

# Retinoid Metabolism in the Prostate: Effects of Administration of the Synthetic Retinoid *N*-(4-Hydroxyphenyl)retinamide

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## ABSTRACT

We have carried out a series of complementary *in vivo* and *in vitro* studies to better understand the metabolism of vitamin A by the prostate gland. Male Sprague-Dawley rats were fed either a control diet sufficient in vitamin A [CON group; 0.8  $\mu\text{g}$  retinol equivalents (RE)/g diet] or a CON diet supplemented with the synthetic retinoid *N*-(4-hydroxyphenyl)-retinamide (4-HPR; CON+4HPR group; 1173  $\mu\text{g}$  of 4-HPR/g diet). After an i.v. injection of a physiological radiolabeled dose of retinol, the vitamin A content and radioactivity of plasma and a number of tissues, including the prostate glands, were monitored for time periods ranging between 30 min and 41 days. On the basis of the results of these vitamin A turnover studies, we developed tissue subsystem models to describe vitamin A dynamics in the prostates of both the CON and CON+4HPR groups. There was a gradual decrease in the vitamin A content of the prostates of the 4-HPR-treated group as compared with the control, such that by the end of the study period, the CON+4HPR group averaged  $0.166 \pm 0.0827$  (mean  $\pm$  SD) REs, whereas the CON group was  $0.732 \pm 0.190$  REs. The fraction of vitamin A exiting the prostate each day was not significantly different in the CON as compared with the CON+4HPR group [ $0.149 \pm 0.103$  versus  $0.155 \pm 0.191 \text{ h}^{-1}$  (mean  $\pm$  FSD), respectively]; however, the average amount of vitamin A turning over from the CON+4HPR group prostates (0.0885  $\mu\text{g}/\text{day}$ ) was nearly three times less than that of the CON group (0.243  $\mu\text{g}/\text{day}$ ). To obtain more detailed information on the mechanisms that might be involved in the changes in vitamin A kinetics observed in our *in vivo* studies, we used both a normal human prostate cell line (PrEC) and a human prostate adenocarcinoma cell line (LNCaP) to monitor *in vitro* retinol and 4-HPR dynamics. Cells were treated with 4-HPR for different time periods up to 48 h (PrEC) or 96 h (LNCaP). Retinol in the media was taken up readily by both PrEC and LNCaP cells, and there was conversion of retinol to the major storage esters of vitamin A, retinyl palmitate and retinyl stearate, as well as several minor retinyl esters, in a pattern indicative of normal retinoid esterification activity. Although 4-HPR was taken up readily and over time accumulated in both cell lines, conversion of 4-HPR to its major metabolite, *N*-[4-methoxyphenyl]retinamide, as well as several other metabolites of 4-HPR was apparent only in the LNCaP cells. Our findings would suggest that a study design that includes appropriately designed complementary *in vivo* and *in vitro* experimental systems represents a useful approach to better understanding possible mechanisms involved in basic retinoid functioning and interactions in the prostate as well as in other organs and related tissue culture systems.

## INTRODUCTION

The synthetic retinoid 4-HPR<sup>2</sup> has been shown to be effective in inhibiting cancer in a number of tissues including the breast, prostate, seminal vesicle, urinary bladder, ovary, and cervix (1–4). The most

frequently reported side effects involve visual and ophthalmological problems (5–11); however, the relatively low pharmacological toxicity of 4-HPR as compared with other retinoids has made it an attractive candidate for a number of trials in which it has been studied alone or in conjunction with other chemotherapeutic agents (4, 12). The principal cellular target and the precise mechanisms of action of 4-HPR remain to be delineated. It is unclear as to whether the cancer preventive and/or therapeutic actions of 4-HPR are occurring through nuclear retinoid receptors (13, 14) or via receptor-independent pathways (15–17). It has been suggested that an important role for 4-HPR might be as a prooxidant that generates reactive oxygen species, which in turn are involved in the induction of apoptosis (16, 17). Recent studies in neuroblastoma cell lines indicate that 4-HPR increases levels of both reactive oxygen and the second messenger ceramide, both of which are able to regulate induction of apoptotic pathways (18). The interactions between 4-HPR and ceramide in regard to a regulatory role in apoptosis have yet to be clarified. In the present study, we have focused on a related area of investigation involving 4-HPR, and that is how this synthetic retinoid is interacting with native retinoids in the system as well as how it might be affecting the normal functioning of the latter compounds. Our laboratory has been conducting a series of complementary *in vivo* and *in vitro* studies to examine in more detail the mechanisms involved in retinoid:drug: nutrient interactions and their role in carcinogenic processes. We are particularly interested in discerning the control mechanisms that are involved in the normal *in vivo* metabolism of the native retinoid forms in the system and how these are affected by various physiological, nutritional, and pharmacological perturbations. The work presented herein focuses on *in vivo* studies of vitamin A metabolism in the rat prostate gland and complementary *in vitro* studies using both a normal and an immortalized prostate cell line.

## MATERIALS AND METHODS

**Chemicals and Isotopes.** Retinoids used in analyses were either obtained commercially (retinol, retinyl acetate, and retinyl palmitate; Sigma Chemical Co., St. Louis, MO) or synthesized (retinyl stearate) according to published methods (19). The 4-HPR and its methoxy derivative, 4-MPR, were kindly provided by Dr. Vernon E. Steele of the National Cancer Institutes Division of Cancer Prevention and Control. Retinyl palmitate added to diet preparations was purchased from Teklad (Teklad, Madison, WI). Tritiated retinol (11,12-<sup>3</sup>H)(*N*)-retinol; specific activity,  $\sim 180 \mu\text{Ci}/\mu\text{g}$ ) was obtained from New England Nuclear (Boston, MA). All procedures involving retinoids were carried out under gold fluorescence lighting. Chemicals and solvents were of reagent or HPLC grade, and those used for extraction of retinoids from plasma, tissues, cell culture, and media included butylated hydroxytoluene (5  $\mu\text{g}/\text{ml}$ ) as an antioxidant.

**Animals and Diets.** Weanling male Sprague-Dawley rats (Harlan-Sprague Dawley, Inc., Frederick, MD) weighing between 50 and 60 g were housed individually in wire-bottomed stainless steel cages in an isolated room with automatically controlled temperature (22–26°C), humidity ( $\sim 60\%$ ), and 12-h light/dark cycles (6 a.m.–6 p.m./6 p.m.–6 a.m.). Food and water were provided *ad libitum*. Rats between 13 and 14 weeks of age were fed either a control diet [CON group; 0.8 RE/g vitamin A-free diet (Teklad) as retinyl palmitate;  $n = 28$ ] or the same control diet supplemented with 4-HPR (CON+4HPR group; 1173  $\mu\text{g}$  of 4-HPR/g diet;  $n = 28$ ). After adaptation to their respective

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<sup>2</sup> The abbreviations used are: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; 4-MPR, *N*-(4-methoxyphenyl)retinamide; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; ROH, retinol; ARAT, acyl-CoA:retinol acyltransferase; LRAT, lecithin:retinol acyltransferase; BPH, benign prostatic hypertrophy; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid binding protein; RE, 1  $\mu\text{g}$  retinol equivalent; FSD, fractional SD.

diets for 6–7 days, animals received an i.v. injection of plasma (average, ~0.8 ml) that contained a physiological dose of  $^3\text{H}$ -labeled retinol obtained from a donor animal (20). Animals were anesthetized (methoxyflurane; Pittman-Moore, Washington Crossing, NJ) and killed at 0.5, 2, and 10 h and 1, 3, 7, 15, 25, and 41 days after dosing. Selected tissue samples including the prostate were removed after whole body perfusion (0.9% saline) and frozen immediately in liquid nitrogen. Vitamin A and radioactivity levels were measured in plasma samples collected throughout the different study periods. Plasma and tissue samples were purged well with nitrogen prior to being stored and protected from light at  $-80^\circ\text{C}$ . The experiments, care, and use of the animals in the work described herein were approved by the National Cancer Institute and Frederick Cancer Research and Development Center Animal Use and Care Committee.

**Tissue Culture.** Normal prostate epithelial cells (PrEC) obtained from Clonetics Corp. (San Diego, CA) were seeded and maintained in their proprietary serum-free media to which the recommended proprietary supplements (human epidermal growth factor, epinephrine, transferrin, gentamicin sulfate, amphotericin-B, bovine pituitary extract, insulin, hydrocortisone, triiodothyronine, and retinoic acid) were added according to the manufacturer's instructions. Upon reaching 80% confluence, cells were harvested, and ~187,500 cells were seeded in 75-cm<sup>2</sup> flasks in the media described above. Human adenocarcinoma (LNCaP) prostate cells (American Type Tissue Collection, Rockville, MD) were seeded to a density of  $4 \times 10^6$  cells in 75-cm<sup>2</sup> flasks (Corning, Corning, NY) in RPMI 1640 supplemented with FBS (Life Technologies, Inc., Gaithersburg, MD). Cells were incubated at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . At 72 h before beginning the experiments, the FBS supplement was switched to a 10% dextran-coated charcoal FBS (DCC-FBS; Hyclone, Logan, UT).

**Cell Growth and Viability Assays.** The effects of a range of concentrations of 4-HPR (0.5–5  $\mu\text{M}$ ) in the presence or absence of retinol on the growth and viability of PrEC cells were assessed by a modified colony-forming assay (21). Briefly, cells were conditioned with 1  $\mu\text{M}$  retinol or vehicle for 72 h, fresh media containing various concentrations of 4-HPR with 0.1  $\mu\text{M}$  retinol or vehicle were added, cells were harvested at 24 or 48 h, and 2000 cells/well were plated in six-well plates. Cells were maintained in media containing 4-HPR with retinol or vehicle for 18 days; viable cells were then fixed with 10% trichloroacetic acid and stained with sulforhodamine B. The dye was eluted in 10 mM Tris, and absorbance at 530 nm was determined (22, 23). For LNCaP cells, the effects over time of a range of concentrations of retinol or 4-HPR (retinol, 0.5–4.0  $\mu\text{M}$ ; 4-HPR, 0.625–5  $\mu\text{M}$ ) on growth and viability were assessed using a commercially available assay kit (Promega Corp., Madison, WI). The assay measured the conversion of a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to formazan (24, 25).

**Retinoid Treatments of Cells.** Media for both the PrEC and LNCaP cells were supplemented with 1  $\mu\text{M}$  retinol (Sigma) for 72 h prior to beginning the experiments. PrEC cells were then treated with media containing either 0.100  $\mu\text{M}$  retinol plus 1.0  $\mu\text{M}$  4-HPR in absolute ethanol or 0.100  $\mu\text{M}$  retinol plus absolute ethanol vehicle. Cells were harvested immediately prior to addition of 4-HPR and then at 3, 6, 24, and 48 h after treatment. After the 72-h pretreatment period, LNCaP cells were treated with media containing either 0.125  $\mu\text{M}$  retinol plus 2.5  $\mu\text{M}$  4-HPR in absolute ethanol or 0.125  $\mu\text{M}$  retinol plus absolute ethanol vehicle. Cells were harvested immediately prior to addition of 4-HPR and then at 48 and 96 h after treatment.

**Plasma and Tissue Processing.** Prior to extraction, a retinyl acetate internal standard in absolute ethanol was added to all plasma or tissue samples. For plasma, after the addition of 2 ml of 50% aqueous ethanol and 4.5 ml of hexane, samples were vortexed (1 min) and centrifuged (6 min; 2400 rpm), and appropriate aliquots were removed from the upper organic layer for determination of retinoid mass and radioactivity. Prostates were extracted three times using a scaled-down version of a hexane:isopropanol extraction system reported earlier (20). Prostates were first ground in small glass:glass homogenization tubes (16  $\times$  100 mm) with 2.0 ml of hexane:isopropanol (3:2, v/v). Samples were then vortexed for 2 min. After the addition of 1.0 ml of an aqueous sodium sulfate wash solution (6.7%, w/v), samples were vortexed for 1 min and centrifuged (2100 rpm; 6 min). After centrifugation, the upper organic phase was removed and put aside. The second and third extraction steps were done using 0.5 ml of hexane:isopropanol (7:2, v/v). All extracts

were pooled and adjusted to an appropriate volume, and aliquots were removed for measurement of total retinoid mass and radioactivity.

**Processing of PrEC and LNCaP Cells.** For extraction of retinoids from cell pellets, frozen pellets were first dried in a centrifugal concentrator (SC200 Speed Vac; Savant Instruments, Farmingdale, NY) equipped with a nitrogen feed. Dried pellets were ground with a small Teflon pestle and extracted using a hexane:isopropanol-based system in a similar manner as outlined above for the prostate glands. The residue in the aqueous phase was dried using the concentrator, and the protein content was determined (Bio-Rad DC protein assay kit; Bio-Rad Laboratories, Hercules, CA). Briefly, samples were solubilized in 1.0 ml of 0.3 N NaOH and 1% SDS by incubating ( $37^\circ\text{C}$ ) for 1–5 h and then quantified in triplicate following the manufacturer's directions for microplate assay. Standards consisted of BSA dried and solubilized in 1.0 ml of 0.3 N NaOH and 1% SDS as above.

**Quantitation of Retinoid Mass and Radioactivity.** For analysis of either retinoid mass or radioactivity, aliquots of total extract were first evaporated to dryness in the concentrator in a manner similar to that described above. For analysis of retinoid mass, dried samples were resolubilized in absolute ethanol with butylated hydroxytoluene prior to quantitation by reverse-phase HPLC [Resolve C<sub>18</sub> guard and analytical (4.6 mm  $\times$  15 cm) columns; 600E System Controller; 717 Autosampler; 490E Detector (for analysis of prostates) or 996 Photodiode Array Detector (PDA; for analysis of PrEC and LNCaP cells); Waters Corp., Milford, MA]. For prostates, retinol and retinyl esters were detected by UV absorbance at 325 nm. For PrEC and LNCaP cell experiments, retinol, retinyl esters, 4-HPR, 4-MPR, and other retinoid metabolites were detected by UV absorbance at wavelengths between 300 and 390 nm. Peak areas were quantitated by digital integration (Millennium 2010 version 2.15.01; Waters) from standard curves of purified chemical standards included with each sample set. Retinoid content of cell pellets was normalized in terms of protein content (*i.e.*, nmol retinoid/mg protein). For analysis of tritium, dried samples were resolubilized in scintillation fluid (Ready Organic; Beckman Instruments, Fullerton, CA) prior to quantitation by liquid scintillation spectrometry (LS 5000TA; Beckman).

**Kinetic Analysis and Development of Mathematical Model.** The SAAM/CONSAM computer modeling programs (26, 27) were used to carry out initial kinetic analysis and model development. An updated Windows 95-based version of these programs (NIH WinSAAM; Version 1.0.9; kindly provided by Dr. Peter Greif, Laboratory of Experimental and Computational Biology/Molecular Structure Section, NCI, Bethesda, MD) was used to generate the kinetic data presented in the present report. Plasma tracer data were used as a "forcing function" to model vitamin A kinetics in the prostate (26–28). Briefly, for the present study, we used the plasma tracer data from the CON and CON+4HPR groups as a forcing function, along with the corresponding tracer response data in the prostate glands of these groups, to model vitamin A kinetics in the prostate. An advantage of using a forcing function approach for development of a tissue or subsystem model is that it allows one to focus on a particular tissue of interest without having to develop a model to describe the entire system. Our use of this approach has been described in detail in a recent report (29). We began development of a compartmental model to describe retinol metabolism in the prostate by first assuming that exchange of vitamin A was taking place between the prostate and the plasma but that there was no direct exchange of prostate vitamin A with other organs in the system. Mean plasma retinol pool masses were estimated as described previously (20), and input of retinol to the prostate from the plasma was estimated by calculating the product of the fractional transfer coefficient for input of retinol into the prostate from the plasma and the mean plasma retinol masses during the course of the 41-day experimental period. The output of vitamin A from the prostate was assumed to be equal to the input from the plasma. Output from the prostate included both reversible recycling to the plasma and irreversible utilization. Thus, a portion of the total output from the prostate represents vitamin A that may be recycled through the plasma to other tissues as well as the prostate (*i.e.*, reversible recycling), whereas a portion of the output represents that used by the prostate (*i.e.*, irreversible utilization or disposal).

**Statistical Analyses.** One-way ANOVA and tests of repeated measures were performed using SPSS for Windows (version 8.0) to determine significant differences ( $\alpha$  level of 0.05 or less) between mean values for plasma, tissue, or cell retinoid content (30). Chemically determined data are presented

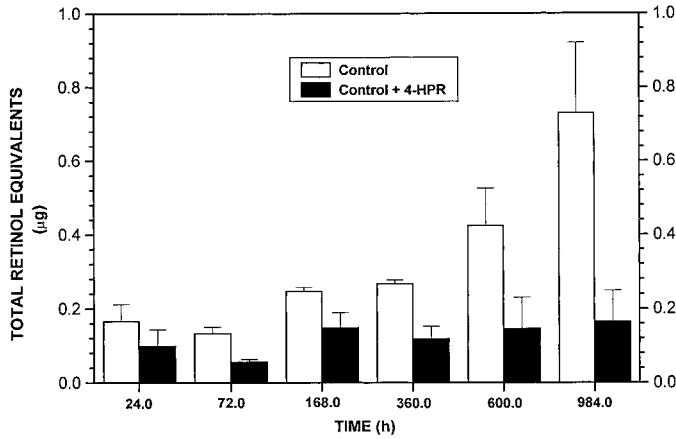


Fig. 1. Mass of vitamin A in prostate glands of CON and CON+4HPR groups during the 41-day turnover study period. Data are presented as means  $\pm$  SD. The CON+4HPR group values were significantly lower than the corresponding CON group values throughout the sampling period ( $\alpha$  level of 0.05 or less). Each collection represents three animals from each of the dietary groups, except for the 41-day point for which there were four and five animals each for the CON and CON+4HPR groups, respectively. Animals were adapted to their respective diets for 1 week prior to beginning the turnover study. A description of treatments is found in the text.

as mean  $\pm$  SD, and model-derived data are presented as mean  $\pm$  FSD (SD/mean).

**RESULTS**

The growth curves of animals in both control and 4-HPR-treated groups were similar, and animals appeared to be in good health throughout the course of the experiments. As described in an earlier report (20), plasma retinol levels during the course of the 41-day turnover study period were significantly decreased in the 4-HPR-treated group as compared with the control ( $22.9 \pm 7.7$  and  $66.1 \pm 9.9$   $\mu\text{g}/\text{dl}$ , respectively). The total mass of vitamin A in the prostates of

both groups during the experimental period is presented in Fig. 1. As compared with the CON group, the vitamin A content of the prostates of the CON+4HPR group was consistently lower throughout the course of the turnover study period.

Models proposed to describe vitamin A metabolism in the prostate glands of the CON and CON+4HPR groups are presented in Fig. 2A, top, along with the corresponding tracer response curves (Fig. 2B, bottom). Data are presented as group average mean values. Error estimates for the parameters presented in the model are expressed as FSD. For both CON and CON+4HPR groups, a minimum of two compartments were required to fit the data obtained from the prostates. The fraction of vitamin A leaving the prostate (*i.e.*, model-derived fractional transfer coefficient; expressed as a group mean value  $\pm$  FSD) on an hourly basis was found to be similar in both groups [ $0.149 \pm 0.103$  and  $0.155 \pm 0.191$   $\text{h}^{-1}$  (mean  $\pm$  FSD) for the CON and CON+4HPR groups, respectively]. The model predicted retinoid mass values for the prostates for both groups were estimated by dividing the output (or turnover) rate of vitamin A ( $\mu\text{g}/\text{h}$ ) from the prostate by the corresponding fractional transfer coefficient for output. The transfer rate ( $\mu\text{g}/\text{h}$ ) from the first or primary compartment to the second compartment was determined by calculating the product of the fractional transfer coefficient from the first to the second compartment and the exchangeable vitamin A mass of the first compartment. The rate of output from the second compartment was assumed to be equal to the input rate. Output from the prostate represents the sum of both reversible recycling to the plasma and irreversible utilization and was assumed to be equal to the input from the plasma. The model-predicted distribution of mass was such that the turnover of vitamin A from the prostates of the 4-HPR-treated group was nearly three times less than that of the control (0.0885 and 0.243  $\mu\text{g}/\text{day}$ , respectively). The model-predicted vitamin A mass in the CON group prostates was 0.688  $\mu\text{g}$ , whereas that of the CON+4HPR group was 0.123  $\mu\text{g}$ . Because most of the increase in the CON prostate vitamin A stores occurred during the latter portion of the experimental study period and

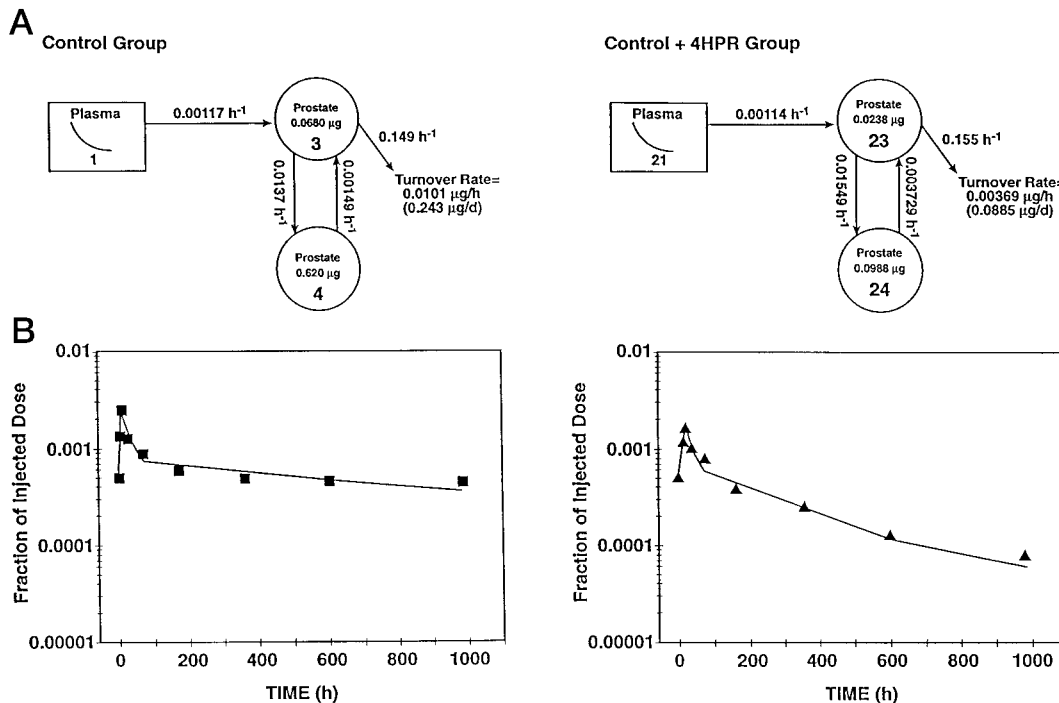


Fig. 2. A, proposed models of vitamin A kinetics in the prostate gland of CON versus CON+4HPR groups showing the fractional rate constants ( $\text{d}^{-1}$ ) and flow of vitamin A mass ( $\mu\text{g}/\text{day}$ ) through the system. B, tracer response curves from the prostate gland of CON ( $\blacksquare$ ) versus CON+4HPR ( $\blacktriangle$ ) groups during the 41-day turnover study period are shown below their respective models.

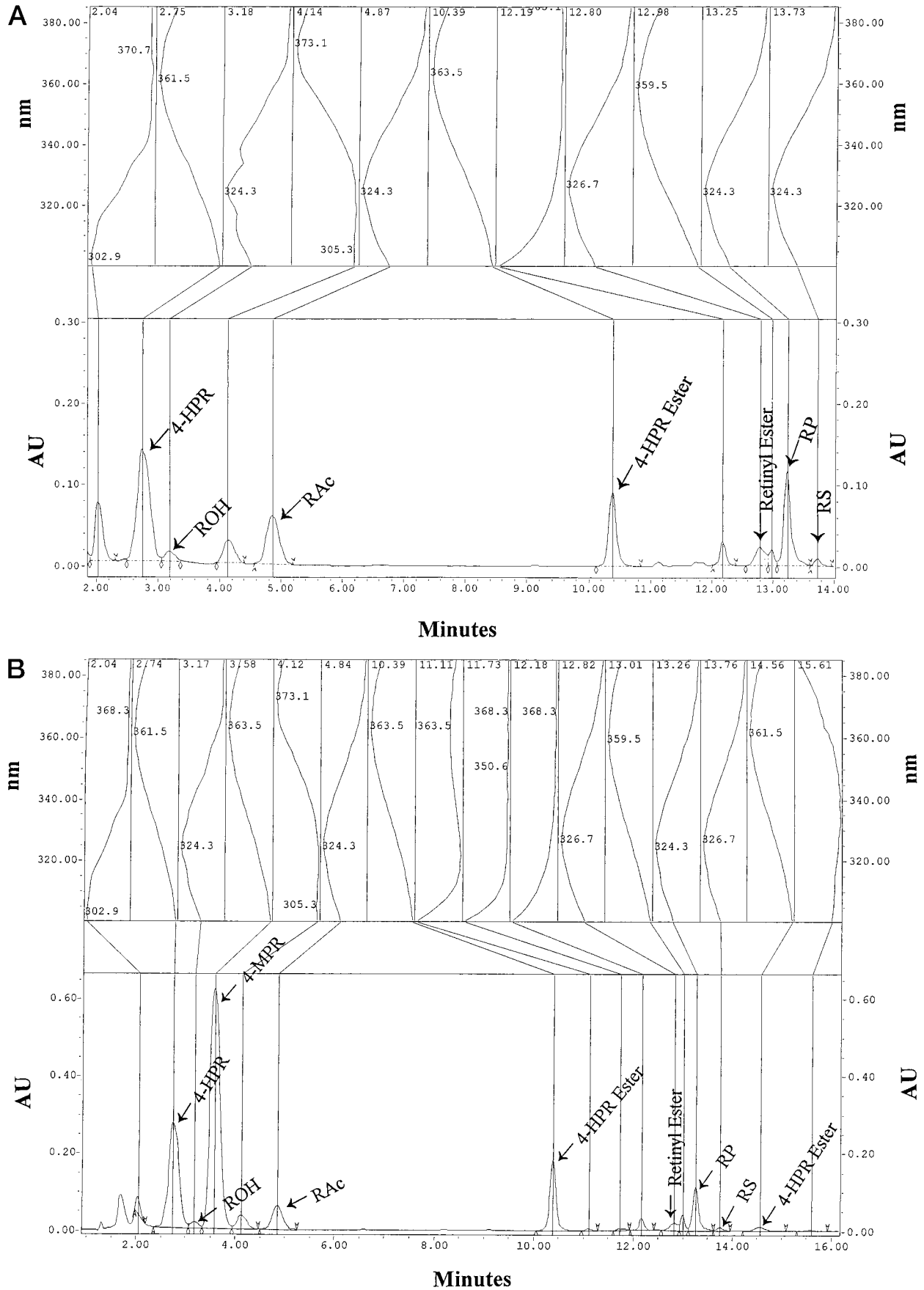


Fig. 3. Representative HPLC/photodiode array spectral index plots of cell retinoid mass in PrEC (A, top) and LNCaP (B, bottom) cells during study period. ROH, retinol; RAc, retinyl acetate; RP, retinyl palmitate; RS, retinyl stearate.

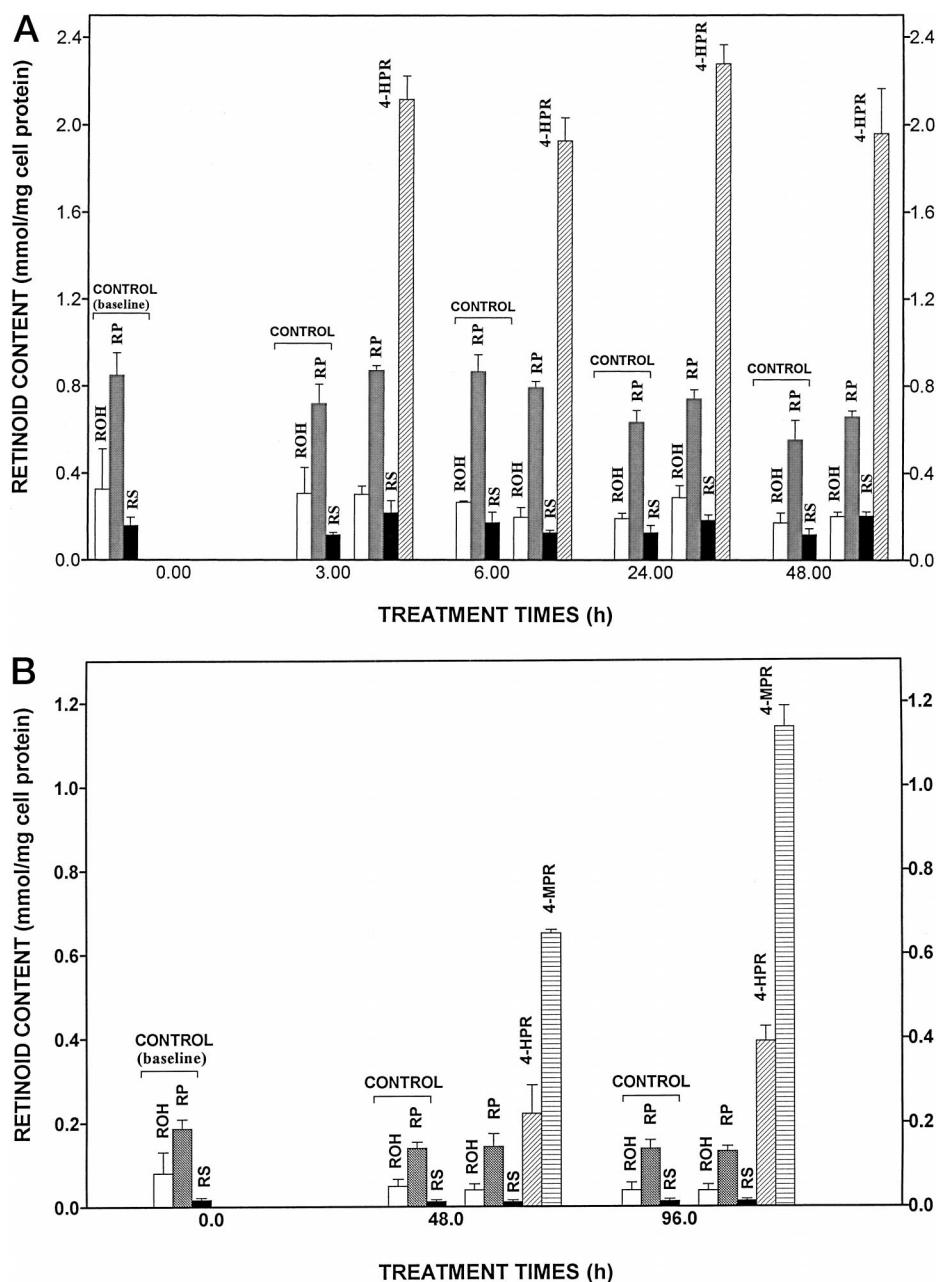


Fig. 4. Normalized retinoid mass (means  $\pm$  SD) identified in PrEC (A, top) and LNCaP (B, bottom) control and 4-HPR-treated cells during the study period. A description of treatments is found in the text. ROH, retinol; RP, retinyl palmitate; RS, retinyl stearate.

the corresponding CON+4HPR group values remained consistently depressed throughout the study period, the terminal 41-day chemically determined values for the two groups were closest to the model-predicted values, averaging  $0.732 \pm 0.189$  and  $0.166 \pm 0.0826 \mu\text{g}$  for the CON and CON+4HPR groups, respectively (Fig. 1).

Residence times, the amount of time a vitamin A molecule, on average, spent in each of the prostate compartments before irreversible loss from the system, were obtained from the inverse matrix calculations derived by the CONSAM program (27). The residence times for vitamin A molecules in the faster turning over compartments for both the CON and CON+4HPR groups were similar, averaging 0.28 day (6.7 h) for compartments 3 and 23. The estimated residence times for compartment 4 of the CON group and 24 of the CON+4HPR group were 30.9 and 13.3 days, respectively, suggesting the possibility that these secondary compartments might be serving as longer term storage compartments for both groups. However, vitamin A molecules entering the secondary compartment of the 4-HPR-

treated prostates (compartment 24) remained there less than half the time as in the corresponding compartment of the control (compartment 4), likely related to the decreased mass of vitamin A in the 4-HPR-treated prostates.

To better understand the mechanisms that might be involved in the alteration of *in vivo* vitamin A kinetics in the prostate that were associated with 4-HPR administration, we carried out *in vitro* studies in normal human prostate cells (PrEC) and human prostate adenocarcinoma cells (LNCaP). Fig. 3 shows representative HPLC/PDA spectral index plots of retinoid mass in PrEC (Fig. 3A, top) and LNCaP (Fig. 3B, bottom) cells over a 48- and 96-h period, respectively. Measurement of intracellular retinoid content of PrEC cells preconditioned with  $1 \mu\text{M}$  retinol and  $0.100 \mu\text{M}$  retinol during the treatment period revealed that cells took up retinol and stored it as such or as a number of retinyl esters, including retinyl palmitate and stearate. Similarly, LNCaP cells preconditioned with  $1 \mu\text{M}$  retinol and  $0.125 \mu\text{M}$  retinol during the treatment period also readily took up retinol, which

was able to be converted to retinyl esters, predominately retinyl palmitate and stearate. We detected several other minor peaks, which based on their spectral index, appeared to be retinyl esters. In both PrEC and LNCaP cells, 4-HPR was readily taken up from the media.

As shown in Fig. 4, during their respective experimental periods, PrEC (Fig. 4A, top) or LNCaP (Fig. 4B, bottom) cells preconditioned with retinol prior to treatment with 4-HPR were not significantly different from their corresponding controls in their stores of retinol, retinyl palmitate, retinyl stearate, and the unidentified "minor" retinyl esters mentioned above. For both the PrEC and LNCaP cells, there was substantial uptake and accumulation of 4-HPR into the cells. In the case of the PrEC cells, there was no apparent further conversion of 4-HPR to its normal methoxy metabolite 4-MPR; however, by 24 h, intracellular accumulation of 4-HPR had reached a point where it was more than 3 and 7 times higher than the corresponding masses for retinyl palmitate and retinol, respectively. In contrast, in the LNCaP cells, there was substantial conversion to and subsequent accumulation of MPR as well as a number of other related metabolites. By the 48-h collection, the total of 4-HPR and its metabolites had accumulated to the point that they were more than four times as high as the total native retinoid stores (*i.e.*, retinol + retinyl esters). By the 96-h collection, the total 4-HPR stores had risen even further, to the point where they were nearly eight times higher than the total native retinoid stores.

## DISCUSSION

A major focus of our work in the area of retinoid metabolism has been a continuing interest in the delineation of homeostatic control mechanisms involved in the regulation of plasma and tissue kinetics of retinoids. Information involving basic aspects of vitamin A metabolism and retinoid interactions is essential to help assure maximal efficacy of any retinoid and/or drug therapy. We are particularly interested in how the administration of certain retinoids found to be useful as chemopreventive and chemotherapeutic agents affect the normal metabolism of native forms of vitamin A in the system. Although the pharmacokinetics of many of the retinoids used for chemopreventive and/or chemotherapeutic purposes have been well studied, the interactive effects that occur among these agents and native retinoid forms that are normally in the system have for the most part not been characterized. A better understanding of the interactions that occur among retinoids will not only help to clarify the numerous interactive effects that occur among retinoids but will also provide a basis for better understanding those interactions that occur among retinoids and other nutrients, hormonal factors, and certain chemotherapeutic drugs.

The work presented here is an expansion of our earlier studies in which we examined the effects of 4-HPR administration on whole body kinetics of vitamin A (20). Using a combination of both *in vivo* and *in vitro* approaches designed to complement one another, the present study was carried out to examine the effects of administration of a synthetic retinoid on metabolism of vitamin A in a particular tissue, the prostate gland. Although for the present work we have focused specifically on the effects of the synthetic retinoid 4-HPR on metabolism of native vitamin A forms in the prostate, the overall approach should be applicable to the study of other synthetic retinoids and retinoid interactions in other organs and related tissue culture systems.

For the *in vivo* portion of our studies, we used a physiologically radiolabeled form of retinol as a tracer and carried out a series of *in vivo* kinetics studies. On the basis of our findings, we developed mathematical/compartamental models to describe vitamin A metabolism in the prostate of animals fed either a normal control diet or a

control diet supplemented with 4-HPR. For the *in vitro* portion of our studies, we have carried out experiments examining retinoid dynamics and interactions in both normal human prostate cells (PrEC) and a human prostate adenocarcinoma cell line (LNCaP).

The results of our *in vivo* tracer kinetics studies indicated that the administration of 4-HPR was associated with decreased vitamin A stores and significant alterations in the kinetics of native vitamin A forms in the prostate over the course of a 41-day experimental period. By the terminal collection point, total retinoid stores in the prostates of the 4-HPR-treated group were more than four times lower than those of the control. This finding was similar to what we had observed for the eyes, which by the end of the study had vitamin A stores that were decreased to approximately five times less than those of the control group (29). The nature of the association of 4-HPR with the effects we observed in these tissues remains to be clarified. In the case of the eyes, we hypothesized that 4-HPR might be interfering with the normal uptake and/or metabolism of vitamin A in this tissue, thus, helping to account for the lower retinoid levels as well as the visual impairment problems that have been reported in human trials (29). It is possible that 4-HPR might interfere in some way with the uptake and/or intracellular transport and metabolism of retinol and related metabolites in the prostate. Our *in vivo* studies did not examine directly whether 4-HPR might be competing and/or interfering with native retinoid forms; however, the results of our *in vitro* studies indicated that both retinol and 4-HPR were taken up readily by prostate cells and able to be stored and/or further metabolized in a similar manner as what one might expect to find *in vivo*.

A number of studies have indicated that 4-HPR affects the activity of several enzymes important in the esterification and storage of native vitamin, including ARAT and LRAT (31–33). For example, the esterification activity of ARAT has been found to be decreased in liver and mammary tissues with 4-HPR treatment (31). In contrast, LRAT activity has been shown to be increased in the liver after administration of 4-HPR (32). The esterification activities of both LRAT and retinal reductase in intestinal cells were found to be inhibited by the administration of 4-HPR (33). The mechanisms involved in these tissue-specific, differential effects of 4-HPR on ARAT and LRAT activity have yet to be delineated. In the present study, a decrease in esterification activity in the prostate with 4-HPR treatment could help to account for the decline in prostate vitamin A stores that we observed in the present study.

The *in vivo* kinetic models we have proposed predict a higher overall turnover of retinol through the prostate of the control group as compared with the 4-HPR-treated group. For both groups, the models describe vitamin A movement in the prostate in terms of a smaller and faster turning over primary compartment (compartments 3 and 23 for the CON and CON+4HPR groups, respectively) and a larger and more slowly turning over secondary compartment (compartments 4 and 24 for the CON and CON+4HPR groups, respectively). The level of vitamin A turning over from the prostate in the 4-HPR-treated group was decreased by nearly three times that of the control. The fraction of the plasma retinol pool entering the prostate as estimated by the model was similar in both the CON and CON+4HPR groups (Fig. 2); however, as was mentioned earlier, the plasma retinol levels of the 4-HPR-treated group averaged only about one-third those of the control. Thus, our model predicts that there is less vitamin A coming into the 4-HPR-treated prostate from the circulation, and ultimately less ends up being stored. No specific tests of prostate function were carried out in the present study; thus, it is unclear whether normal prostate function might have been affected by the lower levels of vitamin A in this tissue and/or by increased levels of a synthetic compound such as 4-HPR, which is not normally found in the system.

It should be noted that a limitation of the forcing function modeling

approach we have used in the present study is that one is not able to clearly identify what portion of the total turnover from the tissue might be recycled back to the tissue. For example, although the model predicted turnover from the control group was nearly three times higher than that of the 4-HPR-treated group, it is unclear as to what portion of this turnover represents actual utilization of vitamin A as opposed to that portion that will be recycled back to the prostate. However, it is reasonable to speculate that most of this turnover represents material that is recycled back to the prostate, because other tissues that have been examined under a variety of feeding regimens and treatments are apparently recycling considerably more vitamin A than is actually used by the tissue (20, 34–37). Thus, for both the control as well as 4-HPR-treated group prostates, it is reasonable to assume that there is likely a higher degree of recycling of vitamin A relative to actual utilization of the vitamin. To be able to distinguish between the amount of vitamin A actually used *versus* that which will be recycled back to the prostate, the prostate data would be modeled with the forcing function removed and in the context of a whole body model of vitamin A metabolism. We are developing such a model at the present time that includes the prostate as well as a number of other tissues that we are in the process of analyzing. Despite some limitations, there are clear advantages to using a forcing function approach to developing a mathematical/compartamental model as we have done. One advantage is that it is possible to derive a reasonably accurate estimate of how a particular tissue of interest is metabolizing vitamin A under a variety of treatment regimens and how it might ultimately end up being modeled in the context of a whole body model, without having to invest the great deal of effort and time that may be required to develop an entire whole body model.

To investigate in more detail the role of 4-HPR in the alteration of *in vivo* vitamin A kinetics in the prostate, we carried out a number of *in vitro* studies using both normal human prostate epithelial (PrEC) and human adenocarcinoma (LNCaP) cells. The importance of comparing differences and similarities between normal and malignant cells in regard to retinoid related processes was highlighted in a recent report by Oridate *et al.* (16) in which they found 4-HPR to be considerably less effective in inducing apoptotic events in normal cervical cells as compared with a carcinoma cell line. Similarly, Guo and Gudas (38) have demonstrated recently that several squamous cell carcinoma cell lines from the human oral cavity and skin have a diminished capacity to esterify retinol as compared with corresponding normal cell lines. Although not measured directly, the results of our *in vitro* studies would suggest that treatment with 4-HPR was not associated with any apparent differences in esterification activity in either the normal human prostate (PrEC) or the human prostate adenocarcinoma (LNCaP) cell lines studied.

We included a low-level retinol preconditioning period for both cell lines, because we had found in our initial pilot studies that without preconditioning these cells with retinol, we were not able to detect any intracellular native retinoid. Moreover, for both cell lines, without retinol preconditioning, there was a decreased uptake of 4-HPR into the cells, as well as a decreased conversion of 4-HPR to 4-MPR and/or related metabolites as compared with that of retinol-treated cells.<sup>3</sup> This finding was of particular interest in light of recent work carried out in several human breast and melanoma cell lines, which indicated that those cell lines that were most responsive to treatment with 4-HPR also contained the 4-MPR metabolite (39). The authors suggested that the metabolism of 4-HPR might be essential for its action, and moreover, that 4-MPR might be a predictor or biomarker of 4-HPR responsiveness. Because an important component of our *in*

*vitro* studies was to monitor cellular dynamics of native retinoid forms as they were affected by treatment with 4-HPR, we reasoned that at a minimum, there should be sufficient levels of native retinoid stores in the cells such that retinol esterification activity could be induced, and that cells should be able to take up 4-HPR. Thus, unless noted otherwise, we included a pretreatment period with retinol for all of the *in vitro* studies discussed in the present report.

We found that the PrEC cells took up and stored relatively large amounts of 4-HPR, whereas the LNCaP cells not only took up and stored 4-HPR but were also capable of converting 4-HPR to its methoxy derivative (4-MPR) as well as a number of related metabolites. Indeed, if 4-HPR and/or its metabolites such as 4-MPR are taken up and accumulate *in vivo* to the extent that we have observed in our experiments using the PrEC and LNCaP prostate cell lines, it is possible that such a large mass of synthetic retinoid in the cell relative to native retinoid could affect normal physiological metabolism and associated kinetics of native retinoids and moreover, might compromise overall cellular functioning in this tissue as well. We did not measure tissue dynamics of 4-HPR and its related metabolites in our *in vivo* studies; however, for future studies of this nature, we now have the methodology in place to simultaneously monitor the *in vivo* uptake and metabolism of 4-HPR as well as native retinoids.

With 4-HPR treatment, we detected no apparent differences in native retinoid stores in either the PrEC or LNCaP cells during the course of the experimental period. Although in contrast to our *in vivo* findings, there are possible explanations for such a disparity aside from the inherent limitations and artificial nature of *in vitro* tissue culture systems in general. For example, along with species differences, there are anatomical and functional differences between rat and human prostates. Anatomically, the rat prostate is made up of three distinct lobes (dorsal, lateral, and ventral), whereas the human prostate appears to be a single, fairly uniform structure (40, 41). Additionally, in the case of the LNCaP cells, these are not obtained from intact human prostate tissues but rather are prostate adenocarcinoma cells isolated from a supraclavicular lymph node to which they have metastasized (42).

Relatively little information is available concerning native *in vivo* vitamin A dynamics in human prostate tissue, and even less is known about how certain retinoid therapies and interactions might affect native vitamin A stores or the flux of the vitamin through this tissue. A recent study in which the levels of retinol and retinoic acid were measured in normal human prostate, BPH, and carcinoma samples reported a 2.5-fold increase in retinol in BPH as compared with normal or carcinoma tissues (43). One explanation the authors suggest is that there might be a more efficient uptake of retinol from the circulation that might be aided by a higher level of CRBP in BPH tissue. It is of interest as well that they found significantly lower levels of retinoic acid in carcinoma tissue as compared with either BPH or normal prostate tissue, a finding they suggest, based on earlier work (44–46), might be related to an increased degradation of retinoic acid, which in turn could be facilitated by increased levels of CRABP. Although the roles of both CRBP and CRABP in the intracellular transport of native retinoid remain to be delineated, in regard to possibility that 4-HPR might interfere in some way with intracellular retinoid transport, *in vitro* binding studies have indicated that both CRBP and CRABP have little or no affinity for 4-HPR (47), although it is unclear as to whether this might be the case *in vivo*. Other retinoid therapies have been shown to influence intracellular binding protein levels and function. For example, in studies with monkeys, treatment with all-*trans* retinoic acid has been shown to be associated with an up-regulation of CRABP, as determined in skin biopsy samples collected during and after the course of administration with this retinoid

<sup>3</sup> K. C. Lewis and J. F. Hochadel, unpublished observations.

(48). Similar effects on intracellular binding proteins with 4-HPR treatment have not been reported.

It remains to be determined how a reduction in stores of native vitamin A in and a decrease in the flux of the native vitamin through the prostate might affect the chemopreventive potential of this tissue. As pointed out above, in those tissues examined, the flux of native vitamin A through the tissue is much greater than is actually used (20, 34–37). The most comprehensive whole body model of native retinoid metabolism that we have to date indicates that even in animals with very low vitamin A status where the utilization rate of vitamin A by the whole body was extremely low (~1.65 RE/day), when this was compared with the plasma turnover rate, the latter was nearly 13 times higher (~21.0 RE/day), the liver turnover rate was more than five times higher (~9.1 RE/day), and the turnover rate from the carcass was nearly six times higher (~9.5 RE/day; Ref. 37). Thus, overall, it would appear that homeostatic control mechanisms are functioning in such a manner so as to assure that there is a relatively high flow of native vitamin through tissues under a wide variety of conditions. One of several objectives in carrying out the types of studies that we have been doing is to gain a better understanding of why this is occurring and to better understand and delineate the specific mechanisms involved. Conceivably, a disruption of this high flux of native vitamin A through the tissue such as is associated with the administration of 4-HPR might over time be detrimental to the overall health and maintenance of the tissue. On the other hand, it is possible that such a decreased flow of native vitamin A through the prostate might serve a protective chemopreventive function, perhaps, for example, by making less native vitamin A available for the formation of precancerous lesions and eventual tumor formation and growth. In our future studies, we plan to examine, in more detail, tissue-specific and differential effects of decreased storage and/or decreased flux of native retinoid through selected tissues and the system in general.

One overall goal of our research efforts has been to develop and test a complementary *in vivo* and *in vitro* modeling systems approach that could be used to assess the chemopreventive and chemotherapeutic potential of retinoid and retinoid/drug combinations. Our *in vitro* studies in PrEC and LNCaP cells represent our initial efforts to design tissue culture systems that would more accurately reflect *in vivo* retinoid metabolism and interactions, which in turn would allow us to more easily focus in detail on selected aspects of our *in vivo* kinetic findings in the prostate. Clearly, inherent limitations of tissue culture systems preclude precise simulation of *in vivo* conditions; however, given the nature of our studies, it was important that we be able to compare at least selected aspects of our *in vivo* and *in vitro* findings. Thus, for our purposes, a tissue culture system had to meet certain minimal requirements. For example, because we were studying retinoid-related events, including factors affecting retinoid storage and dynamics, an important determinant for us in choosing a tissue culture system was that there were at least some native retinoid stores present in the cells and/or the cells were able to process retinoid in a reasonably normal fashion when it was presented to the cell. We found that without preconditioning of both PrEC and LNCaP cells with low levels of retinol prior to beginning our experiments, these cells contained little or no detectable retinoid and appeared to have a reduced ability to metabolize the 4-HPR administered. However, the retinol pretreatment resulted in uptake of retinol and subsequent conversion of retinol to retinyl esters in a pattern normally found in tissues *in vivo*. The retinol pretreatment also enhanced the uptake of 4-HPR into the cells as well as its subsequent conversion to a number of related metabolites.<sup>3</sup> Thus, using the appropriate treatment regimens, we were to some extent able to tailor our system to more closely mimic selected aspects of *in vivo* retinoid metabolism on which we wanted to focus. Nevertheless, for both prostate cell lines we studied, after

treatment with 4-HPR, we did not observe a decrease in total intracellular native retinoid stores similar to what we had found for the intact prostate in our *in vivo* studies. As alluded to earlier, species differences and particularly inherent limitations of *in vitro* tissue culture systems such as we have used in these studies can make direct comparisons among the various systems we are examining problematic. The applicability and translational potential of our findings and the hypotheses that have emerged from our work will need to be evaluated further in future studies.

To our knowledge, a detailed study of normal retinoid kinetics in the prostate such as we have done has not been reported. Although the models we hypothesize need to be tested within the context of a whole body model compartmental model, it is clear that the metabolism of native retinoid in the prostate is altered substantially by the administration of the synthetic retinoid 4-HPR. Moreover, our results highlight the potential utility of a complementary *in vivo* and *in vitro* modeling systems approach such as we have used in the present study. Although the work presented here has focused on the prostate, to better understand metabolism of individual retinoids and various retinoid interactions we are presently applying a similar approach to other target organs and cell systems (both normal and cancerous tissue) including breast, ovarian, cervical, and ocular tissues. We are presently investigating how native retinoid metabolism and retinoid interactions in these systems are affected by different nutrient, hormonal, and pharmacological perturbations.

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