Induction of Apoptosis by *N*-(4-Hydroxyphenyl)retinamide and Its Association with Reactive Oxygen Species, Nuclear Retinoic Acid Receptors, and Apoptosis-Related Genes in Human Prostate Carcinoma Cells

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ABSTRACT

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide (4HPR) has been shown to induce apoptosis in various malignant cells including human prostate carcinoma cells (HPC). We examined several possible mechanisms by which 4HPR could induce apoptosis in HPC cells. 4HPR exhibited concentration- and time-dependent decrease in cell number both in androgen-dependent (LNCaP) and -independent (DU145 and PC-3) cells. The 4HPR concentrations causing 50% decrease in cell number in LNCaP, DU145, and PC-3 cultures were 0.9 ± 0.16, 4.4 ± 0.45, and 3.0 ± 1.0 μM, respectively, indicating that LNCaP cells were more sensitive to 4HPR than the other cells. 4HPR-induced apoptosis in all three cell lines was evidenced by increased enzymatic labeling of DNA breaks and formation of a DNA ladder. 4HPR increased the level of reactive oxygen species, especially in LNCaP cells. 4HPR-induced apoptosis could be suppressed in LNCaP cells by antioxidant and in DU145 cells by a nuclear retinoic acid receptor-specific antagonist, suggesting the involvement of reactive oxygen species or retinoic acid receptors in mediating apoptosis induced by 4HPR in the different HPC cells. Furthermore, 4HPR modulated the expression levels of some apoptosis-related gene (p21, c-*myc*, and c-*jun*), which may also contribute to the induction of apoptosis by 4HPR in HPC cells.

Although progress has been made in the early diagnosis and treatment of prostate cancer in the past 30 years, prostate cancer continues to be the most common cancer and the second leading cause of cancer death in men in the United States (Landis et al., 1998). Therefore, new approaches to the prevention and therapy of prostate cancer are urgently needed.

Retinoids constitute a group of compounds including natural derivatives and synthetic analogs of vitamin A that exert profound effects on the regulation of growth and differentiation of many cell types, especially epithelial cells, both in vivo and in vitro (DeLuca, 1991). Recent studies have demonstrated that some of these compounds can induce apoptosis in several types of cancer cells (Lotan, 1995). As a result, there is an increasing interest in the use of retinoids as potential therapeutic and chemopreventive agents for malignant diseases (Benner et al., 1995).

The synthetic retinoid *N*-(4-hydroxyphenyl)retinamide

(4HPR) was found to be effective against breast, prostate, and ovarian cancers in animal models (Moon et al., 1989; Pollard et al., 1991; Formelli and Cleris, 1993; Pienta et al., 1993). This retinoid is being evaluated for prevention of breast cancer and other malignancies (Costa et al., 1994) and has already shown a potential for preventing ovarian cancer in humans (De Palo et al., 1995). Clinical trials indicate that 4HPR shows minimal toxicity and favorable pharmacokinetic profiles (Costa et al., 1989; Formelli et al., 1993). In recent in vitro studies, 4HPR has been observed to induce apoptosis in malignant hematopoietic cells and neuroblastoma, cervical, breast, ovarian, head and neck, and lung cancer cell lines, including those exhibiting resistance to the effects of the natural vitamin A metabolite all-*trans*-retinoic acid (**ATRA**) (Sun and Lotan, 1998).

Previous studies have shown that 4HPR is effective against prostate cancer in an in vivo animal model (Pollard et al., 1991; Pienta et al., 1993). This retinoid also inhibits cell proliferation and induces apoptosis in some human prostate carcinoma (HPC) cell lines (Igawa et al., 1994; Hsieh and Wu, 1997; Roberson et al., 1997). The present investigation was

ABBREVIATIONS: 4HPR, *N*-(4-hydroxyphenyl)retinamide; **ATRA**, all-*trans*-retinoic acid; HPC, human prostate cancer; ROS, reactive oxygen species; RARs, retinoic acid receptors; RXRs, retinoid X receptors; REs, response elements; BHA, butylated hydroxyanisole; DCF-DA, 2′, 7′-dichlorofluorescin diacetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

designed to address some mechanistic aspects of 4HPR-induced apoptosis in HPC cells, including androgen-dependent and -independent ones.

Apoptosis is a complicated and tightly regulated process, which is genetically controlled by a number of distinct death- or survival-related genes, including oncogenes such as c-*myc*, p53, and p53's downstream target genes that include p21, bcl-2, and bax (Stewart, 1994). Apoptosis can also be induced or modulated by various pharmacological agents (Stewart, 1994). At present, the cellular and molecular mechanisms by which 4HPR induces apoptosis in malignant cells are not well understood. Recently, the generation of reactive oxygen species (ROS) has been implicated in 4HPR-induced apoptosis in some malignant cells (Delia et al., 1997; Oridate et al., 1997). In addition, 4HPR was found to activate retinoic acid receptors (RARs; Fanjul et al., 1996; Kazmi et al., 1996), which are thought to mediate the major effects of retinoids on cells (Chambon, 1996). Furthermore, it is not known which apoptosis-related genes contribute to the process by which 4HPR induces cell death. Therefore, in the present study we examined whether ROS, nuclear retinoid receptors, and apoptosis-related genes such as c-*myc*, c-*fos*, c-*jun*, and p53 as well as p21, bcl-2, and bax are involved in the induction of apoptosis by 4HPR in HPC cells. We found that 4HPR induced apoptosis in either androgen-dependent or -independent HPC cells by distinct mechanisms that involve RARs-, and/or ROS-mediated pathways. In addition, p21, c-*myc*, and c-*jun* might also contribute to apoptosis induced by 4HPR in HPC cells.

Materials and Methods

Retinoids and Other Reagents. 4HPR was obtained from Dr. Ronald Lubet (National Cancer Institute, Bethesda, MD) and **ATRA** was a gift from Dr. Werner Bollag (F. Hoffmann-La Roche, Basel, Switzerland). The RAR antagonistic retinoid CD2366 was kindly provided by Drs. Braham Shroot and Uwe Reichert (CIRD/Galderma, Sophia Antipolis, France). The retinoids were dissolved in DMSO at a concentration of 10 mM and stored under N_2 in the dark at $-80°C$. Stock solutions were diluted to the appropriate final concentrations with growth medium just before use. The antioxidant butylated hydroxyanisole (BHA) was purchased from Sigma Chemical Co. (St. Louis, MO). 2′, 7′-Dichlorofluorescin diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR).

Cells and Cell Culture. The HPC cell lines PC-3, DU145, and LNCaP were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and were grown in monolayer culture in a 1:1 (v/v) mixture of Dubecco's modified Eagle's medium and Ham's F12 medium containing 5% fetal bovine serum (Gibco BRL, Gaithersburg, MD) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C.

Cell Survival Assay. Cells were seeded at densities of 2000 to 6000 cells per well in 96-well cluster tissue culture plates and treated on the next day with different concentrations of retinoids. Control cultures received the same amount of DMSO as treated cultures (0.1%). At the indicated times, cell number was determined using the Alamar Blue reagent (Alamar, Westlake, OH) following the manufacturer's instructions. The drug concentration decreasing cell number by 50% (IC_{50}) was determined from dose-response curves.

DNA Fragmentation Assay. Cells were plated in 10-cm diameter dishes 1 day before initiation of 4HPR treatment. After appropriate times, the cells were harvested and analyzed for the presence of DNA fragments by the TUNEL (terminal deoxynucleotidyl transferase [TdT]-mediated labeling of 3′-hydroxyl ends of DNA fragments formed during apoptosis with fluorescein-tagged dUTP) using

the APO-DIRECT kit (Phoenix Flow Systems, Inc., San Diego, CA) following the manufacturer's protocol. DNA fragmentation was also analyzed by DNA ladder formation in agarose gels as described previously (Oridate et al., 1996).

Measurement of Intracellular ROS. Cells were seeded at 1×10^5/well in 48-well tissue culture plates. The intracellular generation of ROS was measured using of the oxidation-sensitive fluorescent dye DCF-DA as previously described (Delia et al., 1997). Briefly, on the second day after seeding, cells were washed twice with Hank's balanced salt solution (GIBCO BRL) and loaded with 500 μl Hank's balanced salt solution containing 10 μg/ml of DCF-DA and different concentrations of tested compounds (e.g., 4HPR and antioxidants). The cells were then incubated at 37°C for 90 min and the fluorescence intensity was measured at 530 nm after excitation at 480 nm in a CytoFluor 2350 Fluorescence Measurement System (Millipore, Bedford, MA). The increase in fluorescence intensity was used to represent the generation of net intracellular ROS. Four wells were used for each treatment and the mean ± S.D. were determined.

Reporter Plasmids, Transient Transfection, and Luciferase Assay. The retinoic acid response element (RARE)-tk-luc reporter plasmid was kindly provided by Dr. J. Kurie (University of Texas, M. D. Anderson Cancer Center, Houston, TX). It contains a trimer of the RARβ RARE. Wild-type p21 (WAF1) promotor-luciferase reporter (WWP-luc) and p21 promotor deletion mutant (no p53 binding site)-luciferase reporter (DM-luc) plasmids were obtained from Dr. W. Zhang (University of Texas M. D. Anderson Cancer Center). The procedures for plasmid purification, transfection, and luciferase assay were described previously (Sun et al., 1997a).

RNA Purification and Northern Blotting. Purification of total cellular RNA and northern blotting were performed as previously described (Sun et al., 1997b). Jac. 1, a plasmid containing the mouse c-*jun* cDNA, and GST-CIP1, a plasmid containing human p21 (WAF1) cDNA, were obtained from ATCC. pBR28, a plasmid containing human c-*fos* cDNA, and pSVC Myc-1, a plasmid containing mouse c-*myc* cDNA, were obtained from Dr. Paul Chiao (University of Texas, M. D. Anderson Cancer Center). RAR and retinoid X receptors (RXR) cDNA fragments were the same as described previously (Sun et al., 1997b). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a control for RNA loading.

Protein Preparation and Western Blotting. Cells were washed in PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% sodium dodecaylsulfate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. After incubation on ice for 15 min, the lysates were subjected to centrifugation at 12,000 rpm, and the resulting supernatants were collected. Protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA). Each lane of a 10% polyacrylamide slab gel received 80 μg of protein. After electroporesis and transfer to nitrocellulose membrane (Bio-Rad) by electroblotting, blots were probed with specific primary and secondary antibodies and the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL) according to the manufacturer's protocol. Mouse monoclonal anti-p53 antibody (Ab-6) (Calbiochem, La Jolla, CA), mouse monoclonal anti-WAF1 antibody (Ab-1) (Calbiochem), mouse monoclonal anti-bcl-2 antibody (Dako, Carpinteria, CA), rabbit polyclonal anti-bcl-X$_{S/L}$ antibody (S-18) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal ant-bax antibody (Upstate Biotechnology, Lake Placid, NY), and mouse monoclonal anti-β-actin antibody (Sigma) were used for Western blotting.

Results

Effects of 4HPR on Survival of HPC Cells. To evaluate the effects of 4HPR on the growth of HPC cells, we used the three most commonly used cell lines including one that was androgen dependent (LNCaP) and two that were androgen independent (PC-3 and DU145) (Table 1). Treatment of the

three HPC cell lines with 4HPR resulted in a concentration-and time-dependent decrease in cell number compared with control cells (Fig. 1). The IC_{50}s for LNCaP, DU145, and PC-3 were 0.9 ± 0.16 μM, 4.4 ± 0.45 μM and 3.0 ± 1.0 μM, respectively (Table 1), indicating that the LNCaP androgen-dependent cells were more sensitive to 4HPR treatment than DU145 and PC-3 androgen-independent cells. Notwithstanding this difference, 4HPR was able to affect all three cell lines once its concentration and treatment time were increased (Fig. 1B). In contrast with 4HPR, **ATRA** was ineffective in inhibiting the growth of the HPC cells (Fig. 1A). This difference suggests that 4HPR may act by a mechanism that is distinct from the one ascribed to **ATRA**, namely, the nuclear retinoid receptor pathway.

Induction of Apoptosis by 4HPR. Observation under the microscope of 4HPR-treated cultures revealed that cells rounded up, detached, and floated, suggesting that 4HPR caused cell death. Evidence that this cell death was by apoptosis came from the finding of increased DNA fragmentation detected by the TUNEL assay (Fig. 2A) after a 2-day exposure to 5 μM 4HPR (for PC-3 and DU145 cells) or 1 μM 4HPR (for LNCaP cells). Figure 2B shows the formation of a DNA ladder in LNCaP cell line after a 48-h treatment with 5 μM 4HPR. A DNA ladder was also found in PC-3 and DU145 cells treated with 10 μM 4HPR for 2 days (data not shown).

Suppression of 4HPR-Induced Apoptosis by Antioxidants and Stimulation of ROS Production by 4HPR. 4HPR-induced apoptosis could be inhibited in some cells by antioxidants and ROS were implicated in 4HPR-induced apoptosis in human leukemia (Delia et al., 1997) and cervical carcinoma (Oridate et al., 1997) cells. To determine whether ROS are involved in 4HPR-induced apoptosis in HPC cells, we analyzed the effects of antioxidants on 4HPR-induced apoptosis and the effects of 4HPR on ROS generation. Figure 2C shows that the antioxidant BHA was able to suppress the induction of DNA ladder formation in 4HPR-treated LNCaP cells. Furthermore, BHA was able to rescue LNCaP cells from apoptosis induction by 4HPR (Fig. 3A). In contrast, BHA had only a minor effect on 4HPR's ability to induce apoptosis in DU145 and PC-3 cells (Fig. 3A). The differential effect of BHA on cell survival became clearer when the effects of 4HPR on ROS generation were investigated. Figure 3B shows that 4HPR was able to increase ROS levels in all three HPC cell lines by 3- to 4.5-fold. However, the total amount of ROS in 4HPR-treated LNCaP cells was much higher than in the two other cell lines. BHA suppressed 4HPR-induced ROS generation in all three cell lines but affected cell survival only in LNCaP cells (Fig. 3A). Presumably, 4HPR-induced ROS mediated apoptosis in LNCaP cells but not in DU145 and PC-3 cells, in which the absolute amount of increased ROS

may be below a putative threshold required for signaling apoptosis.

Effects of 4HPR on Expression of Nuclear Retinoid Receptors. Because the induction of apoptosis in DU145 and PC-3 cells could not be explained by induction of ROS, it was of interest to explore the possible role of nuclear receptors in this effect. Therefore, we examined the expression of the receptors in HPC cells and determined whether 4HPR exerts any effect on receptor expression. As shown in Fig. 4, the constitutive expression of RARβ mRNA was detected only in LNCaP cell but not in DU145 or PC-3 cells, whereas RARα, RARγ, and RXRα mRNAs were detected in all of the cell lines. 4HPR treatment modulated receptor expression differentially in the three HPC cell lines. Only minor changes in receptor mRNA levels were detected in PC-3 cells, whereas 4HPR increased RARα in DU145 and, to a lesser extent, also in LNCaP cells. 4HPR also increased RARγ and RXRα and suppressed RARβ in LNCaP cells.

Suppression of 4HPR-Induced Apoptosis by an RAR-Specific Antagonist. To determine whether retinoid receptors are involved in the apoptotic signaling pathway of 4HPR in some HPC cells, we used the RAR-α, -β, and -γ-specific antagonist CD2366 (Sun et al., 1997b) to determine whether it could antagonize the induction of apoptosis by 4HPR. First, we demonstrated that 4HPR activated the retinoid receptor signaling pathway as indicated by luciferase induction in DU145 cells transfected with RARE-tk-Luc (Fig. 5A). At a concentration of 2.5 μM, 4HPR activated transcription via RARE but its effect was lower than that of **ATRA**'s (Fig. 5A). This RARE transactivation was almost completely abolished by 2-fold molar excess of CD2366 (Fig. 5A). However, the effect of 2.5 μM 4HPR on cell survival was suppressed only partially in DU145 cells and minimally in LNCaP and PC-3 cells in the presence of 2-fold molar excess of CD2366 (Fig. 5B). These results suggest that RARs may play some role in mediating apoptosis 4HPR-induced apoptosis, at least in DU145 cells.

Modulation of p53 Expression by 4HPR. The three HPC cell lines used in this study differ in their p53 status as shown in Table 1. Western blot analysis indicated that p53 protein was up-regulated by 4HPR in LNCaP cells, which express wild-type p53 but not in DU145 and PC-3 cells, which express mutant p53 (Carroll et al., 1993) (Fig. 6). The induction of p53 protein in LNCaP cells was time dependent (Fig. 6), beginning at 8 h and increasing further through 32 h after exposure to 4HPR.

Modulation of p21 Expression by 4HPR. p21 protein expression was induced by 4HPR at 1 μM in LNCaP cells or at 5 μM in DU145 and PC-3 cells (Fig. 6). The induction of p21 was detected after 8 h and increased further at 16 and

TABLE 1
Characteristics of HPC cell lines used in study

Cell Line	Androgen Dependence	Source	p53 Status[a]	Response to Retinoids (IC_{50})[b]	
				ATRA	4HPR
				μM	
LNCaP	Yes	Metastasis to supraclavicular lymph node	Wild type	>10	0.9 ± 0.16
DU145	No	Metastasis to brain	Mutant	>10	4.4 ± 0.45
PC-3	No	Primary	Mutant	>10	3.0 ± 1.0

[a] Carroll et al., 1993.
[b] From Fig. 1A.

32 h in all three HPC cell lines. Interestingly, induction of p21 mRNA was detected in PC-3 at 4 h and LNCaP cells at 16 h (Fig. 7). In DU145 cells, only a small increase in p21 mRNA was observed at 32 h after 4HPR treatment, which was later than the increase in protein level (Fig. 7). In LNCaP cells, no activation by 4HPR of a luciferase reporter gene containing a fragment of the p21 promoter with p53 binding sites was observed (data not shown).

Modulation of Bcl-2, Bax, and Bcl-x$_L$ Expression by 4HPR. One way by which 4HPR could induce apoptosis is by decreasing the levels of the antiapoptotic proteins bcl-2 and bcl-x$_L$ or increasing the levels of the proapoptotic protein bax

(Adams and Cory, 1998). We found that the HPC cells differed in the constitutive expression of the above proteins. LNCaP and PC-3 cells expressed all of them, whereas DU145
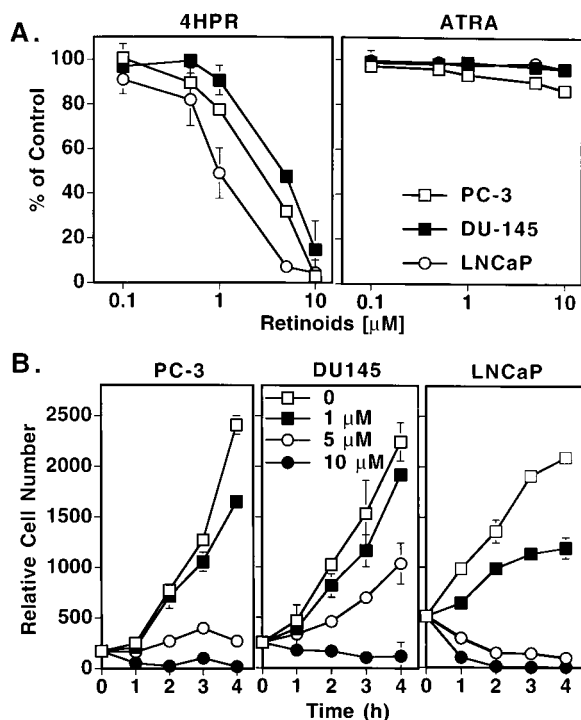

Fig. 1. Concentration- (A) and time- (B) dependent effects of 4HPR on growth of HPC cells. Cells were seeded at 3000 to 6000 cells per well in 96-well culture plates. After 4 days (A) or indicated time (B) of treatment with 4HPR, cell number was determined using Alamar blue reagent. Each point represents mean ± S.D. of quadruplicate determinations.
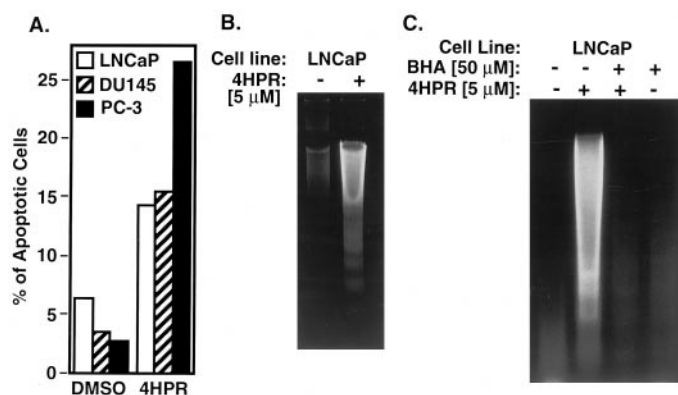

Fig. 2. 4HPR-induced apoptosis evidenced by increased TdT-labeled DNA fragments (A) and by formation of DNA ladder (B and C) in HPC cells. Cells were seeded in 10-cm dishes 1 day before treatment and harvested after 48 h treatment with 4HPR at a concentration of 1 μM (LNCaP) or 5 μM (DU145 and PC-3) for TUNEL assay (A) and 5 μM for DNA ladder analysis (B and C).
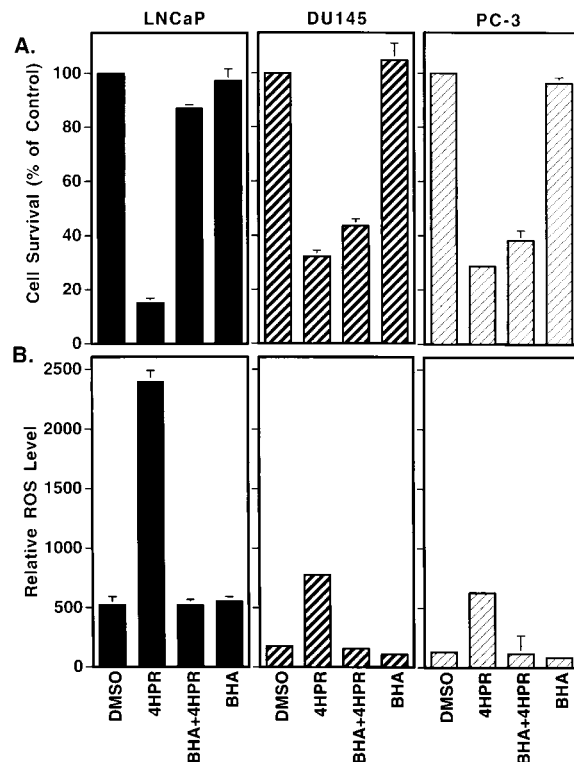

Fig. 3. Stimulation of ROS production by 4HPR and effects of antioxidant BHA on 4HPR-induced ROS production and apoptosis in HPC cells. A, cells were treated with 2.5 μM (LNCaP) or 5 μM (DU145 and PC-3) 4HPR, 50 μM BHA, or BHA plus 4HPR for 3 days. Relative number of cells was analyzed using Alamar blue reagent. Each bar represents mean ± S.D. of quadruplicate determinations. B, cells were exposed to 5 μM 4HPR, 50 μM BHA, or BHA plus 4HPR for 90 min and ROS production was determined by DCF-DA assay. Each bar is mean ± S.D. of quadruplicate determinations.
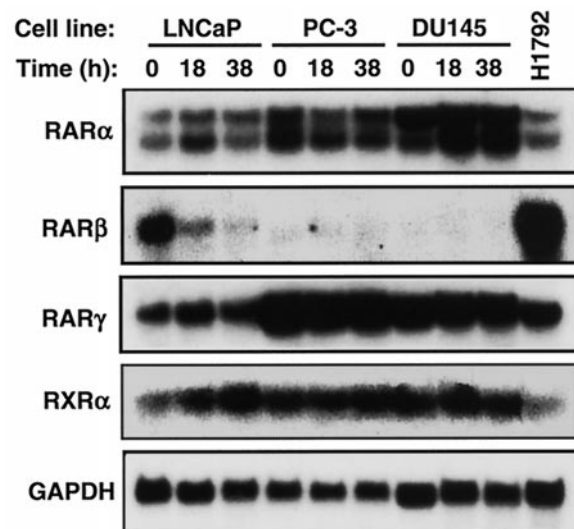

Fig. 4. Constitutive expression of nuclear retinoid receptors and their modulation by 4HPR in HPC cells. Cells were treated 1 day after seeding with 1 μM (LNCaP) or 5 μM (DU145 and PC-3) of 4HPR for indicated times. Total RNA was then isolated and analyzed by Northern blotting using 30 μg RNA per lane. RNA from cell line H1792 was used as a positive control, especially for RARβ.

expressed bcl-x$_L$ but had undetectable levels of bcl-2 and bax. 4HPR treatment failed to modulate bcl-2, bcl-x$_L$, or bax in LNCaP cells (Fig. 6A). However, the levels of bcl-x$_L$ decreased by 4HPR in PC-3 and DU145 cells (Fig. 6, B and C).

Modulation of Oncogenes c-*myc*, c-*jun*, and c-*fos* Expression by 4HPR. c-*myc*, c-*fos*, and c-*jun* have been implicated in apoptosis (Smeyne et al., 1993; Packham and Cleveland, 1995; Preston et al., 1996; Karin et al., 1997). Therefore, we analyzed their expression and modulation by 4HPR in the three HPC cell lines. c-*fos* was not detected in any of the cell lines by Northern blotting of total RNA (Fig. 8A). The levels of c-*myc* mRNA was lower in PC-3 than in DU145 and LNCaP cells and the level of c-*jun* was lower in LNCaP cells than in the two other cell lines. 4HPR did not modulate c-*fos* mRNA expression in all three HPC cell lines. However, 4HPR did modulate c-*myc* and c-*jun* gene expression (Fig. 8). c-*jun* mRNA was up-regulated in all cell lines, especially in PC-3 and LNCaP cells, whereas c-*myc* mRNA was up-regulated in PC-3 and DU-145 cells but down-regulated in LNCaP cells. The modulations of c-*myc* and c-*jun* expression by 4HPR were observed as early as 4 h before

apoptosis happened. Therefore, c-*myc* and c-*jun* might also be important mediators of 4HPR-induced apoptosis in HPC cells.

Discussion

We found that 4HPR induces apoptosis in both androgen-dependent and -independent HPC cells. High concentrations (3–10 μM) of 4HPR were needed to induce apoptosis in the androgen-independent PC-3 and DU145 cells, as was found in human hemopoietic cells, lung, cervical, and head and neck cancer cells (Sun and Lotan, 1998). However, 4HPR induced apoptosis in the androgen-dependent LNCaP cells even at <1 μM.

Recently, enhanced ROS generation has been suggested to mediate 4HPR-induced apoptosis in human cervical cancer (Oridate et al., 1997) and leukemia (Delia et al., 1997) cells. In the present study, 4HPR stimulated ROS production, and the antioxidant BHA completely blocked this effect in LNCaP cells. Furthermore, BHA reversed 4HPR-induced apoptosis. These results indicated that ROS is critical in mediating apoptosis induced by 4HPR in LNCaP cells. In contrast, 4HPR stimulated only a low level of ROS production and BHA had only minimal effects on 4HPR-induced apoptosis in the two androgen-independent cell lines. It has been proposed previously by us (Oridate et al., 1997) that a threshold exists for ROS to trigger apoptosis. This proposal is further supported by our present results. We found that 4HPR induced an approximate 4.5-fold increase in ROS production in all of three HPC cell lines. However, the absolute levels of ROS in DU145 and PC-3 cells were much lower than in LNCaP cells. Because BHA failed to suppress 4HPR-induced apoptosis in DU145 and PC-3 cells, the apoptotic signaling pathway in these two cell lines appears to be independent of ROS production.

It is thought that the biological activities of retinoids are mediated by two classes of nuclear retinoid receptors: the RARs and the RXRs, which are members of the steroid hormone receptor gene superfamily (Chambon, 1996). Both types of receptors include at least three subtypes, which are encoded by three distinct types of gene, α, β, and γ. The RARs bind **ATRA** and 9-*cis*-RA, whereas the RXRs bind only 9-*cis*-RA (Chambon, 1996). These receptors, as well as additional members of the nuclear hormone receptor family, form stable heterodimers, as well as homodimers, to function as ligand-activated transcription factors that regulate the transcription activity of target genes by binding to response elements (REs) specific for RARs (RAREs) or RXRs (RXREs) located in the promoter region of these genes. All three HPC cell lines expressed constitutively RARα, RARγ, and RXRα, whereas
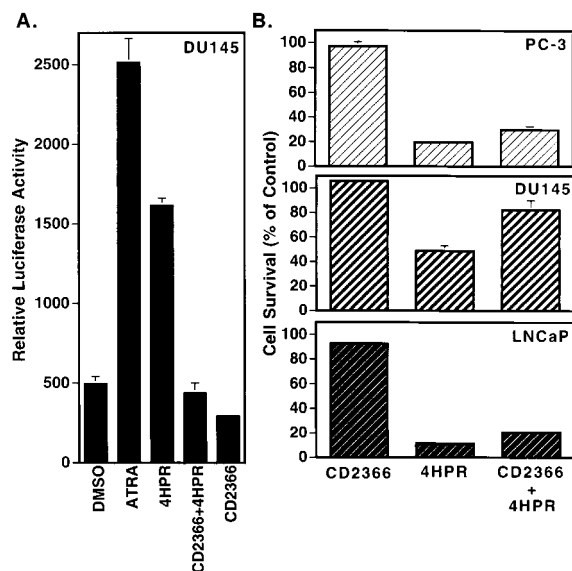
Fig. 5. Effects of RAR-pan antagonist on transactivation of RARE by 4HPR (A) and on 4HPR-induced apoptosis (B). A, DU145 cells were plated in 6-well plates 1 day before transient cotransfection with RARE-tk-luc plasmid and β-gal enzyme-encoding plasmid using lipofectamine reagent. Luciferase activity and β-gal activity were determined after 16 h of treatment with 1 μM **ATRA**, 2.5 μM 4HPR, or 2.5 μM 4HPR plus 5 μM CD2366. Each bar is mean ± S.D. of quadruplicate determinations. B, cells were treated with 2.5 μM 4HPR, 5 μM CD2366, or 4HPR plus CD2366 for 4 days. Cell number was determined using Alamar blue reagent. Each bar is mean ± S.D. of quadruplicate determinations
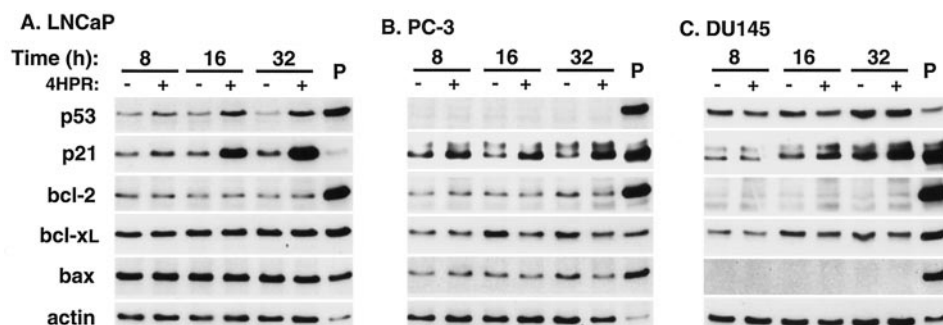
Fig. 6. Modulation of expression of p53, p21, bcl-2, bcl-x$_L$, and bax proteins by 4HPR in HPC cells. Cells were treated 1 day after seeding with 1 μM (LNCaP) or 5 μM (DU145 and PC-3) 4HPR for indicated times. Whole cell lysates were prepared and the proteins were analyzed by Western blotting. The same membrane was repeatedly blotted, stripped, and reblotted with different antibodies. Level of actin was used as a control for loading. Lane on the right (P) was an extract of retinoid CD437-treated H460 cells that serves as a positive control for the different proteins.

only LNCaP cells expressed RARβ. However, it is not likely that RARβ expression was important for the enhanced response of LNCaP cells to 4HPR treatment because 4HPR suppressed RARβ expression. This result is different from what was observed in other cell types in which 4HPR increased RARβ (Swisshelm et al., 1994). The mechanism by which 4HPR suppressed RARβ in LNCaP cells is not clear. It is noteworthy that in another cell type, NIH-3T3 fibroblasts, retinoic acid suppressed the level of RARα mRNA (Scita et al., 1996).

There is no direct evidence that 4HPR binds to any of the known retinoid receptors. For example, Sheikh et al. (1995) failed to identify binding of 4HPR or its major metabolite *N*-(4-methoxyphenyl)retinamide (**4 MPR**) to RARs in vitro. Nonetheless 4HPR was able to transactivate RARE through all three RARs at 1 to 10 μM in human breast carcinoma cells, although this transactivation was weaker than by **ATRA** (Sheikh et al. 1995; Kazmi et al., 1996). In our study, 4HPR (2.5 μM) induced transcriptional activity of RARE in DU145 HPC cells, although it was less potent than 1 μM **ATRA** (Fig. 5). Because the RARE transcriptional activity induced by 4HPR could be completely abolished by the RAR-specific antagonist CD2366 (Fig. 5), we suggest that RARs are involved in the RARE transactivation by 4HPR. Furthermore, 4HPR-induced apoptosis could be suppressed by CD2366 in DU145 cells, indicating that RARs are also involved in mediating 4HPR-induced apoptosis in HPC DU145 cells.

ROS and RARs may be important mediators of 4HPR-induced apoptosis in different HPC cells. However, this does not exclude other mechanisms by which 4HPR induces apoptosis in HPC cells because neither antioxidant nor receptor antagonist exerted marked suppressive effects on 4HPR-induced apoptosis in PC-3 cells. To further understand the apoptotic mechanism of 4HPR, the expression of apoptosis-related genes such as c-*myc*, c-*jun*, p53, and p53's downstream targeted genes p21, bcl-2, bcl-x, and bax, and their modulation by 4HPR was investigated. LNCaP cells with wild-type p53 (Carroll et al., 1993) were found to be much more sensitive to 4HPR treatment than PC-3 and DU145 cells with mutant p53 (Carroll et al., 1993). p53 protein was only up-regulated by 4HPR in LNCaP cells but not in Du145 and PC-3 cells (Fig. 6). These results suggest that wild-type p53 may be involved in 4HPR-induced apoptosis in LNCaP cells.

p21, which has been shown to induce G1 arrest by inhibition of cyclin-dependent kinase and proliferating cell nuclear antigen-dependent DNA replication, was recently identified as one of the wild-type p53-regulated gene (El-Deiry et al., 1993; Li et al., 1994). Exposure of cells to DNA-damaging agents leads to p53-dependent induction of p21 expression, resulting in growth arrest or apoptosis (Fan et al., 1994). In addition, induction of p21 can occur by p53-independent mechanisms in response to mitogenic stimuli, differentiation, or in tumor cells with mutant p53 (Sheikh et al., 1994). 4HPR markedly increased the expression of p21 protein in all three HPC cell lines (Fig. 6) albeit by different mechanisms. Whereas in PC-3 cells, p21 mRNA increased earlier than the increase in protein, in LNCaP cells, and even more so in DU145 cells, the increase in p21 mRNA was slower than the increase in protein, suggesting that both transcriptional and post-transcriptional mechanisms may account for the effects
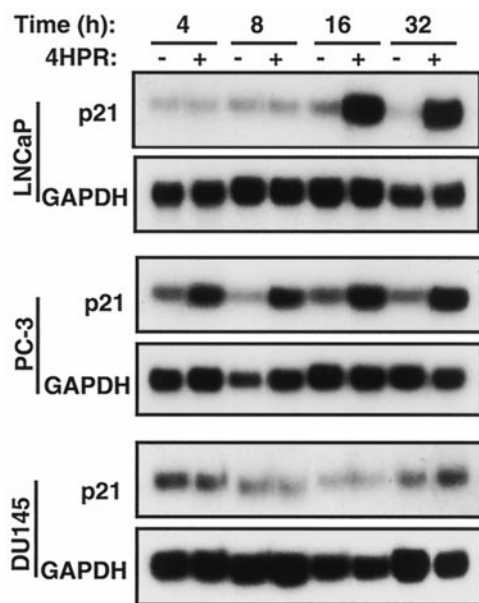

Fig. 7. Modulation of p21 mRNA by 4HPR in HPC cells. Cells were treated 24 h after seeding with 1 μM (LNCaP) or 5 μM (DU145 and PC-3) 4HPR for indicated times. Total RNA was isolated and subjected to Northern blotting (30 μg/lane). GAPDH mRNA level was determined to compare loading and transfer in different lanes.
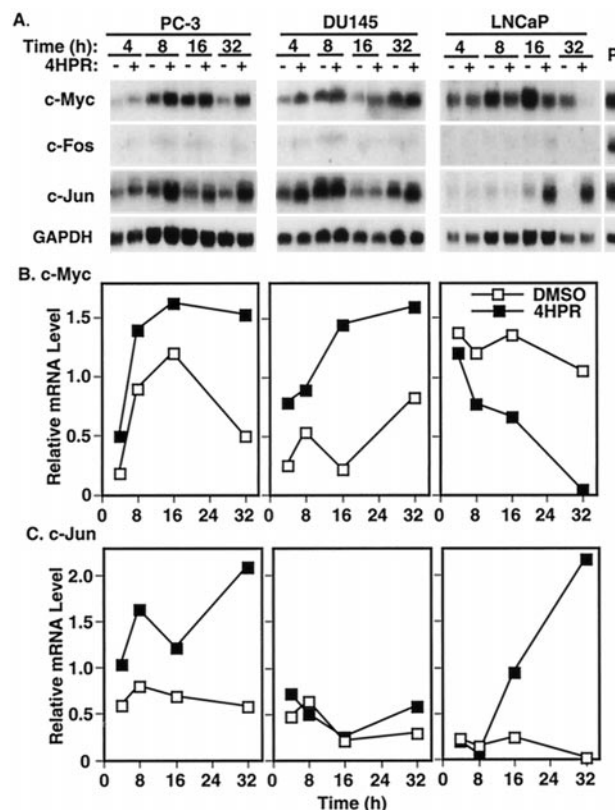

Fig. 8. Modulation of c-*myc*, c-*fos*, and c-*jun* mRNAs by 4HPR in HPC cells. Cells were treated on second day after seeding with 1 μM (LNCaP) or 5 μM (PC-3 and DU145) 4HPR for indicated times. Total RNA was isolated and subjected to Northern blotting using 30 μg RNA per lane. A, Northern blot results; B, quantitation of c-*myc* mRNA that is normalized by GAPDH mRNA; C, quantitation of c-*jun* mRNA that is normalized by GAPDH mRNA.

of 4HPR on p21. The induction of p21 by 4HPR occurred before the induction of apoptosis, suggesting that it may be an important event in 4HPR signaling of apoptosis. The induction of p21 by 4HPR in PC-3 and DU145 cells with mutant p53 was wild-type p53-independent. However, even in LNCaP cells that contain wild-type p53, the fold induction of p53 protein by 4HPR was much less than the increase of p21 protein level and 4HPR did not increase the luciferase activity controlled by p21 promoter with p53 binding sites in LNCaP cells (data not shown). Therefore, the increase in p21 expression by 4HPR in LNCaP cells might be also p53 independent.

Bcl-2, bcl-x_L, and bax have been implicated as major players in the control of apoptosis pathway (Adams and Cory, 1998). Bcl-2 and bcl-x_L promote cell survival, whereas bax promotes cell death (Adams and Cory, 1998). Bax was shown to homodimerize (bax:bax), as well as to form heterodimers with bcl-2 (bax:bcl-2), and it was suggested that the ratio of bcl-2 to bax determines survival or death following a apoptotic stimulus (Adams and Cory, 1998). 4HPR did not modulate the expression of bcl-2 and bax in any of the cell lines, suggesting that these genes are not involved in the induction of apoptosis by 4HPR in HPC cells. The level of bcl-x_L protein was down-regulated by 4HPR in PC-3 and DU145 androgen-independent cells. Because this protein plays an antiapoptotic role, its decrease by 4HPR may also contribute to the induction of apoptosis by 4HPR in androgen-independent HPC cells.

The oncogenes c-*myc*, c-*fos*, and c-*jun* have also been implicated in induction of apoptosis in some cell systems (Smeyne et al., 1993; Packham and Cleveland, 1995; Preston et al., 1996; Karin et al., 1997). c-*myc* has an established role in the promotion of cell proliferation, differentiation, and transformation (Packham and Cleveland, 1995). However, recent studies have demonstrated that increased expression of c-*myc* increased cellular susceptibility to apoptosis induction (Stewart, 1994; Packham and Cleveland, 1995). 4HPR enhanced c-*myc* expression in DU145 and PC-3 cells, which occurred early before apoptosis happens. Therefore, it may also contribute to induction of apoptosis by 4HPR in HPC cells. In contrast with DU145 and PC-3 cells, 4HPR treatment of LNCaP cells decreased c-*myc* level. Interestingly, down-regulation of c-*myc*, which leads to induction of apoptosis was reported in other cells (Davidoff and Mendelow, 1993; Sonenshein, 1997).

c-*jun* and c-*fos* that form either homodimers or heterodimers and bind to the DNA consensus sequence TGA(C/G)TCA (named the 12–0-tetradecanoylphorbol 13-acetate RE) in the promoter regions of several genes are found to be very important factors in induction of apoptosis (Smeyne et al., 1993; Preston et al., 1996; Karin et al., 1997). 4HPR did not modulate c-*fos* gene expression in any of the HPC cell lines. However, 4HPR up-regulated c-*jun* expression, especially in LNCaP and PC-3 cells. This induction seemed to correlate with the induction of apoptosis in the three HPC cell lines. These data suggest that c-*jun* may be another important mediator of apoptosis by 4HPR in HPC cells.

In conclusion, the induction of apoptosis by 4HPR in the three HPC cell lines appears to be mediated by several mechanisms that are distinct and specific for each of the cell lines. Thus, in LNCaP cells, the increase in ROS may be a major pathway leading to apoptosis with some contribution from increased p53, p21, c-*jun*, and decreased c-*myc*. In DU145 cells, 4HPR-induced apoptosis may involve RARs, an increase in p21, a decrease in bcl-X_L, and an increase in c-*myc*. Lastly, 4HPR-induced apoptosis in PC-3 cells may be mediated by increased p21, decreased bcl-X_L, and increased c-*myc* and c-*jun*. Whereas this study is the first to show that 4HPR can modulate c-*myc* and c-*jun*, additional more direct studies are needed to assess the role of these molecules in apoptosis induction.

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