CGG CCA GCC AGG TCC AGA CG-3′	

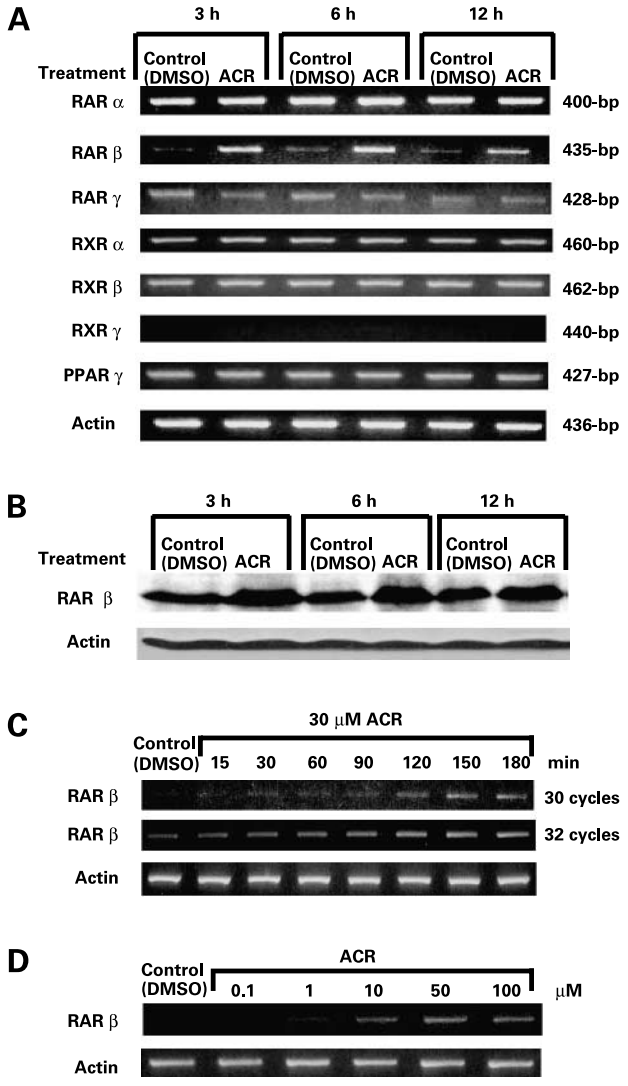

Figure 2. Effects of ACR on expression of nuclear receptors. **A,** RT-PCR analysis of nuclear receptor mRNA. HepG2 cells were treated with DMSO (*Control*) or ACR (30 μM) for 3, 6, and 12 h. RNA samples were then analyzed by RT-PCR using the pairs of primers shown in Table 1. Actin was used as an internal control. **B,** RARβ protein expression in HepG2 cells after treatment with DMSO (*Control*) or ACR. Cells were treated as described above and protein extracts were examined for RARβ expression by Western blot analysis. **C,** RT-PCR analysis of RARβ mRNA. RNA was prepared from HepG2 cells treated with DMSO (*Control*) or ACR for the indicated periods of time (15–180 min). **D,** RT-PCR analysis of RARβ mRNA. RNA was prepared after treating cells with DMSO (*Control*) or with the indicated concentrations of ACR for 3 h. Similar results were obtained in repeated studies.

this increase persisted at the subsequent 6 and 12 h time points (Fig. 2A). Similar results were obtained in samples obtained at 24 and 48 h after drug treatment (data not shown). In contrast, no major changes in the band intensities of the other four nuclear RARs and of PPARγ were seen in cells treated with the same dose of ACR, although some decrease in RARγ was seen at 3 and 6 h (Fig. 2A). The expression of RXRγ mRNA was not detected in these cells (Fig. 2A).

In view of our findings that ACR caused an increase in the level of expression of RARβ mRNA and the fact that RARβ expression is often induced by its own ligand (3, 9), we then examined if ACR also affects the levels of expression of the RARβ protein by using Western blot analysis (Fig. 2B). We treated HepG2 cells with ACR (30 μM) and protein extracts were prepared at 3, 6, 12, and 24 h. We found that within 3 h there was a marked increase in the RARβ protein and this increase persisted at the 6, 12, and 24 h time points (Fig. 2B, but the 24 h time point is not shown).

We then examined how early the induction of RARβ mRNA expression occurs by performing RT-PCR assays in RNA extracts prepared for HepG2 cells at 15, 30, 60, 90, 120, and 180 min after treatment with ACR (20 μM; Fig. 2C). An increase in the RARβ band was apparent after 120 min with both 30 and 32 cycles of the PCR when compared with that of untreated cells. We also investigated the dose response effect of ACR on the levels of expression of RARβ mRNA (Fig. 2D). HepG2 cells grown in DMEM plus 10% FBS were treated with increasing doses (0.1–100 μM) of ACR, as indicated, and total RNA was isolated at 3 h after addition of the drug. ACR caused an increase in the levels of expression of RARβ mRNA in a dose-dependent manner. An increase could be detected with as little as 1 μM and the induction was saturated with about 50 μM ACR (Fig. 2D). Thus, treatment of HepG2 cells with ACR causes a rapid (within 2 h) increase in cellular levels of RARβ. This induction increases further at 3 h and persists for at least 24 h. Furthermore, this effect is dose dependent and can be detected with as little as 1 μM ACR. The increase in RARβ mRNA apparently leads to a rapid and persistent increase in cellular levels of RARβ protein (Fig. 2B). As mentioned above, RARβ, when activated by a ligand, can stimulate further expression of RARβ (3, 9), ACR can apparently induce a positive feedback loop with respect to RARβ synthesis.

ACR Causes a Rapid Increase in the Levels of Expression of $p21^{CIP1}$ mRNA and This Induction Occurs via a p53-Independent Mechanism

In the above studies, we found that ACR caused a rapid induction of RARβ mRNA and protein within 3 h after addition of the drug to HepG2 cells. Because we reported previously that ACR induces an increase in the protein $p21^{CIP1}$ in these cells (8), in the current study, we examined whether ACR affects the levels of expression of $p21^{CIP1}$ mRNA under the same conditions in which ACR induced the expression of RARβ. HepG2 cells were treated with ACR (20 μM) and RNA samples were prepared at 15, 30, 60, 90, 120, and 180 min and assayed by RT-PCR using primers for $p21^{CIP1}$ mRNA (Fig. 3A). We detected an increase in the intensity of the RARβ band within 90 min after the addition of ACR, and the intensity of this band increased further at 180 min (Fig. 3A). To confirm and extend these results, we also did Northern blot analyses for $p21^{CIP1}$ mRNA. We found a marked increase in this mRNA within 3 h after the addition of ACR. Further increases were seen at 6, 12, and 24 h. At 48 h, the level declined but still remained relatively high (Fig. 3B). The

induction of $p21^{CIP1}$ expression can occur via p53-dependent and p53-independent mechanisms (26), and HepG2 cells contain a wild-type *p53* gene (27). Therefore, we did parallel time course studies on the effects of treatment of HepG2 cells with ACR on cellular levels of the $p21^{CIP1}$ and p53 proteins. Although ACR led to an increase in the level of the $p21^{CIP1}$ protein within 3 h and this effect persisted for at least 48 h, during the same time course, there was no significant change in cellular levels of the p53 protein (Fig. 3C). RT-PCR assays for p53 mRNA also indicated no change in cellular levels of this mRNA during the same time course of treatment with ACR (Fig. 3C).

RARβ Expression Markedly Stimulates RARE-CAT Activity in the Presence of ACR

Various retinoids appear to exert their antitumor effects through distinct retinoid receptors. Thus, ATRA and 13-*cis*-retinoic acid appear to act mainly through RARβ (28), but 9-*cis*-RA is a high-affinity ligand and activates RXRα (29, 30). However, it is not known with certainty whether ACR mediates its action through a specific nuclear receptor (3).

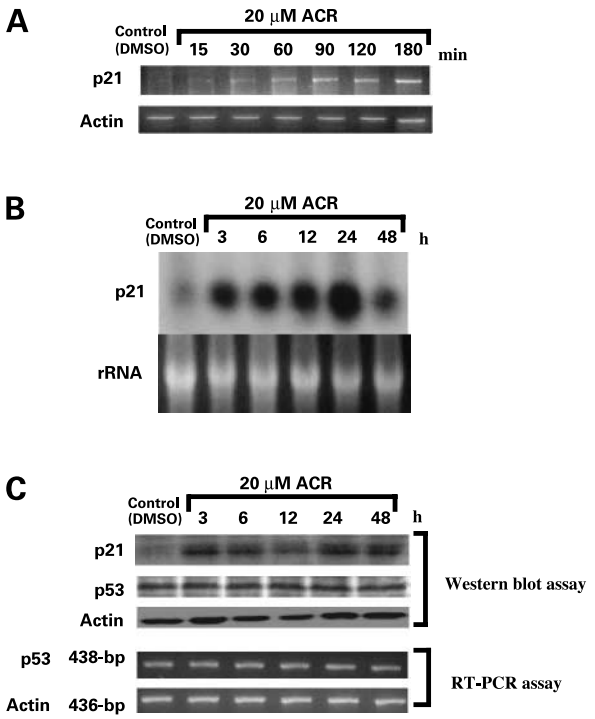

Figure 3. Effects of ACR on expression of $p21^{CIP1}$ and p53. **A,** RT-PCR analysis of $p21^{CIP1}$ mRNA. HepG2 cells were treated with DMSO (*Control*) or ACR (20 μM) for the indicated times (15 – 180 min). RNA was extracted and analyzed by RT-PCR using the primers for $p21^{CIP1}$ mRNA. **B,** Northern blot analysis of $p21^{CIP1}$ mRNA. HepG2 cells were treated with DMSO (*Control*) or ACR (20 μM) for the indicated times (3 – 48 h). RNA was extracted and examined by Northern blot analysis using a [α-^{32}P]dCTP-labeled $p21^{CIP1}$ probe. rRNA was used as a loading control. **C,** Western blot analysis for the $p21^{CIP1}$ and p53 proteins (*upper panels*) and RT-PCR analysis of p53 mRNA (*lower panels*). HepG2 cells were treated with DMSO (*Control*) or ACR (20 μM) for the indicated times (3 – 48 h). Protein extracts were examined by Western blot analysis for the $p21^{CIP1}$ and p53 proteins and RNA extracts were examined by RT-PCR for p53 mRNA. Actin was used as a loading control. Similar results were obtained in repeated studies.

Therefore, to determine whether the ability of ACR to stimulate the transcriptional activity of a RARE is enhanced by specific retinoid receptors, we did transient transfection reporter assays in which a RARE-CAT reporter was transfected into HepG2 cells together with expression vectors of a series of retinoid receptors. We found that RARβ expression markedly stimulated CAT activity by about 3-fold after the addition of 30 μM ACR (Fig. 4). The expression of RARs α and γ and RXRα caused about a 1.5–2-fold stimulation of CAT activity in the presence of ACR (Fig. 4). However, the CAT activity in the presence of ACR was only about 2-fold higher than that in the absence of ACR when cells were cotransfected with either RARs α or γ or RXRα. The ACR-induced increases in CAT activity obtained with all four of these expression vectors were statistically significant by Student's *t* test (each *P* value < 0.004). These findings, together with our evidence that treatment of HepG2 cells with ACR markedly increases the expression of RARβ but not the other retinoid receptors tested (Fig. 2A), suggest that RARβ plays an important role in the molecular mechanisms of growth inhibition induced by ACR. They do not, however, exclude the possibility that other nuclear receptors also play a role in this process.

Discussion

Retinoids exert their molecular effects by binding to and activating the transcriptional activity of specific nuclear receptors in mammalian cells (9). Several studies demonstrate that human HNSCC cell lines and oral premalignant and malignant lesions express little or no RARβ, whereas they do express RARs α and γ mRNA (14–17). ATRA induces increased expression levels of the RARβ protein in immortalized human bronchial epithelial cell lines and in human HNSCC cell lines (31–34). Yamada *et al.* (35) reported that in PLC/PRF/5 and Huh7 human hepatoma cell lines the novel retinoid ACR inhibits proliferation and induces up-regulation of RARβ mRNA after 3–5 days of treatment with this compound. In recent studies, we found that ACR inhibits the growth of HepG2, Hep3B, and Huh7 human hepatoma cell lines, with IC_{50} values of about 60, 30, and 4 μM, respectively, when the cells were grown in DMEM plus 10% FBS (8). In the present study, we focused on the roles of specific nuclear retinoid receptors and other early retinoid-related events involved in this mechanism of growth inhibition using the well-characterized HepG2 cell line for these detailed studies.

We found that treatment of HepG2 cells with ACR caused a rapid (within 2 h) increase in the expression level of RARβ mRNA, whereas there were no increases in the levels of expression of RARs α and γ, RXRs α and β, or PPARγ mRNA (Fig. 2A). There was also a rapid increase in the levels of expression of the RARβ protein (Fig. 2B). These effects of ACR on RARβ expression were both time and dose dependent (Fig. 2, C and D). Within 3 h of the addition of ACR to HepG2 cells, there was also a marked and persistent increase in cellular levels of the cell cycle inhibitor protein $p21^{CIP1}$ and its related mRNA (Fig. 3).

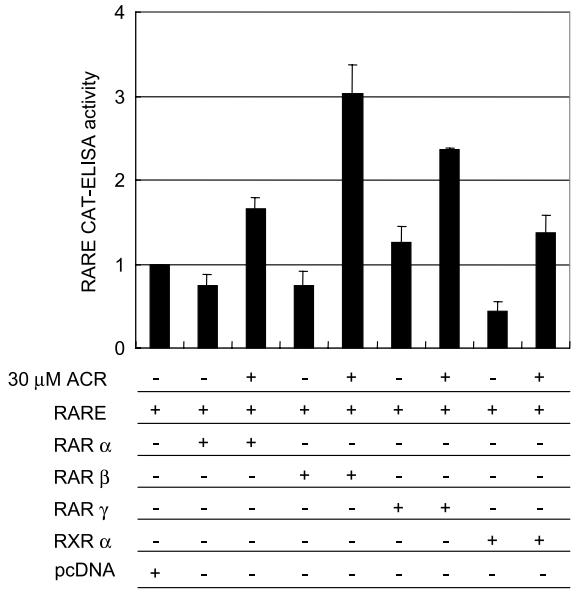

Figure 4. Effects of ACR on the transcriptional activity of RARs. The RARE-CAT reporter (2.0 μg) was transfected into HepG2 cells together with control pcDNA3 or RARs α, β, and γ and RXRα expression vectors. The transfected cells were treated for 24 h with 0.1% DMSO (-) or 30 μM ACR (+) in DMEM plus 10% FBS. Extracts were then examined for CAT activity. In these assays, a cytomegalovirus promoter β-gal plasmid was also cotransfected with the RARE-CAT reporter, and β-gal activity was measured to normalize the data for transfection efficiency. All assays were done in triplicate. *Columns,* means; *bars,* SD.

This induction of $p21^{CIP1}$ was not associated with cellular levels of p53 mRNA and protein (Fig. 3C), suggesting that ACR induces $p21^{CIP1}$ expression by a p53-independent mechanism. Furthermore, we recently reported that the proliferation of HepG2 cells is markedly inhibited by ACR (8). Taken together, these findings suggest that both RARβ and $p21^{CIP1}$ play critical roles in the molecular mechanisms by which ACR inhibits the growth of human hepatoma cells. Because of the novel open-ring structure of ACR, prior to the present studies, it was not obvious that it inhibits the growth of tumor cells by acting through retinoid receptors. Our finding that, like the physiologic retinoid ATRA (14–17), ACR causes an early and marked induction of RARβ and our evidence that in HepG2 cells it induces the transcriptional activity of a RARE-CAT reporter when the cells are cotransfected with RARβ expression plasmid (Fig. 4) provide strong evidence that the action of ACR is mediated via retinoid receptors. We have not, however, excluded the possibility that some of the growth inhibitory effects of ACR are mediated via other mechanisms. Another possible mechanism relates to the apparent ability of RARs to inhibit the activity of the transcription factor AP-1 (36). RARβ exhibits a strong ATRA-independent inhibition of AP-1 activity, whereas inhibition of AP-1 activity by RARs α and γ is ATRA dependent (37). In HepG2 cells, we found that ACR inhibits both AP-1 and c-Fos promoter activity in a dose-dependent manner and we also found similar effects of ACR in a human squamous cell carcinoma cell line (unpublished data). Thus, the growth inhibitory effects by ACR might also occur through inhibition of AP-1 and/or c-Fos activity.

Several studies demonstrate that exogenous expression of RARβ increases the growth inhibition exerted by ATRA on cervical carcinoma (38, 39), renal carcinoma (40), and breast carcinoma cells (41–43). In a recent study, we found that Huh7 human hepatoma cells are more sensitive to growth inhibition by ACR than HepG2 cells (8), and in unpublished studies, we found that the former cells express higher levels of the RARβ protein (data not shown). Therefore, the level of expression of RARβ in hepatoma cells may influence their sensitivity to the growth inhibitory effects of ACR. Other studies have found that increased expression of RARγ but not RARβ in the SqCC/Y1 human SCC cell line increased the sensitivity of these cells to growth inhibition by ATRA and 9-*cis*-RA (34, 44). In addition, appreciable levels of RARβ are expressed in some retinoic acid-resistant tumor cell lines (45). Therefore, the sensitivity of specific hepatoma and other types of tumor cells to growth inhibition by ACR may be influenced by factors other than basal or inducible levels of RARβ.

The promoter region of the $p21^{CIP1}$ gene contains a RARE to which both RARs and RXRs can bind (36). Through this element, retinoid receptors can activate the transcription of the $p21^{CIP1}$ gene (23). The $p21^{CIP1}$ promoter sequence from residues −1212 to −1194 defines a functional RARE that is required to mediate the induction by ATRA of $p21^{CIP1}$ transcription (23). Several inducers of myeloid cell differentiation also up-regulate $p21^{CIP1}$ expression (24, 46). These include 12-*O*-tetradecanoyl phorbol-13-acetate, okadaic acid, sodium butyrate, DMSO, 1,25-dihydroxyvitamin D3, and retinoids. Thus, the $p21^{CIP1}$ promoter contains multiple response elements involved in cell cycle arrest, growth inhibition, and/or differentiation. This may explain why we found that ACR causes rapid induction of $p21^{CIP1}$ expression in HepG2 cells (Fig. 3). Presumably, this occurs through the RARE in the promoter region of $p21^{CIP1}$ and is mediated at least in part by RARβ. This speculation is consistent with evidence that treatment of HL-60 human leukemia cells with ATRA also causes rapid induction of $p21^{CIP1}$ mRNA (24, 46). In addition, a novel synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene and the conventional retinoid 9-*cis*-RA caused a rapid and dramatic increase in the levels of $p21^{CIP1}$ mRNA and protein in human MCF-7 and MDA-MB-231 breast carcinoma and TMK-1 gastric carcinoma cell lines (47, 48). Our finding that the induction of $p21^{CIP1}$ by ACR in HepG2 cells occurs via a p53-independent mechanism (Fig. 3C), although these cells carry a wild-type *p53* gene (27), is consistent with evidence that induction of $p21^{CIP1}$ by ATRA in HL-60 cells (24, 46) and induction by a novel synthetic retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene of G_1 arrest and apoptosis in the MDA-MB-231 and MCF-7 cell lines (48) also occur via p53-independent mechanisms.

In our previous studies, we found that treatment of HepG2 cells with ACR caused a rapid (within 3 h) decrease in cellular levels of the hyperphosphorylated (inactivated) form of the retinoblastoma protein (8). Increased expression of $p21^{CIP1}$ often occurs during differentiation (24). In addition, decreased phosphorylation of retinoblastoma protein and cell cycle arrest in G_1 also occur during the induction of differentiation in HL-60 cells (49). Therefore, it is of interest that previous studies found that treatment of Huh7, Hep3B, and the PLC/PRF/5 human hepatoma cell lines with ACR induced expression of albumin mRNA, a putative marker of differentiation in these cells (35), although ATRA repressed albumin expression in these cells (50). More detailed studies are required to further characterize possible effects of ACR on the differentiation of hepatoma cells.

The present studies provide evidence that the growth inhibitory effects of ACR in human hepatoma cells are mediated at least in part via retinoid receptors, especially RARβ, and the RARE present in the $p21^{CIP1}$ gene. It seems likely that RAR elements in other retinoid-responsive genes also play a role in this process. Despite its unusual chemical structure, the molecular mechanisms of action of ACR resemble those of several retinoids. A serious limitation in the clinical use of ATRA and other retinoids in cancer chemoprevention and therapy is their adverse effects, particularly with prolonged administration. These can include dryness of the skin and lips, skin rash, nasal congestion, and, in some cases receiving high doses, a severe ''retinoic acid syndrome'' (51, 52). On the other hand, thus far, clinical studies with ACR have not revealed the adverse effects seen with conventional retinoids (4, 5). The reasons why ACR appears to be better tolerated are not known, and this aspect warrants further study.

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