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Effects of N-(4-hydroxyphenyl) Retinamide on Urokinase-type Plasminogen Activator and Plasminogen Activator Inhibitor-1 in Prostate Adenocarcinoma Cell Lines

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ABSTRACT

Previous investigations have demonstrated that a synthetic retinoid, N-(4-hydroxyphenyl) retinamide (4-HPR), inhibits the invasion of prostate adenocarcinoma *in vitro*. Urokinase-type plasminogen activator (uPA) is a prerequisite for tumor invasion. The purpose of this study was to evaluate the effects of 4-HPR on uPA and plasminogen activator inhibitor-1 (PAI-1) in prostate cancer.

Human prostate adenocacinoma cell lines, TSU-PR1 and PC3, were grown in serum-free media containing 4-HPR. Cellular mRNA and protein were subsequently extracted. Northern blot analysis, enzyme-linked immunosorbent assay (ELISA) and chromogenic functional analysis were performed on the samples.

Administration of 10^{-6}M 4-HPR for 3 days resulted in an increase in uPA mRNA expression (TSU-PR1: 391%, PC3: 356%), and a simultaneous increase in PAI-1 mRNA expression (TSU-PR1: 217%, PC3: 235%) was observed. ELISA concomitantly demonstrated a significant increase ($p<0.05$) in uPA protein in the conditioned media (TSU-PR1: 134%, PC3: 139%) and cell lysates (TSU-PR1: 284%, PC3: 255%). Both cell lines demonstrated a significant increase ($p<0.05$) in PAI-1 protein in the conditioned media (TSU-PR1: 152%, PC3: 167%) and cell lysates (TSU-PR1: 170%, PC3: 222%). Concentrations below 10^{-6}M failed to alter the protein production of either uPA or PAI-1. The functional uPA assay demonstrated a reduction of the proteolytic activity of uPA (TSU-PR1: 13%, PC3: 7%) in cell lysates of 10^{-6}M 4-HPR ($p<0.05$), while there was minimal uPA activity in the conditioned media.

4-HPR stimulates a paradoxical increase in uPA and PAI-1, but the anti-invasive effects of 4-HPR are consistent with the increase in both uPA and PAI-1, resulting in an overall reduction of functional uPA activities.

Key words: *Prostate cancer, 4-HPR, uPA, PAI-1*

Retinoids are a group of vitamin A metabolites and synthetic analogues which act as transcriptional regulators within the nucleus and have been shown to promote the differentiation and growth of cells. Recently retinoids have been demonstrated to suppress the development of numerous human and experimental tumors[12,16,21]. Furthermore, N-(4-hydroxyphenyl) retinamide (4-HPR) is one of the retinoids, which was synthesized in the United States in the late 1960's as both preventive and therapeutic agents against mammary carcinoma[15]. 4-HPR has demonstrated chemopreventive effects against malignant epithelial cells such as breast, bladder, ovarian and prostate carcinoma[4,5,14,18]. The inhibition of *in vitro* invasion of prostate cancer cell lines by 4-HPR has been previously reported[8].

Tumor invasion has many stages, including degradation of the extracellular matrix[13]. Urokinase-type plasminogen activator (uPA) is primarily responsible for plasmin generation in processes involving degradation of the extracellular matrix[17]. The tissue of primary cancers and metastatic tumors of the breast, ovary, prostate, cervix uteri, lung and gastrointestinal tracts have been reported to contain high amounts of uPA compared to benign control tissues[19]. These findings suggest that aberrations in the normally regulated expression of uPA occur in tumor cells and that uPA may be necessary for tumor invasion. uPA has several types of inhibitors, one of which is plasminogen activator inhibitor-1 (PAI-1). uPA and PAI-1 were found to be abundant in many tumors and, generally, areas that contained uPA also contained PAI-1[1].

The present study focused on uPA and PAI-1 to investigate the anti-invasive effect of 4-HPR in prostate adenocarcinoma cell lines. Furthermore, the mechanism of expressing the effects of 4-HPR on uPA and PAI-1 in prostate cancer was sur-

mised.

MATERIALS AND METHODS

Materials

4-HPR was generously supplied by R.W. Johnson Pharmaceutical Research Institute (Spring House, PA). It was prepared as a sterile stock solution of 10mM in dimethyl sulfoxide (DMSO) and stored at –70°C for less than 3 weeks. Cycloheximide (CHX) and Actinomycin D (ACD) were obtained from Sigma (St. Louis, MO). CHX (15μM final concentration) and ACD (10μM final concentration) were added in fresh medium either alone or in combination with 10^{-6}M 4-HPR in the experiments with CHX and ACD.

Cell culture

Human invasive prostate adenocarcinoma cell lines, TSU-PR1 (the gift of Dr. Iizumi, the University of Tsukuba, Ibaraki, Japan)[9] and PC-3 (ATCC, Rockville, MD)[10], were routinely grown and maintained in a humid atmosphere of 5% CO_2 / 95% air at 37°C in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 10% or 14% fetal bovine serum, 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. The cells were placed in RPMI-1640 media containing a serum-free media additive of ITS+ (Collaborative Research, Bedford, MA), which contains insulin, transferrin, selenious acid, bovine serum albumin and linoleic acid, prior to conducting the experiments, and 4-HPR in concentrations ranging from 10^{-6}M to 10^{-8}M was added for the indicated time. The final concentration of DMSO in the media was always below 0.1% (V/V).

Northern blot analysis

mRNA was extracted with a Quick Prep mRNA Purification Kit (Pharmacia, Piscataway, NJ). Northern blot analysis was performed as described previously[6,20]. Equal amounts of mRNA were subjected to electrophoresis through a denaturing formaldehyde / agarose gel and transferred to a nylon membrane. Then the membrane was hybridized with uPA and PAI-1 probe labeled with ^{32}P by random priming (Multiprime DNA labelling system, Amersham, Arlington Heights, IL) and washed according to the protocols. The autoradiographs generated were analyzed using a densitometer (Molecular Dynamics, La Jolla, CA). β-actin probe was used to serve as the control for RNA loading.

Enzyme-linked immunosorbent assay (ELISA) for uPA and PAI-1

To determine uPA and PAI-1 protein, the conditioned media and cell lysates were analyzed for uPA and PAI-1 antigen using IMUBIND ELISA Kits (American Diagnostica, Greenwich, CT). After treatment with 4-HPR, the conditioned media were collected following removal of cell debris by centrifugation. The cell lysates were prepared by adding cell lysis buffer (1% Triton X-100 (Sigma) in 10mM Tris-HCl: pH 8.0), mixed vigorously and centrifuged for 3 min at 1500*g*. The supernatants were stored at –80°C as samples before assay. ELISA was performed according to the manufacturer's protocol and absorbance was measured on a microplate reader (Biorad, Melville, NY). The corresponding concentration in ng/ml was obtained from the standard curve and the results were expressed in ng/mg cellular protein, which was determined by the method of the Pierce BCA Protein Assay (PIERCE, Rockford, IL) for normalization of cell numbers.

Functional uPA activity assay

The uPA activity was determined utilizing the chromogenic substrate of SPECTROZYME UK (American Diagnostica), which is directly metabolized by uPA and generates chromophore. The assay was performed in microplate. 50μl of buffer (50nM Tris pH 8.8 with 0.01% Tween 80 and 10KIU/ml sterile aprotinin), 50μl of the chromogenic substrate and 50μl of the conditioned media or cell lysates were added to each well and incubated for 15 min at room temperature. The absorbance at 405nm was then determined using a microplate reader (Biorad). The absorbances were converted to international units (IU) by comparison with the standard curve generated from purified LMW-uPA (American Diagnostica). The results were normalized in the same way as ELISA.

Statistics

Every protein assay was done in triplicate, and statistical analyses were performed in the protein assay using Student's t test for all experimental conditions relative to control cells. Significant values were defined as a p value<0.05.

RESULTS

Effects of 4-HPR on uPA and PAI-1 mRNA expression

Northern blot analysis demonstrated that the 3 day treatment of TSU-PR1 and PC3 cell lines with 4-HPR resulted in an increased expression of uPA and PAI-1 mRNA. Treatment of 10^{-6}M 4-HPR enhanced uPA mRNA production by 391% and 356% in TSU-PR1 and PC3 cells relative to untreated cells. A simultaneous increase in PAI-1 mRNA expression by 217% and 235% in TSU-PR1 and PC3 respectively was observed at 10^{-6}M 4-HPR. Enhancement of uPA and PAI-1 mRNA expression was obtained dose-dependent, with the lower concentration of 4-HPR producing a less significant increase in mRNA production in both cell lines (Fig. 1 A, B).

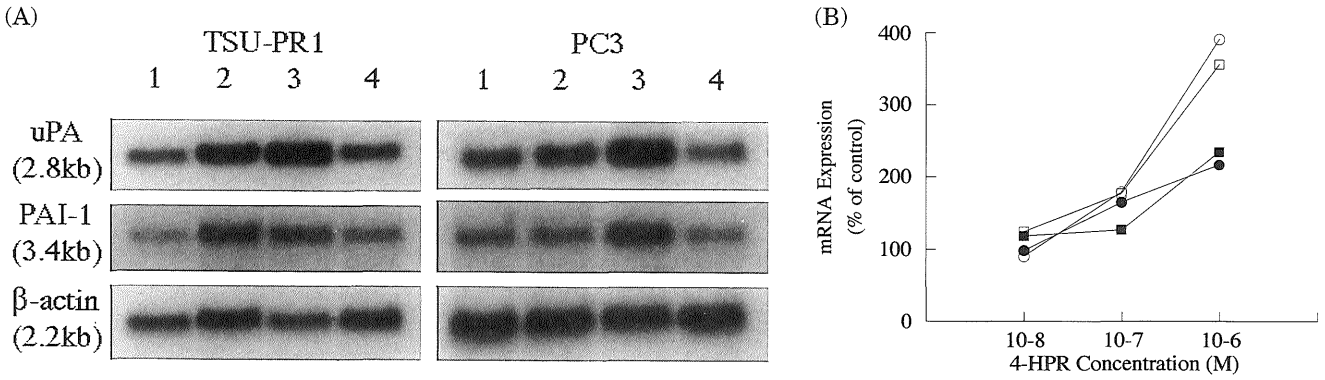

Fig. 1 A, B. mRNA expression of uPA and PAI-1 with 3 day exposure of 4-HPR:
(A) Northern blots of TSU-PR1 and PC3: lane 1 was 10^{-8}M 4-HPR, lane 2 was 10^{-7}M 4-HPR, lane 3 was 10^{-6}M HPR and lane 4 was control in each blot.
(B) Densitometer analyses: (○) uPa and (●) PAI-1 in TSU-PR1, (□) uPA and (■) PAI-1 in PC3

Table 1. uPa and PAI-1 protein of TSU-PR1 and PC3 with 3 day exposure of 4-HPR

Cell line	Sample	4-HPR concentration (M)	uPA (ng/mg)	PAI-1 (ng/mg)
TSU-PR1	Conditioned media	Control	217.2 ± 7.6	17.53 ± 0.16
		10^{-8}	218.7 ± 4.7	18.31 ± 0.45
		10^{-7}	209.8 ± 2.3	20.64 ± 0.30*
		10^{-6}	290.4 ± 10.4*	26.64 ± 0.36*
	Cell lysate	Control	141.8 ± 5.5	6.06 ± 0.20
		10^{-8}	136.9 ± 3.0	6.36 ± 0.11
		10^{-7}	129.8 ± 7.6	6.23 ± 0.13
		10^{-6}	402.0 ± 5.8*	10.31 ± 0.18*
PC3	Conditioned media	Control	148.2 ± 4.5	11.28 ± 1.37
		10^{-8}	120.1 ± 5.3	11.54 ± 0.51
		10^{-7}	143.5 ± 3.3	12.52 ± 0.34
		10^{-6}	206.2 ± 15.3*	18.82 ± 0.06*
	Cell lysate	Control	135.6 ± 4.1	2.22 ± 0.09
		10^{-8}	141.5 ± 4.3	3.02 ± 0.26*
		10^{-7}	147.4 ± 5.4	2.79 ± 0.23
		10^{-6}	345.6 ± 5.9*	4.93 ± 0.15*

Values are the means ± SD of triplicate samples.
* statistically significant ($p<0.05$)

Effects of 4-HPR on uPA and PAI-1 protein

ELISA concomitantly demonstrated that 3 day treatment of both cell lines with 4-HPR at a concentration of 10^{-6}M resulted in a significant increase of uPA and PAI-1 protein in the cell lysates as well as in the conditioned media. 10^{-6}M 4-HPR increased uPA protein in the conditioned media (TSU-PR1: 134%, PC3: 167%) and cell lysates (TSU-PR1: 284%, PC3: 255%) and PAI-1 protein in the conditioned media (TSU-PR1: 152%, PC3: 167%) and cell lysates (TSU-PR1: 170%, PC3: 222%). In comparison, a lower concentration of the drug (10^{-8}M and 10^{-7}M) failed to alter the production of uPA and PAI-1 protein in the cell lysates and conditioned media of either cell line (Table 1). Sequence-timed analyses revealed that these uPA and PAI-1 protein increases were observed at every time point (1 day, 2 days and 3 days) (Table 2).

Effects of 4-HPR on functional uPA activity

The uPA chromogenic assay for the cell lysates of TSU-PR1 and PC3 cells treated with 10^{-6}M 4-HPR demonstrated a mild but significant reduction of proteolytic activity (TSU-PR1: 13%, PC3: 7%), while concentrations below 10^{-6}M had no detectable effect on uPA activity in either cell line (Table 3). There was minimal uPA activity in the conditioned media. Sequence-timed analyses revealed that these decreases of functional uPA activity in TSU-PR1 and PC3 were observed at every time point (Table 4).

Effects of CHX on uPA and PAI-1 mRNA increases induced by 4-HPR

In order to evaluate whether 4-HPR-induced increases in uPA and PAI-1 mRNA levels are direct or require the intermediate induction of cellular regulatory proteins, the effect of blocking

Table 2. Sequence-timed analyses for uPA and PAI-1 protein of TSU-PR1 and PC3 with 4-HPR exposure

Cell line	Sample	Time (day)	uPA (ng/mg)		PAI-1 (ng/mg)	
			Control	10^{-6}M 4-HPR	Control	10^{-6}M 4-HPR
TSU-PR1	Conditioned media	1	14.23 ± 0.04	14.98 ± 0.05*	15.51 ± 1.10	19.01 ± 3.35
		2	58.10 ± 2.45	69.25 ± 2.51*	18.47 ± 0.81	21.24 ± 4.64
		3	91.78 ± 3.62	112.78 ± 4.07*	20.36 ± 0.51	27.06 ± 1.33*
	Cell lysate	1	12.05 ± 0.12	15.41 ± 0.28*	5.55 ± 1.01	8.97 ± 2.65
		2	30.36 ± 0.26	70.73 ± 0.93*	4.50 ± 0.37	6.26 ± 2.01
		3	36.87 ± 0.56	104.51 ± 8.28*	5.50 ± 0.12	8.02 ± 1.68
PC3	Conditioned media	1	10.90 ± 0.35	9.55 ± 0.20	7.80 ± 0.99	10.95 ± 3.14
		2	37.60 ± 1.76	47.66 ± 0.46*	9.56 ± 1.01	11.98 ± 2.64
		3	47.92 ± 0.59	66.51 ± 1.15*	9.71 ± 0.26	14.41 ± 1.24*
	Cell lysate	1	12.58 ± 0.54	19.04 ± 0.35*	1.34 ± 1.32	5.25 ± 3.81
		2	27.51 ± 0.85	50.80 ± 0.81*	1.62 ± 0.81	3.88 ± 2.61
		3	31.36 ± 1.33	53.23 ± 1.17*	2.77 ± 0.56	5.04 ± 2.35

Values are the means ± SD of triplicate samples.
* statistically significant ($p<0.05$)

Table 3. Functional uPA activity in cell lysates of TSU-PR1 and PC3 with 3 day exposure of 4-HPR

Cell line	4-HPR concentration (M)	uPA activity (IU/mg)
TSU-PR1	Control	450.9 ± 14.9
	10^{-8}	424.7 ± 7.0
	10^{-7}	447.5 ± 5.9
	10^{-6}	391.8 ± 4.2*
PC3	Control	464.6 ± 10.0
	10^{-8}	471.0 ± 2.2
	10^{-7}	449.8 ± 3.7
	10^{-6}	433.5 ± 0.9*

Values are the means ± SD of triplicate samples.
* statistically significant ($p<0.05$)

Table 4. Sequence-timed analyses of functional uPA activity in cell lysates of TSU-PR1 and PC3 with 4-HPR exposure

Cell line	Time (day)	uPA activity (IU/mg)	
		Control	10^{-6}M 4-HPR
TSU-PR1	1	135.5 ± 5.0	112.4 ± 2.7*
	2	340.4 ± 7.7	293.5 ± 6.8*
	3	450.9 ± 14.9	391.8 ± 4.2*
PC3	1	384.9 ± 7.4	362.4 ± 3.9*
	2	431.8 ± 11.5	430.7 ± 3.6
	3	508.4 ± 5.6	451.9 ± 2.8*

Values are the means ± SD of triplicate samples.
* statistically significant ($p<0.05$)

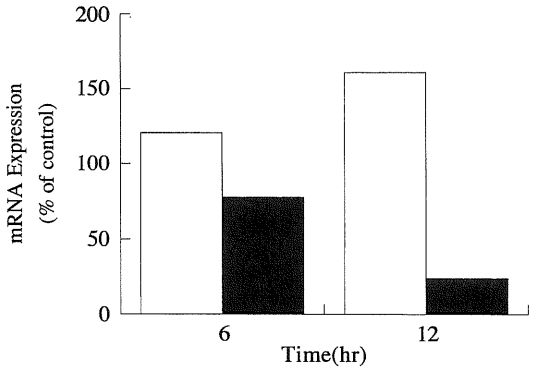

Fig. 2. Effects of CHX on 4-HPR-induced increase in uPA mRNA expression of TSU-PR1: (□) exposure of 10^{-6}M 4-HPR alone; (■) exposure of 10^{-6}M 4-HPR with CHX.

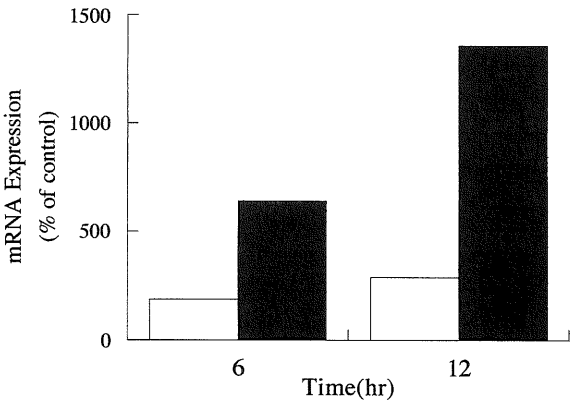

Fig. 3. Effect of CHX on 4-HPR-increase in PAI-1 mRNA expression of TSU-PR1: (□) exposure of 10^{-6}M 4-HPR alone; (■) exposure of 10^{-6}M 4-HPR with CHX.

protein synthesis by CHX was proven in the mRNA expression of TSU-PR1. The treatment of TSU-PR1 cells with 15μM of CHX resulted in nearly complete inhibition of the uPA mRNA increase induced by 10^{-6}M 4-HPR (Fig. 2). This result indicated that the effect of 4-HPR is indirect and that protein synthesis is required for 4-HPR-mediated induction of uPA mRNA. In PAI-1 northern blot analyses, it was observed that the addition of CHX causes a marked increase in the PAI-1 mRNA of TSU-PR1 than 10^{-6}M 4-HPR alone (Fig. 3).

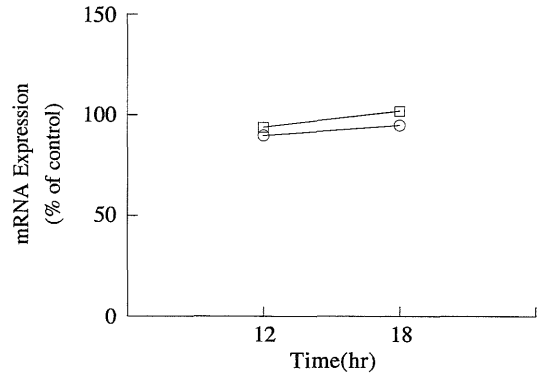

Fig. 4. Effects of 4-HPR on mRNA stability of uPA and PAI-1 in TSU-PR1: (○) uPA and (□) PAI-1 were treated with 10^{-6}M 4-HPR and ACD

Effects of 4-HPR on mRNA stability of uPA and PAI-1

In order to evaluate the effects of 4-HPR on the mRNA stability of uPA and PAI-1, 10μM ACD was added to TSU-PR1, preventing transcription for more mRNA production, and the sequence-timed mRNA levels were analyzed. Both the uPA and PAI-1 mRNA expression of TSU-PR1 treated with 10^{-6}M 4-HPR was almost similar to those of the control cells at each time point (12 hr and 18 hr) (Fig. 4). This result revealed that 4-HPR has no effect on the mRNA stability of uPA and PAI-1.

DISCUSSION

Although initially secreted as an enzymatically inactive proenzyme by tumor cells, uPA exerts its proteolytic function after being bound to a specific cell surface receptor. The uPA receptor on cells is the reaction site for uPA-mediated plasminogen activation in solid tumors[19]. Then plasmin degrades the components of the tumor stroma (fibrin, fibronectin, proteoglycan and laminin) and may activate type IV collagenase and stromelysin, which also degrade the extracellular matrix[17]. Thus uPA promotes the extracellular matrix dissolution which is a prerequisite for tumor invasion. PAI-1 is synthesized as an active molecule but it is converted to a latent form in the conditioned media of cultured cells. Active PAI-1 is stabilized by the extracellular matrix to bind uPA[2]. Therefore, in this study, although 4-HPR increased uPA and PAI-1 mRNA expression and caused uPA and PAI-1 protein increases in both the conditioned media and cell lysates, the uPA and PAI-1 in the conditioned media were inactive and only the uPA and PAI-1 in the cell lysates contributed to the actual uPA activity.

The increase of PAI-1 denied the uPA increase and the uPA increase in the data would not have been expected. However, when receptor-bound uPA is inhibited by PAI-1, rapid internalization of a trimeric complex (uPA receptor, uPA and PAI-1) occurs and this internalization may be the trigger for normal cell proliferation[19]. This internalization of the complex may possibly cause the beginning of another cascade for expression of cellular protein in prostate cancer cells.

The results of this study suggest that 4-HPR reduces functional uPA activity in prostate adenocarcinoma cells. However, this mild reduction of uPA activity could not explain all of the 4-HPR's anti-invasive effects against prostate cancer and seemed to be only one of the factors for the anti-invasive effects. Actually 4-HPR reduced the capacity of prostate cancer cell lines to adhere to the basement membrane matrix as well as inhibiting cellular motility[11]. Further investigations of 4-HPR's effects on other proteolytic enzymes that degrade extracellular matrix in tumor invasion, such as matrix metalloproteinases, serine proteinases and heparanase, will be required. It seems that many of these factors compose the anti-invasive effect of 4-HPR.

Concerning the mechanism of expressing retinoids' effects, retinoids alter the gene expression of the target protein at the transcription level[3]. Specific retinoid-binding proteins in the cellular nucleus, retinoid receptors, generally control gene expression, which generates other cellular regulatory proteins, and those cellular regulatory proteins induce the target gene expression that apparently receives the effect of the retinoids[7]. Because the 4-HPR-induced increase in uPA mRNA was inhibited by blocking protein synthesis with CHX administration, it may be inferred that 4-HPR bound the nuclear retinoid receptor and it stimulated synthesis of the protein which controlled uPA transcription. Also PAI-1 northern blot analysis with CHX was employed to investigate the mechanism of 4-HPR's inducing PAI-1 mRNA expression and the result revealed that CHX caused a marked increase in PAI-1 mRNA. This marked increase in PAI-1 mRNA by CHX is known in many cell lines as superinduction and failed to give clues to trace the mechanism of 4-HPR's effect on PAI-1 mRNA[22]. However, some reports suggest that the PAI-1 mRNA increase may have been partly caused by the increase in the PAI-1 gene transcription rate[1]. 4-HPR's induction of PAI-1 mRNA expression might require synthesis of the protein that increases the PAI-1 transcription rate in the same way as the induction of uPA mRNA. The supposition that the retinoid receptor bound with 4-HPR induces uPA and PAI-1 mRNA transcription is compatible with the result of northern blot analysis with ACD which revealed no effect of 4-HPR on uPA and PAI-1 mRNA stability.

In conclusion, the synthetic retinoid, 4-HPR, stimulates a paradoxical increase in uPA mRNA and protein in both TSU-PR1 and PC3 cell lines. This increase in uPA protein is associated with a concomitant increase in PAI-1 protein. The anti-

invasive effects of 4-HPR are consistent with increases in both uPA and PAI-1, resulting in the overall reduction of functional uPA activities. The effect of 4-HPR on uPA increase requires synthesis of cellular regulatory proteins, but 4-HPR has no effect on the mRNA stability of uPA and PAI-1. It was thus surmised that the anti-invasive effects of 4-HPR were expressed through the nuclear retinoid receptor. This study further supports the continued examination of 4-HPR as a clinically useful anti-invasive agent against prostate cancer.

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