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N-(4-hydroxyphenyl)retinamide (Fenretinide) and nephrectomy alter normal plasma retinol-binding protein metabolism

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We have reported previously that injecting vitamin A–deficient rats with N-(4-hydroxyphenyl)retinamide causes a significant reduction in the liver retinol-binding protein concentration and a 2 fold rise in the kidney retinol-binding protein concentration. This presumably reflects a rapid translocation of retinol-binding protein from the liver to the kidney through the plasma, although no rise in plasma retinol-binding protein is detected. In the present studies, nephrectomized rats were used to determine if retinol-binding protein accumulating in kidneys passes through the plasma. Bilateral nephrectomy in control rats caused the plasma retinol-binding protein concentration to approximately double by 5 hr postsurgery. However, nephrectomy plus N-(4-hydroxyphenyl)retinamide treatment did not result in an increase in the plasma retinol-binding protein concentration. Therefore, the lowering of liver retinol-binding protein concentration in response to N-(4-hydroxyphenyl)retinamide treatment was not accounted for by an accumulation of retinol-binding protein in the plasma compartment. Interestingly, the muscle retinol-binding protein concentration increased with nephrectomy plus N-(4-hydroxyphenyl)retinamide treatment. The ratio of muscle retinol-binding protein:plasma retinol-binding protein in vitamin A–deficient nephrectomized rats treated with N-(4-hydroxyphenyl)retinamide was significantly higher than in comparable rats treated with either carrier or retinol. We conclude that in vivo N-(4-hydroxyphenyl)retinamide induces the secretion of retinol-binding protein from the liver. Since the N-(4-hydroxyphenyl)retinamide–retinol-binding protein complex does not bind with transthyretin it rapidly leaves the plasma. In non-nephrectomized rats this complex is rapidly filtered by the kidney. Nephrectomizing rats causes the retinol-binding protein secreted in response to N-(4-hydroxyphenyl)retinamide to diffuse into interstitial fluid.
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Introduction

In recent years there has been revived interest in the efficacy of retinoids, compounds similar in structure to retinol, in the prevention and treatment of cancers. Modifications to the chemical structure of retinol have produced over 2,000 analogs with varying target organ specificity and chemopreventive properties.[1] Unfortunately most of these retinoids are toxic, preventing their approval for cancer treatment. One promising analog with a low level of toxicity, N-(4-hydroxyphenyl)retinamide (Fenretinide; HPR) is known to inhibit effectively cancers of mammary tissue,[2] urinary bladder,[3] skin,[4] prostate gland,[5] and ovarian tissue.[6]

Although HPR is relatively nontoxic, it has caused night blindness, a symptom of vitamin A–deficiency, in patients given a dose of 2 mmol/day.[7] The symptoms appear to result from a depression of plasma retinol concentration

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thus causing less retinol to be available for the visual process. A dose of 0.51 mmol of HPR/day decreases the plasma retinol concentration by about 40%[8,9] while the plasma retinol pool is not turned over more rapidly during HPR treatment.[10] The indication from these data is that the lower plasma retinol concentration is caused by a decreased secretion of the retinol/retinol-binding protein complex (holoRBP) into the plasma following HPR treatment.

Vitamin A–deficient rats accumulate liver retinol-binding protein (RBP) and rapidly secrete it into plasma upon retinol repletion.[11] Schaffer et al.[12] demonstrated that the accumulated RBP in vitamin A–deficient rats decreases about 60% within 150 min after HPR treatment while kidney RBP concentration rises 29 fold higher than control rats. An increase in serum RBP concentrations was not observed after the administration of HPR, although presumably the accumulated RBP in the kidney was transported from the liver through the blood.

In the present study, nephrectomized rats were used to determine if liver RBP secreted in response to HPR is being translocated through the plasma and rapidly removed by the kidney. Nephrectomy causes retinol to accumulate in the general circulatory system.[13] In rats of normal vitamin A status, greater than 95% of plasma retinol is associated with RBP. Therefore, presumably the plasma RBP concentration parallels the plasma retinol rise. The nephrectomized rat model provides an appropriate system to determine if the RBP accumulating in the kidneys after HPR treatment passes through the plasma.

Methods and materials

All procedures were conducted in compliance with guidelines of The Pennsylvania State University Animal Care and Use Committee.

Animals and diets

Male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) 21 days of age and weighing 40 to 50 g were used for all experiments. Upon arrival animals were individually housed in stainless steel wire-mesh cages in a room with controlled temperature (22–24°C), humidity (40 to 60%), and lighting (0700 to 1900 hr). All animals had free access to food and water.

The vitamin A–sufficient rats used in study 1 were fed a purified casein-based diet[12] containing 2.63 μmol of retinyl palmitate/kg of diet. The vitamin A–deficient rats used in study 2 were fed the same purified diet except that it was vitamin A–free.[12] After the vitamin A–deficient rats had a plasma vitamin A concentration of <0.15 μmol/L, after approximately 50 days, they were divided into clusters with nearly the same body weight, and then rats from each cluster were randomly assigned to treatments.

Preparation of HPR, retinol, and Tween 40 solutions

The HPR, retinol, and carrier solutions were prepared fresh on the day of injection by the method of Schaffer et al.[12] under gold lighting. An appropriate amount of HPR or retinol was suspended in Tween 40 (Sigma Chemical Co., St. Louis, MO, USA) and diluted with water (1:4 vol/vol). The HPR solution (18 mmol/L) was administered in a dose of 2.85 mL/kg of body weight (BW) providing 51 μmol of HPR/kg of BW. The retinol solution (0.52 mmol/L) was administered as a 1.0 mL injection. The control carrier solution for both studies contained Tween 40 and water (1:4, vol/vol).

Study 1

This study was conducted with three separate groups of vitamin A–sufficient rats that were obtained from the vendor several months apart. The animals were divided randomly among the four treatment groups: nephrectomized and injected with Tween 40 carrier solution (NEPH-CARRIER, $n = 7$); nephrectomized and injected with HPR (NEPH-HPR, $n = 7$); sham operated and injected with HPR (SHAM-HPR, $n = 7$); and sham operated and injected with Tween 40 carrier solution (SHAM-CARRIER, $n = 7$). All of the rats in the SHAM-CARRIER treatment were in the third group of rats obtained from the vendor. Data from animals that did not survive during the 5 hr post-treatment were excluded from analysis.

After obtaining a baseline blood sample via a tail vein, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (0.16 mmol/kg of BW; A. J. Buck & Son, Owings Mills, MD, USA). The animals were maintained under anesthesia for the duration of the experiment with additional injections of sodium pentobarbital (0.016 mmol/kg of BW) as needed. Following anesthesia, using aseptic techniques, the NEPH-HPR and NEPH-CARRIER groups were bilaterally nephrectomized and the SHAM-HPR and SHAM-CARRIER groups were sham operated. All rats were injected intravenously via the dorsal vein of the penis with the appropriate treatment solution. At 0.5, 1, 2, 3, 4, and 5 hr, blood samples were taken from the jugular vein[14] and mixed with 5 μL of 0.4 mol/L of disodium ethylenediamine-tetraacetic acid (EDTA). Plasma samples were removed and stored at −20°C under N_2 until analyzed for retinol, RBP, and transthyretin (TTR). After 5 hr, rats were exsanguinated via the abdominal aorta. Perfusion of the animals was performed with 250 mL of Hanks' balanced salt solution, pH 7.2,[15] administered via the left ventricle of the heart. Liver, kidneys, and a portion of the external abdominal oblique muscle were excised, blotted, and stored frozen at −20°C for subsequent analysis.

Study 2

Vitamin A–deficient rats were divided into three groups: vitamin A–deficient, nephrectomized, and injected with Tween 40 carrier solution (A^-NEPH-CARRIER, $n = 6$); vitamin A–deficient, nephrectomized, and injected with HPR (A^-NEPH-HPR, $n = 6$); and vitamin A–deficient, nephrectomized, and injected with retinol (A^-NEPH-RETINOL, $n = 6$). The experimental protocols and parameters analyzed in study 2 were as described for study 1. Data collected from animals that did not survive for the entire 5 hr post-treatment period were excluded from analysis.

Radioimmunoassays

One gram of liver, kidney, and muscle were separately homogenized in 4 vol (wt:vol) of 0.25 mol/L of sucrose using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY, USA). Tissue homogenates were diluted with an equal volume of Triton X-100 (61.9 mmol/L), and diluted to the most sensitive range of our radioimmunoassays (0.93 to 2.33 nmol/L for RBP and 0.18 to 0.91 nmol/L for TTR). At the time of analysis, plasma samples were thawed, diluted appropriately, and analyzed for RBP[16] and TTR[17] by radioimmunoassays.

Retinoid determinations

Gold lighting was used for any procedure that involved samples that would be analyzed for vitamin A. Prior to treatment, serum

vitamin A concentrations in the vitamin A–deficient rats were determined fluorometrically[18] using a blood sample taken from the jugular vein. In both studies plasma samples were analyzed for retinol using the high performance liquid chromatography (HPLC) method of Schaffer et al.[12] employing a stainless steel 3.9 mm × 15 cm, 5 μm reverse-phase C_{18} column (Nova-Pak, Millipore, Milford, MA, USA) using a mobile phase of methanol-water (88: 12, vol/vol) at a flow rate of 1.0 mL/min. Liver samples were saponified by the method of Green et al.[19] prior to HPLC analysis for retinol.

Statistical analysis

Values are reported as mean ± the population standard error. For most of the data, comparisons within and between data sets were made using one-way analysis of variance (ANOVA) followed by comparisons among treatment mean differences using Tukey's honestly significant difference test for multiple comparisons.[20] Because the initial plasma values in study 1 differed greatly from animal to animal, but the animals responded to the treatments in a consistent manner, the plasma values in study 1 were analyzed by a paired Student's *t*-test. Linear regression was used to determine the rate of change in the concentration of plasma retinol and plasma RBP. Statistics were calculated with the SYSTAT software package, DOS version 5.0 (Systat, Inc., Evanston, IL, USA). A minimum probability of significance was assigned at $P < 0.05$.

Results

Study 1. Nephrectomy plus HPR treatment alters plasma, liver, kidney, and muscle vitamin A metabolism in vitamin A–sufficient rats

Differences in liver RBP and TTR are indicated in *Table 1*. In addition to its role in retinol metabolism, TTR functions in the transport of thyroxin in the plasma. We used TTR as a general plasma protein metabolism marker. Consistent with our previous findings,[10,12] all rats receiving HPR had a significantly higher concentration of liver RBP compared with all rats receiving the Tween 40 carrier solution 5 hr after treatment. In contrast, nephrectomy did not produce a significant change in liver RBP concentration. Interestingly, the liver TTR concentrations of the rats injected with HPR were approximately 1.5 times the concentrations found in the rats given the Tween 40 carrier. Nephrectomy did not change the concentrations of liver TTR.

Kidney RBP and TTR concentrations in sham operated rats are presented in *Table 2*. Data from the kidneys of nephrectomized rats are not presented since those organs were not perfused, thus allowing blood to remain in the tissue and giving concentrations that are not strictly representative of the tissue itself. Five hours post-treatment, kidney RBP concentrations in SHAM-HPR treated rats were about three times that of SHAM-CARRIER. Kidney TTR concentrations were unchanged by HPR treatment.

To determine if RBP (mol. wt. = 21,422)[21] was diffusing into the extravascular-extracellular interstitial space after nephrectomy and HPR treatment, the RBP concentration in a portion of the external abdominal oblique muscle was measured along with the concentration of a marker protein, TTR (mol. wt. = 54,792).[22] Absolute concentrations for RBP and TTR in muscle and the ratio between the two proteins are indicated in *Table 3*. TTR concentrations were used as a measure for general plasma protein concentrations in the interstitial fluid. The concentrations of TTR did not vary significantly among treatment groups. After 5 hr, muscle RBP was significantly higher in NEPH-HPR treated rats compared with that of SHAM-HPR treated rats.

The extent of RBP diffusion into the interstitial fluid was determined by the ratio of muscle to plasma RBP. The muscle:plasma ratio of RBP concentration was significantly higher in the NEPH-HPR treated rats than in any of the other treatments (*Table 3*).

Changes in plasma retinol and RBP over the 5 hr course

Table 1 Effects of N-(4-hydroxyphenyl)retinamide treatment and nephrectomy on liver RBP and TTR concentrations*

Group	*n*	RBP	TTR
Vitamin A–sufficient, study 1		(nmol/g of wet wt.)	
NEPH-CARRIER	5	3.2 ± 0.3	0.91 ± 0.11[a]
NEPH-HPR†	6	4.4 ± 0.5	1.3 ± 0.1[b]
SHAM-CARRIER	5	3.3 ± 0.1	0.90 ± 0.05[a]
SHAM-HPR†	6	3.9 ± 0.4	1.6 ± 0.1[b]
Vitamin A–deficient, study 2		(nmol/g of wet wt.)	
A⁻NEPH-CARRIER	4	9.3 ± 1.5[a]	0.52 ± 0.09[a]
A⁻NEPH-HPR	4	5.4 ± 0.5[b]	1.1 ± 0.1[b]
A⁻NEPH-RETINOL	5	1.9 ± 0.2[c]	0.50 ± 0.07[a]

*Values are means ± SE. Rats were either nephrectomized or sham operated and then injected with either 51 μmol of HPR/kg of BW, 0.52 μmol of retinol (study 2), or Tween 40 carrier solution. Livers were collected 5 hr after treatment. Within studies, values in the same column having the same superscript are not significantly different, $P > 0.05$. One-way ANOVA followed by Tukey's honestly significant difference test was used for statistical analysis.
†In study 1, when the RBP data from both HPR and CARRIER treatment groups were separately pooled and compared by Student's *t*-test, the HPR treatment was significantly higher than the CARRIER treatment, $P < 0.05$. Mean liver weight was not significantly different within each study and was 12.2 ± 0.4 g for vitamin A–sufficient rats (study 1) and 9.1 ± 0.3 g for vitamin A–deficient rats (study 2). Mean body weight was not significantly different within each study and was 423 ± 69 g for vitamin A–sufficient rats (study 1) and 276 ± 21 g for vitamin A–deficient rats (study 2).
RBP = retinol-binding protein, TTR = transthyretin, HPR = N-(4-hydroxyphenyl)retinamide.

Table 2 Effect of N-(4-hydroxyphenyl)retinamide treatment on kidney RBP and TTR concentrations*

Group	RBP	TTR
Vitamin A–sufficient, study 1	(nmol/g of wet wt.)	
SHAM-CARRIER	3.9 ± 0.6[a]	1.1 ± 0.1
SHAM-HPR	12 ± 1[b]	1.3 ± 0.2

*Values are means ± SE. The number of animals per treatment groups is the same as reported in *Table 1*. Rats were sham operated and then injected with either 51 μmol of HPR/kg of BW or Tween 40 carrier solution. Kidneys were collected 5 hr after treatment and were not significantly different in mass between treatments. The mean mass of both kidneys was 2.85 ± 0.094 g for vitamin A–sufficient rats. Student's *t*-test was used for statistical analysis. Values in the same column having the same superscript are not significantly different, $P > 0.05$.
RBP = retinol-binding protein, TTR = transthyretin, HPR = N-(4-hydroxyphenyl)retinamide.

Table 3 Effect of N-(4-hydroxyphenyl)retinamide treatment and nephrectomy on muscle RBP and TTR concentrations*

Group	Muscle			RBP muscle:plasma ratio†
	RBP	TTR	RBP:TTR ratio	
Vitamin A–sufficient, study 1		(nmol/g of wet wt.)		
NEPH-CARRIER	0.11 ± 0.03^{a,b}	0.17 ± 0.03	0.60 ± 0.09^{a,b}	0.019 ± 0.006^{b}
NEPH-HPR	0.18 ± 0.03^{a}	0.22 ± 0.02	0.81 ± 0.1^{a}	0.099 ± 0.02^{a}
SHAM-CARRIER	0.12 ± 0.03^{a,b}	0.20 ± 0.03	0.59 ± 0.1^{a,b}	0.025 ± 0.007^{b}
SHAM-HPR	0.061 ± 0.03^{b}	0.15 ± 0.02	0.39 ± 0.03^{b}	0.049 ± 0.005^{b}
Vitamin A–deficient, study 2		(nmol/g of wet wt.)		
A^{-}NEPH-CARRIER	0.14 ± 0.03^{b}	0.44 ± 0.02	0.31 ± 0.04^{a}	0.043 ± 0.007^{a}
A^{-}NEPH-HPR	0.24 ± 0.02^{a}	0.44 ± 0.05	0.56 ± 0.05^{b}	0.10 ± 0.02^{b}
A^{-}NEPH-RETINOL	0.20 ± 0.02^{a,b}	0.41 ± 0.03	0.48 ± 0.02^{b}	0.025 ± 0.003^{a}

*Values are means ± SE. The number of animals per treatment is as reported in *Table 1*.
†Determined by dividing the muscle RBP expressed as μmol/kg by the 5 hr plasma RBP expressed as μmol/L. Rats were either nephrectomized or sham operated and then injected with either 51 μmol HPR/kg of BW, 0.52 μmol retinol (study 2), or Tween 40 carrier solution. Within studies, values in columns having the same superscript are not significantly different, $P > 0.05$. Tukey's honestly significant difference test was used for statistical analysis.
RBP = retinol-binding protein, TTR = transthyretin, HPR = N-(4-hydroxyphenyl)retinamide.

of study 1 are depicted in *Figures 1* and *2*, respectively. Although initially plasma retinol was higher in the control, SHAM-CARRIER animals, its level did not change significantly over time. Plasma retinol and RBP in the NEPH-CARRIER rats rose significantly during the sampling time to concentrations that were about twice the baseline levels. SHAM-HPR rats displayed about a 50% drop in both plasma retinol and RBP concentrations 5 hr post-treatment. Interestingly, the plasma retinol and RBP concentration in

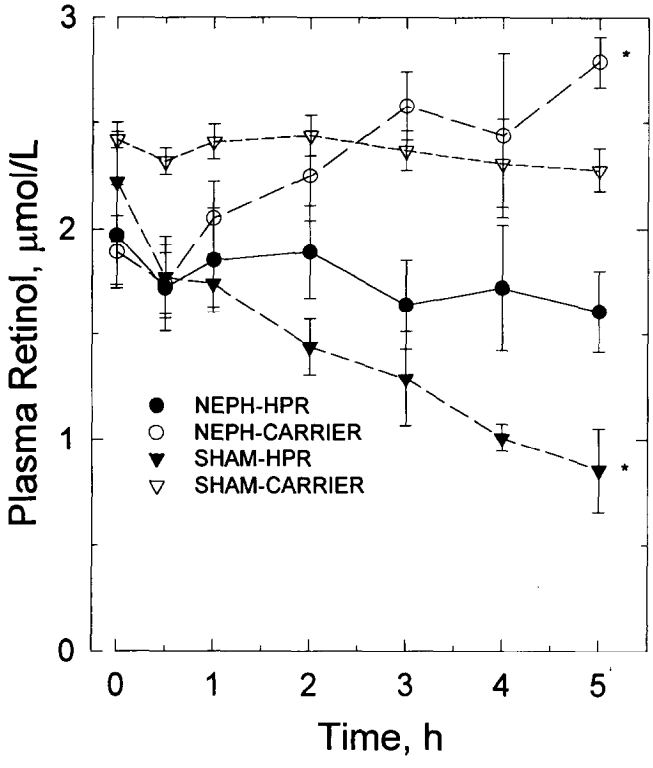

Figure 1 Study 1: Plasma retinol concentrations of vitamin A–sufficient rats after nephrectomy plus Tween 40 injection (NEPH-CARRIER), nephrectomy plus HPR injection (51 μmol/kg of BW) (NEPH-HPR), sham operated plus Tween 40 injection (SHAM-CARRIER), and sham operated plus HPR injection (51 μmol/kg of BW) (SHAM-HPR). Results are presented as means ± SE. The 0 hr, values for each treatment group were separately compared with the respective 5 hr values by a paired Student's *t*-test; *$P < 0.05$. The number of animals per treatment is as reported in *Table 1*. HPR = N-(4-hydroxyphenyl)retinamide.

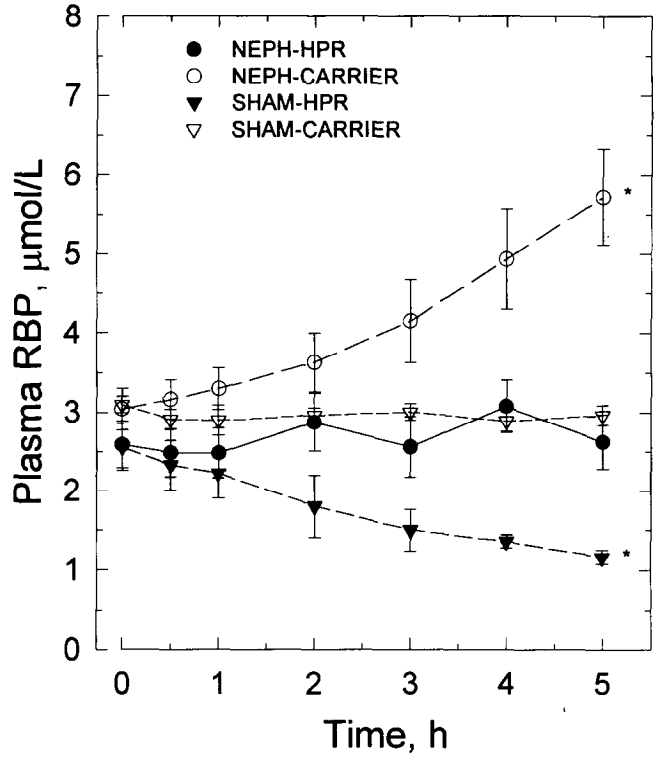

Figure 2 Study 1: Plasma retinol-binding protein (RBP) concentrations of vitamin A–sufficient rats after nephrectomy plus Tween 40 injection, nephrectomy plus HPR injection (51 μmol/kg of BW), sham operated plus Tween 40 injection, and sham operated plus HPR injection (51 μmol/kg of BW). Results are presented as means ± SE. The 0 hr values of each treatment group were separately compared with the respective 5 hr values by a paired Student's *t*-test; *$P < 0.05$. The number of animals per treatment is as reported in *Table 1*. HPR = N-(4-hydroxyphenyl)retinamide.

NEPH-HPR treated rats did not change significantly over the course of the study.

Plasma TTR (data not shown) was used as a marker of general protein distribution and metabolism. No significant changes were observed within or between treatment groups over the 5 hr sampling period.

Study 2. Vitamin A deficiency plus HPR treatment alters plasma, liver, kidney, and muscle retinol and RBP

The effect of vitamin A deficiency and HPR treatment on liver RBP and TTR is indicated in *Table 1*. Significantly less liver RBP was present following A⁻NEPH-RETINOL treatment as compared with A⁻NEPH-CARRIER or A⁻NEPH-HPR treatment. A⁻NEPH-HPR treated animals had 35% less accumulated RBP present following treatment relative to A⁻NEPH-CARRIER treatment, while A⁻NEPH-RETINOL treatment caused 80% less RBP to be present. A significantly higher concentration of liver TTR was noted with HPR treatment, similar to that observed in vitamin A–sufficient animals (study 1).

Changes in the plasma retinol and RBP concentration over the 5 hr study period are indicated in *Figures 3* and *4*, respectively. We have previously reported that administration of retinol to vitamin A–deficient rats causes the plasma concentrations of retinol and RBP to increase transiently to twice normal concentrations.[11,12,16] In this study, the administration of retinol to vitamin A–deficient nephrectomized rats caused a rise in plasma retinol and RBP to a higher concentration than in any of our previous studies, greater than 3 fold. Plasma retinol did not rise in either the A⁻NEPH-CARRIER or the A⁻NEPH-HPR treated groups, but plasma RBP rose in both groups to significantly higher concentrations by 5 hr. The plasma RBP concentrations at 5 hr were within the range normally observed in vitamin A–sufficient rats. There was no significant difference in plasma RBP between carrier and HPR treated rats.

Muscle RBP and TTR concentrations of vitamin A–deficient rats were measured to determine if nephrectomy plus HPR treatment changed the profile of proteins in the interstitial fluid relative to plasma. Absolute values for RBP and TTR and the ratio between the two proteins are reported for each treatment group in *Table 3*. The concentration of RBP in the external abdominal oblique muscle was significantly greater in both A⁻NEPH-HPR and A⁻NEPH-RETINOL treated rats over that of A⁻NEPH-CARRIER treated rats. HPR did not produce a greater RBP concentration in interstitial fluid than retinol. As we observed in study 1, the ratio of RBP:TTR demonstrated that RBP will diffuse into inter-

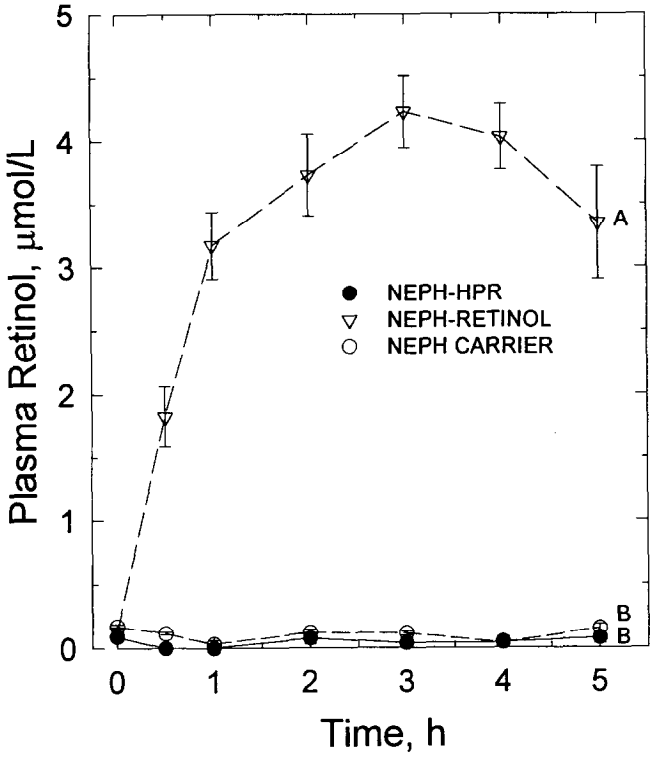

Figure 3 Study 2: Plasma retinol concentrations of vitamin A–deficient rats after nephrectomy plus Tween 40 injection, nephrectomy plus HPR injection (51 µmol/kg of BW), nephrectomy plus retinol injection (0.52 µmol). Results are presented as means ± SE. Data points at 5 hr having the same superscript are not significantly different ($P < 0.05$) Tukey's honestly significant difference test was used for statistical analysis. The number of animals per treatment is as reported in *Table 1*. RBP = retinol-binding protein, HPR = N-(4-hydroxyphenyl)retinamide.

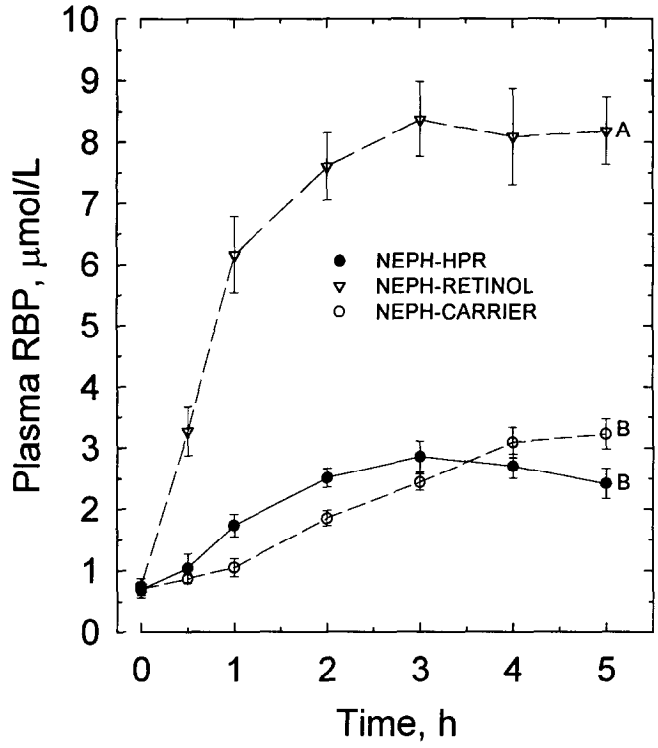

Figure 4 Study 2: Plasma retinol-binding protein (RBP) concentrations of vitamin A–deficient rats after nephrectomy plus Tween 40 injection, nephrectomy plus HPR injection (51 µmol/kg of BW), nephrectomy plus retinol injection (0.52 µmol). Results are presented as means ± SE. Data points at 5 hr having the same superscript are not significantly different ($P > 0.05$). All of the 5 hr values were significantly different ($P < 0.05$) from the respective time 0 values. Tukey's honestly significant difference test was used for statistical analysis. The number of animals per treatment is as reported in *Table 1*. HPR = N-(4-hydroxyphenyl)retinamide.

stitial fluid. Additionally, the ratio of muscle RBP:plasma RBP indicates that RBP preferentially partitions into interstitial fluid during NEPH-HPR treatment. The muscle TTR concentration was not significantly different due to any of the treatments.

As in study 1, plasma TTR (data not shown) was used as a marker of plasma protein status. No significant changes were observed within or between vitamin A–deficient treatment groups for the 5 hr sampling period.

Discussion

Vitamin A–deficient rats accumulate a large excess of RBP in the liver which can be rapidly secreted into the plasma when the animal is repleted with retinol.[11] Recently we reported the effects of HPR on the metabolism of RBP in vitamin A–deficient rats.[12] When the rats were given HPR their liver RBP concentrations dropped but not to the extent that was obtained with 1/30th the amount of retinol. The kidney RBP concentrations increased to about 29 times the concentrations in the deficient control rats. These concentrations were higher than would be expected in normal rats. However, in this study, we were intrigued that the plasma RBP concentrations did not rise even transiently following HPR treatment. In the present study our purpose was to construct a model that would delineate whether HPR accumulating in kidneys of HPR treated rats was being translocated from the liver through the plasma.

Berni and Formelli[23] have previously demonstrated that RBP bound HPR, but the HPR-RBP complex did not bind to TTR. Since holoRBP (mol. wt. = 21,422)[21] normally circulates in plasma as a protein–protein complex with TTR (mol. wt. = 54,792),[22] the large complex protects the holoRBP from rapid glomerular filtration in the kidney. Smaller molecular weight proteins (mol. wt. <25,000) like RBP are rapidly filtered as they flow through the kidney glomerulus.[24–26] The renal metabolism of these filtered proteins is unclear, but it is hypothesized that they are either reabsorbed or catabolized by the renal tubular cells and thus do not appear in the urine.[27] Based on the binding affinities published by Berni and Formelli,[23] if an HPR-RBP complex was formed in the liver and secreted into the plasma it would not be expected to bind to TTR. Therefore, an HPR-RBP complex would be rapidly cleared from the plasma by glomerular filtration at the kidneys. If we nephrectomize the animals, we would expect an HPR-RBP complex to accumulate in the plasma following HPR treatment. Relatively small molecular weight proteins have the ability to diffuse through fenestrations in the capillary walls into the extravascular-extracellular interstitial fluid.[28] Therefore, another possibility is that without the ability to filter rapidly a small molecular weight HPR-RBP complex, it may diffuse more rapidly out of the circulatory system in nephrectomized rats.

RBP that does not have retinol bound (apoRBP) has a lower binding affinity for TTR than holoRBP.[24] While most of the holoRBP is bound to TTR, the binding affinity is such that at physiological concentrations about 5% of the holoRBP circulates free of TTR. Non-TTR bound RBP passing through the kidneys is rapidly filtered, providing a major route for plasma retinol and RBP turnover. Ikegami and Zile[13] demonstrated that nephrectomy raises the plasma retinol concentration in vitamin A–sufficient rats to about twice that of sham operated animals. As expected, nephrectomy of vitamin A–deficient rats failed to produce an increase in the plasma retinol concentration. We have confirmed these observations in our studies. Using linear regression analysis, we determined that retinol accumulates in the plasma of vitamin A–sufficient NEPH-CARRIER rats at a rate of 4.1 ± 0.5 nmol/hr (R^2 = 0.93). This is 26% of the plasma retinol turnover rate observed in non-nephrectomized rats of normal vitamin A status, 15.9 nmol/hr.[29] In rats having normal vitamin A status, the output of plasma retinol to the kidneys has been estimated to equal the input of retinol to the plasma from the kidneys.[30] Therefore, nephrectomy alone should not produce a rise in plasma retinol. In order to explain our observed rise in plasma retinol we must assume that either the input of retinol and RBP to plasma increased or the output from plasma decreased. Since over the 5 hr course of this study we did not observe a change in the general plasma protein concentration during nephrectomy, our observations imply, as suggested by Ikegami and Zile,[13] that the kidney is mechanistically involved in controlling plasma retinol homeostasis.

Since HPR appears to cause the mobilization of liver RBP[12] and nephrectomy causes an accumulation of retinol in the plasma,[13] we anticipated that administration of HPR to nephrectomized rats would increase the plasma RBP concentration even higher than nephrectomy alone. However, the plasma concentrations of retinol did not change over the course of study, and the large increase in plasma RBP concentration, expected as an HPR-RBP complex, was not observed. Even in nephrectomized vitamin A–deficient rats given HPR, the plasma RBP concentrations did not rise above those seen in the nephrectomized vitamin A–deficient animals. The form of plasma RBP in A⁻NEPH-CARRIER would be of the apo form since there is very little plasma retinol in the system, and the form of RBP in A⁻NEPH-HPR is assumed to be a combination of apo and an HPR-RBP complex. The observed parallel rises between vitamin A–deficient nephrectomized control and HPR-treated animals reflect the combined effects of diminished secretion of holoRBP into the plasma during HPR treatment and a lack of rapid RBP filtration by the kidney during HPR treatment.

Since there was no transient rise in plasma RBP following HPR treatment combined with nephrectomy, we investigated the possibility that the HPR-RBP complex was diffusing out of the circulatory system into the interstitial fluid. We used the RBP concentrations of muscle as a representative of interstitial fluid. Since the external abdominal oblique muscle had been well perfused, contamination from plasma RBP was minimal. Muscle contains less than 1% of the amount of RBP mRNA present in liver, and it has not been demonstrated to have plasma membrane receptors for RBP.[31] Therefore, any accumulated RBP remaining in the muscle following perfusion is representative of interstitial fluid RBP. Using a tissue having the ability to synthesize RBP endogenously would yield a tissue RBP concentration representing both synthesis by the tissue and accumulation in interstitial fluid. Thus, detecting a significant RBP concentration difference due to treatment, reflecting RBP originating from the plasma, would have been virtually impossible in tissues having the ability to synthesize RBP.

We observed a significant rise in the concentration of RBP in the muscle of nephrectomized rats treated with HPR. This increase in muscle RBP occurred in both vitamin A–sufficient and vitamin A–deficient rats. We realize the limitations of extrapolating the muscle RBP content to reflect the total body interstitial fluid RBP content. However, the tissue RBP concentration[32] correlates well with the interstitial fluid volume.[28] By assuming that 6.7% of muscle wet weight is interstitial fluid[28] and that 12.4% of BW is interstitial fluid,[33] the rise in the interstitial fluid RBP content between A⁻NEPH-CARRIER and A⁻NEPH-HPR (53.1 nmol) is greater than the decrease in the total amount of liver RBP (35.6 nmol). Therefore, the interstitial fluid may also represent the sum of the HPR-RBP complex that was formed in the liver plus other tissues that are smaller producers of RBP. If the HPR was affecting the synthesis of RBP in muscle we should have observed a rise in non-nephrectomized animals. A rise was not observed. RBP requires a ligand for secretion from the liver,[11,16,32] HPR rapidly lowers accumulated liver RBP[12] in vitamin A–deficient rats, and in this study we detect RBP in a greater total amount in interstitial fluid than the observed decrease in the total liver RBP.

Based on the totality of these findings we can conclude that an HPR-RBP complex is secreted from the liver into the plasma and that this complex (mol. wt. $< 22{,}000$) passes through the plasma and is rapidly cleared from the circulation by the kidneys. However, since in our studies we used 30× the amount of HPR as retinol, retinol is much more effective than HPR at inducing secretion of RBP from the liver. Therefore, a simple competition alone, between retinol and HPR, for binding to RBP does not appear to account for all of the inhibition in holoRBP secretion observed during HPR treatment.

Interestingly, HPR, a synthetic derivative of retinoic acid, has not been reported to produce retinoid toxicity symptoms at high doses, rather it produces retinol deficiency symptoms. This phenomenon of high doses of a retinoid producing retinol deficiency occurs even in vitamin A–sufficient humans and laboratory animals. A rapid decline of plasma retinol and RBP concentrations following HPR treatment is observed in studies investigating this mechanism.[9,10,12] The use of HPR for the treatment of cancer in humans requires daily dosages for several years. Using HPR in long-term cancer treatment causes a continued depression of the plasma retinol concentration.[34] Delineating the process by which the plasma retinol concentration is depressed during HPR treatment is necessary before a protocol can be developed for combatting this effect. Our current observations that accumulated liver RBP is significantly lowered following HPR treatment and our detection of a rise in an extrahepatic pool of RBP, which would more than account for this loss, provides plausible evidence that HPR is binding to RBP in the liver producing a complex that no longer follows the normally observed pattern of RBP-retinol metabolism. In the long-term administration of HPR, the altered retinol metabolism could prove additionally harmful by increasing the prevalence of vitamin A deficiency signs and symptoms in HPR-treated patients.

In the course of these studies, two unexplained findings were obtained. First, treatment with HPR increased the liver levels of TTR. It is not known whether RBP secretion from liver affects the amount of TTR secreted. Second, bilateral nephrectomy of vitamin A–deficient rats produced a continuous rise in plasma RBP, the form of which must be apo since a parallel rise in retinol was not observed. If, as we have previously suggested,[11,16,32] vitamin A deficiency prevents liver RBP secretion, the source of this RBP is unknown. We speculate that the liver may continually secrete a basal level of apoRBP, or since extrahepatic tissues do not accumulate RBP during vitamin A deficiency,[32] perhaps extrahepatic tissues continue to secrete apoRBP during vitamin A deficiency.

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References

1 Lippman, S.M., Kessler, J.F., and Meyskins, F.L. Jr. (1987). Retinoids as preventative and therapeutic anticancer agents (part I). *Cancer Treat. Rep.* **71,** 391–405

2 Moon, R.C., Thompson, H.J., Becci, P.J., Grubbs, C.J., Gander, R.J., Newton, D.L., Smith, J.M., Phillips, S.L., Henderson, W.R., Mullen, L.T., Brown, C.C., and Sporn, M.B. (1979). N-4-Hydroxyphenyl retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res.* **39,** 1339–1346

3 Moon, R.C., McCormick, D.L., Becci, P.J., Shealy, Y.F., Frickel, F., Paust, J., and Sporn, M.B. (1982). Influences of 15 retinoic acid amides on urinary bladder carcinogenesis in the mouse. *Carcinogenesis* **3,** 1469–1472

4 McCormick, D.L. and Moon, R.C. (1986). Antipromotional activity of dietary N-(4-hydroxyphenyl)retinamide in two-stage skin tumorigenesis in CD-1 and SENCAR mice. *Cancer Lett.* **31,** 133–138

5 Pollard, M. and Luckert, P.H. (1991). The inhibitory effect of 4-hydroxyphenyl retinamide (4-HPR) on metastasis of prostate adenocarcinoma - III cells in Lobund-Wistar Rats. *Cancer Lett.* **59,** 159–163

6 Formelli, F. and Cleris, L. (1993). Synthetic retinoid Fenretinide is effective against a human ovarian carcinoma xenograft and potentiates cisplatin activity. *Cancer Res.* **53,** 5374–5376

7 Kaiser-Kupfer, M.I., Peck, G.L., Caruso, R.C., Jaffe, M.J., DiGiovanna, J.J., and Gross, E.G. (1986). Abnormal retinal function associated with Fenretinide, a synthetic retinoid. *Arch. Opthal.* **104,** 69–70

8 Dimitrov, N.V., Meyer, C.J., Perloff, M., Ruppenthal, M.M., Phillipich, M.J., Gilliland, D., Malone, W., and Minn, F.L. (1990). Alteration of retinol-binding protein concentrations by the synthetic retinoid Fenretinide in healthy human subjects. *Am. J. Clin. Nutr.* **51,** 1082–1087

9 Formelli, F., Carsana, R., and Costa, A. (1987). N-(4-Hydroxyphenyl)retinamide (4-HPR) lowers plasma retinol levels in rats. *Med. Sci. Res.* **15,** 843–844

10 Smith, J.E., Lawless, D.C., Green, M.H., and Moon, R.C. (1992). Secretion of vitamin A and retinol-binding protein into plasma is depressed in rats by N-(4-hydroxyphenyl)retinamide (Fenretinide). *J. Nutr.* **122,** 1999–2009

11 Smith, J.E., Muto, Y., Milch, P.O., and Goodman, D.S. (1973). The effects of chylomicron vitamin A on the metabolism of retinol-binding protein in the rat. *J. Biol. Chem.* **248,** 1544–1549

12 Schaffer, E.M., Ritter, S.J., and Smith, J.E. (1993). N-(4-hydroxyphenyl)retinamide (Fenretinide) induces retinol-binding protein secretion from liver and accumulates in the kidney of rats. *J. Nutr.* **123,** 1497–1503

13 Ikegami, S. and Zile, M.H. (1992). Plasma vitamin A in nephrectomized rats. *Nutr. Res.* **12,** 265–277

14 Phillips, W.A., Stafford, W.W., and Stuut, J., Jr. (1973). Jugular vein technique for serial blood sampling and intravenous injection in the rat. *Proc. Soc. Exp. Biol. Med.* **143,** 733–735

15 Hanks, J.H. and Wallace, R.E. (1949). Relation of oxygen and temperature in the preservation of tissues by refrigeration. *Proc. Soc. Exp. Biol. Med.* **71,** 196–200

16 Smith, J.E., Deen, D.D., Jr., Sklan, D., and Goodman, D.S. (1980). Colchicine inhibition of retinol-binding protein secretion by rat liver. *J. Lipid Res.* **21,** 229–237

17 Navab, M., Smith, J.E., and Goodman, D.S. (1977). Rat plasma prealbumin: metabolic studies on effects of vitamin A status and on tissue distribution. *J. Biol. Chem.* **252,** 5107–5114

18 Thompson, J.N., Erody, P., Brien, R., and Murry, T.K. (1971). Fluorometric determination of vitamin A in human blood and liver. *Biochem. Med.* **5,** 67–89

19 Green, M.H., Uhl, L., and Green, J.B. (1985). A multicompartmental model of vitamin A kinetics in rats with marginal liver vitamin A status. *J. Lipid Res.* **26,** 806–818

20 Steel, R.G.D. and Torrie, J.H. (1980). Multiple comparisons. In *Principles and Procedures of Statistics: A Biometrical Approach,* 2nd ed., p. 185–186, McGraw-Hill, New York, NY USA

21 Sundelin, J., Laurent, B.C., Anundi, H., Trägårdh, L., Larhammar, D., Björck, L., Eriksson, U., Åkerström, B., Jones, A., Newcomer, M., Peterson, P.A., and Rask, L. (1985). Amino acid sequence homologies between rabbit, rat, and human serum retinol-binding proteins. *J. Biol. Chem.* **260,** 6472–6480

22 Sundelin, J., Melhus, M., Das, S., Eriksson, U., Lind, P., Trägårdh, L., Peterson, P.A., and Rask, L. (1985). The primary structure of rabbit and rat prealbumin and a comparison with the tertiary structure of human prealbumin. *J. Biol. Chem.* **260,** 6481–6487

23 Berni, R. and Formelli, F. (1992). In vitro interaction of Fenretinide with plasma retinol-binding protein and its functional consequences. *FEBS Lett.* **308,** 43–45

24 Smith, F.R. and Goodman, D.S. (1971). The effect of diseases of the liver, thyroid, and kidneys on the transport of vitamin A in human plasma. *J. Clin. Invest.* **50,** 2426–2436

25 Rask, L. and Peterson, P.A. (1974). Structure and function of the retinol-binding protein: a protein characteristic of tubular proteinuria. *Protides Biol. Fluids* **21,** 485–491

26 Vahlquist, A., Peterson, P.A., and Wibell, L. (1973). Metabolism of the vitamin A transporting protein complex. I. Turnover studies in normal persons and patients with chronic renal failure. *Eur. J. Clin. Invest.* **3,** 352–362

27 Mogielnicki, R.P., Waldmann, T.A., and Strober, W. (1971). The renal handling of low molecular weight proteins. 1. L-chain metabolism in experimental renal disease. *J. Clin. Invest.* **50,** 901–909

28 Wiig, H., DeCarlo, M., Sibley, L., and Renkin, E.M. (1992). Interstitial exclusion of albumin in rat tissues measured by a continuous infusion method. *Am. J. Physiol.* **263,** H1222–H1233

29 Green, M.H. and Green, J.B. (1987). Multicompartmental analysis of whole body retinol dynamics in vitamin A-sufficient rats. *FASEB J.* **46,** 1011 (abstract 4047)

30 Lewis, K.C., Green, M.H., Green, J.B., and Zech, L.A. (1990). Retinol metabolism in rats with low vitamin A status: a compartmental model. *J. Lipid Res.* **31,** 1535–1548

31 Soprano, D.R. and Blaner, W.S. (1994). Plasma retinol-binding protein. In *The Retinoids, Biology, Chemistry and Medicine,* 2nd ed. (Sporn, M.B. Roberts, A.B., and Goodman, D.S., eds.), p. 257–281, Raven Press, New York, NY USA

32 Smith, J.E., Muto, Y., and Goodman, D.S. (1975). Tissue distribution and subcellular localization of retinol-binding protein in normal and vitamin A-deficient rats. *J. Lipid Res.* **16,** 318–323

33 Durkot, M.J., Martinez, O., Brooks-McQuade, D., and Francesconi, R. (1986). Simultaneous determination of fluid shifts during thermal stress in a small-animal model. *J. Appl. Physiol.* **61,** 1031–1034

34 Formelli, F., Clerici, M., Campa, T., Di Mauro, M.G., Magni, A., Mascotti, G., Moglia, D., De Palo, G., Costa, A., and Veronesi, U. (1993). Five-year administration of Fenretinide: Pharmacokinetics and effects on plasma retinol concentration. *J. Clin. Oncol.* **11,** 2036–2042