Pergamon

European Journal of Cancer Vol. 32A, No. 10, pp. 1803–1808, 1996
Published by Elsevier Science Ltd
Printed in Great Britain
0959-8049/96 $15.00+0.00

PII: S0959-8049(96)00197-9

Original Paper

Effects of Chronic Administration of *N*-(4-hydroxyphenyl)retinamide (4-HPR) in Rats on Vitamin A Metabolism in the Eye

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The retinoid *N*-(4-hydroxyphenyl)retinamide (4-HPR) effectively inhibits cancer in a variety of tissues. In contrast to many other retinoids, the toxicity problems associated with administration of 4-HPR have been found to be minimal or absent. However, the effects of 4-HPR upon normal metabolism of native physiological forms of vitamin A *in vivo* have not been adequately investigated. To understand better the interaction between 4-HPR and the native physiological forms of vitamin A, the present study examines the effects of long-term administration of 4-HPR upon normal vitamin A metabolism in the eyes. Male Sprague–Dawley rats were fed either a control diet sufficient in vitamin A (CON group; 0.8 retinol equivalents [RE]/g diet; $n = 28$) or a CON diet supplemented with 4-HPR (CON + 4-HPR group; 1173 μg 4-HPR/g diet; $n = 28$). Following an i.v. dose of physiologically radiolabelled retinol, associated with its normal plasma transport complex, the vitamin A content and radioactivity of the plasma and eyes were examined at different times over a 41 day period. Mean plasma retinol levels measured during the study period were significantly reduced in the CON + 4-HPR group as compared with the CON group (23.5 ± 7.0 and 50.3 ± 5.3 [mean ± S.D.]μg/dl, respectively). From approximately 7 days post-dosing, vitamin A levels in the eyes of the 4-HPR-treated group steadily decreased such that by the end of the study, they were only approximately one-fifth those of the CON group (0.098 ± 0.075 and 0.50 ± 0.053 RE, respectively). Kinetic analysis of vitamin A turnover in the eyes indicated that there was no apparent down-regulation of the fraction of vitamin A leaving this tissue on a daily basis; these values were found to be similar in both groups, averaging 0.104 ± 0.0393 and 0.113 ± 0.0373 per day (mean ± fractional standard deviation [F.S.D.]) for the CON and CON + 4-HPR groups, respectively. At the same time, the flow of vitamin A through the eyes was significantly decreased in the CON + 4-HPR group eyes (0.0162 ± 0.101 μg/day) as compared with the CON group (0.0604 ± 0.0672 μg/day). Our results suggest that compensatory mechanisms that would normally function to conserve depleting ocular vitamin A stores may be blocked in the 4-HPR-treated animals and further, that the 4-HPR itself appears to be interfering with the normal uptake and/or metabolism of vitamin A in the eye. These findings may help to provide at least a partial explanation for the visual impairment problems that have been reported in human trials that include long-term administration of 4-HPR. Published by Elsevier Science Ltd

Key words: *N*-(4-hydroxyphenyl)retinamide, vitamin A metabolism, retinoid supplementation, kinetic analysis, compartmental modelling

Eur J Cancer, Vol. 32A, No. 10, pp. 1803–1808, 1996

INTRODUCTION

THE RETINOID *N*-(4-hydroxyphenyl)retinamide (4-HPR) has been shown to be a useful agent in preventing cancer in a number of tissues [1]. It has been shown to be particularly effective in inducing cellular differentiation and the inhibition of chemically induced mammary tumours in rodents [2, 3]. Recent evidence from animal studies suggests the chemopreventive potential of 4-HPR in prostate cancer [4–6] and ovarian cancer [7]. As compared to other retinoids, the toxicity problems encountered with chronic administration of 4-HPR are reported to be minimal [2]. The effectiveness of 4-HPR in the

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Received 18 Dec. 1995; revised 15 Mar. 1996; accepted 26 Mar. 1996.

prevention of mammary tumorigenesis in animals has led to its present use in a series of clinical trials to assess the feasibility of long-term administration of this compound for the purpose of preventing mammary cancer in humans [8, 9]. Although no major toxicity symptoms have been reported thus far, a substantial lowering of plasma retinol and/or retinol-binding protein has been observed [8–15], in addition to several reported cases of impaired visual function, usually nyctalopia or night blindness [13–17]. The overt visual problems appear to be reversed when the 4-HPR treatment is discontinued. The current approach being used in human trials is to include a 3 day "drug holiday" period each month during which the 4-HPR is withheld [8, 9, 18]. We recently reported that long-term administration of 4-HPR is associated with marked alterations of normal vitamin A kinetics and overall metabolism of the vitamin [19]. Using eye samples which were collected during the course of the latter study, in order to better understand the role of 4-HPR in the visual problems that have been reported, the present study focuses specifically upon the effects of long-term administration of this retinoid upon metabolism of vitamin A in ocular tissue.

MATERIALS AND METHODS

Chemicals and isotopes

All chemicals and solvents were of reagent or HPLC grade. Solvents, used for extraction of retinoids from plasma and tissues, were made up to include butylated hydroxytoluene (BHT; 5 μg/ml) as an antioxidant. The 4-HPR was kindly provided by Dr Vernon E. Steele of the National Cancer Institutes Division of Cancer Prevention and Control. Other retinoids used in analyses were either obtained commercially (retinol, retinyl acetate and retinyl palmitate; Sigma, St. Louis, Missouri, U.S.A.) or synthesised (retinyl stearate) in our laboratory according to published methods [20]. Retinyl palmitate added to diet preparations was purchased from Teklad (Teklad, Madison, Wisconsin, U.S.A.). Tritiated retinol (11, 12-[^{3}H](*N*)-retinol; specific activity approximately 175 μCi/μg) was obtained from New England Nuclear (Boston, Massachusetts, U.S.A.). All procedures involving retinoids were carried out under gold fluorescent lighting.

Animals and diets

Weanling male Sprague–Dawley rats (Harlan Sprague–Dawley, Inc., Frederick, Maryland, U.S.A.) weighing 50–60 g were housed individually in wire-bottomed stainless steel cages in an isolated room with automatically controlled temperature (22–26°C), humidity (approximately 60%), and 12-h light/dark cycles (0600–1800/1800–0600 h). The experiments, care and use of the animals were approved by the National Cancer Institute and Frederick Cancer Research and Development Center Animal Use and Care Committee. Food and water were provided *ad libitum*. Animals between 13–14 weeks of age were fed either a control diet (CON group; 0.8 RE/g diet vitamin A free diet [Teklad] as retinyl palmitate; $n = 28$) or a control diet supplemented with 4-HPR (CON + 4HPR group; 1173 μg 4-HPR/g diet; $n = 28$). Following a 1 week adaptation period, animals received an i.v. injection of plasma containing [^{3}H]-labelled retinol associated with its normal plasma transport complex (i.e. retinol-binding protein:transthyretin (RBP:TTR). This plasma containing the radiotracer dose was prepared in donor animals as described earlier [19]. Following injection, animals were killed at 0.5, 2, 6 h and 1, 3, 7, 15, 25 and 41 days post-dosing and eyes, selected tissue samples, and a large terminal blood sample was collected. Body weights, food consumption and plasma vitamin A levels were monitored throughout the study. Aliquots of plasma were stored at −70°C under nitrogen atmosphere and protected from light until analysis. Following removal of a terminal blood sample, rats were anaesthetised with methoxyflurane. The whole body was then perfused by gravity feed (1.9 m) of 0.9% saline (containing 0.2% D-isoascorbic acid (Sigma) as an antioxidant) infused into the right auricle, tissues removed, blotted dry, weighed and stored in a similar manner as the plasma samples.

Plasma and tissue processing

Plasma and tissue samples were analysed as reported earlier [19]. Briefly, plasma samples had an internal standard, retinyl acetate in absolute ethanol, added prior to extraction. Plasma samples were extracted from 2 ml of 50% aqueous ethanol into 4.5 ml of hexane. Following mixing (1 min) and centrifugation (6 min; 2400 rpm), appropriate aliquots were removed for determination of retinoid mass and radioactivity.

Eyes were first ground in small glass:glass homogenisation tubes (16 × 100 mm) with 2.0 ml of hexane:isopropanol (3:2, v/v). After homogenisation, tubes were vortexed for 1 min and 1.0 ml of sodium sulphate wash was added, followed by another 1 min vortexing. Following centrifugation at low speed (approximately 2000 rpm) for 4–5 min, the upper organic phase was removed and set aside. Two additional extractions were carried out using 0.5 ml of hexane:isopropanol (7:2, v/v) and the latter extracts were pooled with the first. Appropriate aliquots were taken for determination of retinoid mass and radioactivity.

Analysis and quantitation of retinoid mass and radioactivity

For analysis of retinoid mass, samples were prepared by first evaporating solvent from aliquots of lipid extract in a 37°C water bath using a gentle stream of nitrogen. After drying, samples were immediately resolubilised with absolute ethanol prior to HPLC injection. Retinoids were quantitated by reverse-phase HPLC using a methanol:water-based mobile phase [19]. For analysis of radioactivity, solvent from aliquots of lipid extract was evaporated and samples were resolubilised in scintillation fluid prior to quantitation by liquid scintillation spectrometry [19].

Kinetic analysis of vitamin A in the eyes

A forcing function approach [21–23] was used to obtain an estimate of vitamin A turnover in the eyes. This approach is based on the rationale that a mathematical description of the plasma tracer response and the tracer response data in a particular tissue of interest, along with the corresponding measures of retinoid mass (i.e. tracee data), can be used to model the kinetics of a traced substance in that tissue. For the present study, we used the plasma tracer data from the CON and CON + 4HPR groups that was described in detail in an earlier report [19]. We assumed that the eyes exchanged vitamin A directly with the plasma but not with other organs. The mean observed plasma radioactivity data for each of the groups was normalised to the fraction of injected dose, and the SAAM/CONSAM computer modelling programs [21, 22] were used to fit these data by a weighted least-squares regression to a multicomponent exponential equation of the general form:

$$y_t = \sum_{i=1}^{n} I_i e^{-g_i t}$$

where y_t is the fraction of the dose in the plasma at time t; I_i and g_i (per day) are constants equal to the intercept and fractional slope, respectively, of each component, and n is the number of exponential terms in the equation. Standard deviations of the group average at each point were used as weighting factors in the regression analysis. The equation describing the plasma tracer response curve for each group was used as a "forcing function". In conjunction with the tracer response, information obtained from the eyes, and the tracee data from both the plasma and eyes, this function was used to develop a subsystem model to estimate vitamin A turnover in ocular tissue.

Statistical analyses

Analysis of variance along with Duncan's multiple range test [24] was used to determine significant differences (alpha level of 0.05 or less) between mean values for plasma and tissue retinoid content. Unless otherwise noted, chemically determined data are presented as mean ± standard deviation (S.D.) and model-derived data are presented as mean ± fractional standard deviation (F.S.D.; S.D./mean).

RESULTS

Animals in both groups grew normally and appeared to be in good health throughout the entire study period. There were no apparent differences in food consumption between groups. Plasma retinol levels sampled at different time points throughout the course of the 41 day turnover study period were decreased in the 4-HPR treated group as compared to the control (Figure 1). Plasma retinol levels expressed as an average over the course of the study were significantly depressed in the CON + 4-HPR group (23.5 ± 7.0 μg/dl) as compared to the CON group (50.3 ± 5.3 μg/dl). The changes in total mass of vitamin A in the eyes over time are presented in Figure 2. The vitamin A content of the eyes of the CON + 4-HPR group was lower than that of the CON group throughout the turnover study period. By as early as the 3 day collection point (i.e. 10 days on the 4-HPR containing diet), the vitamin A content of the eyes had decreased significantly in the 4-HPR treated group as compared to the control. With regard to the vitamin A content of the eyes, there was no apparent effect of time for the CON group as indicated by linear regression analysis, whereas there was a highly significant effect ($P < 0.0001$) of time for the CON + HPR group.

The group mean observed plasma tracer concentration data for the CON and the CON + 4-HPR groups were each able to fit to a four component exponential equation. As described above, these equations were used as forcing functions along with the corresponding tracer responses obtained from the eyes of each of the groups (Figure 3a) to model the kinetics of vitamin A metabolism in the eyes and obtain estimates of several relevant kinetic parameters associated with this tissue. The data are presented as group average mean for each of the data points. An estimate of error for these data expressed as F.S.D. ranged between 0.02504 to 0.04054 for the CON group and between 0.01681 to 0.05707 for the CON + 4-HPR group. For each of the groups, one compartment was able to adequately fit the data obtained from the eyes (Figure 3b). As a reflection of vitamin A turnover in the eyes, the fraction of vitamin A leaving this tissue (i.e. model derived fractional rate constant expressed as a group mean value ± F.S.D.) on a daily basis was found to be similar in the CON + 4-HPR group (0.113 ± 0.0393 per day) as compared to the CON group (0.104 ± 0.0373 per day). At the same time, the flow of vitamin A through the eyes was significantly decreased in the CON + 4-HPR group eyes (0.0162 ± 0.101 μg/d) as compared to the CON group (0.0604 ± 0.0672 μg/d).

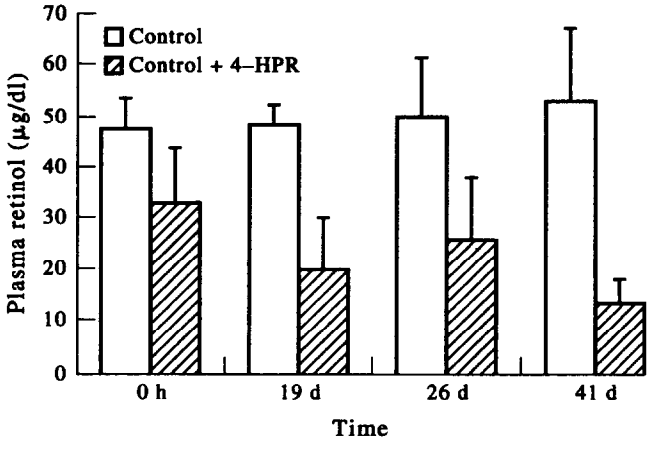

Figure 1. Plasma retinol levels of 41 day CON ($n = 4$) and CON + 4-HPR ($n = 5$) groups during the turnover study period. Data are presented as mean ± S.D. CON group values were significantly higher (alpha level of 0.05 or less) than the corresponding CON + 4-HPR group values throughout the sampling period. The 0 h collection represents a baseline collection conducted prior to beginning the turnover study and following a 1 week adaptation period to the diets. A description of treatments is found in the text.

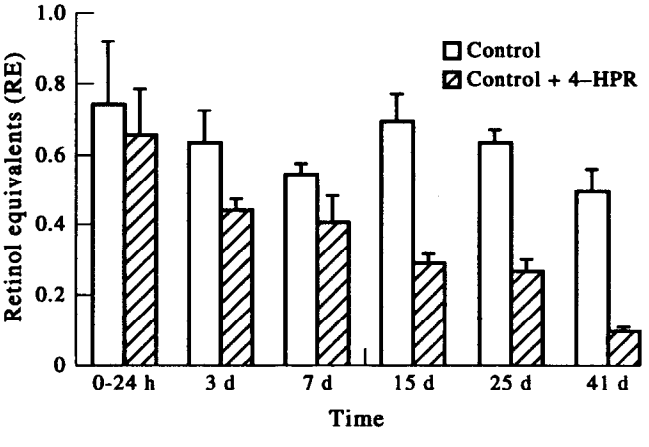

Figure 2. Mass of vitamin A in eyes of CON and CON + 4-HPR groups during the 41 day turnover study period. Data are presented as mean ± S.D. CON group values were significantly higher (alpha level of 0.05 or less) than the corresponding CON + 4-HPR group values throughout the sampling period. The 0–24 h point represents samples pooled from 0.5, 2, 6 and 24 h collections. 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 + 4-HPR 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.

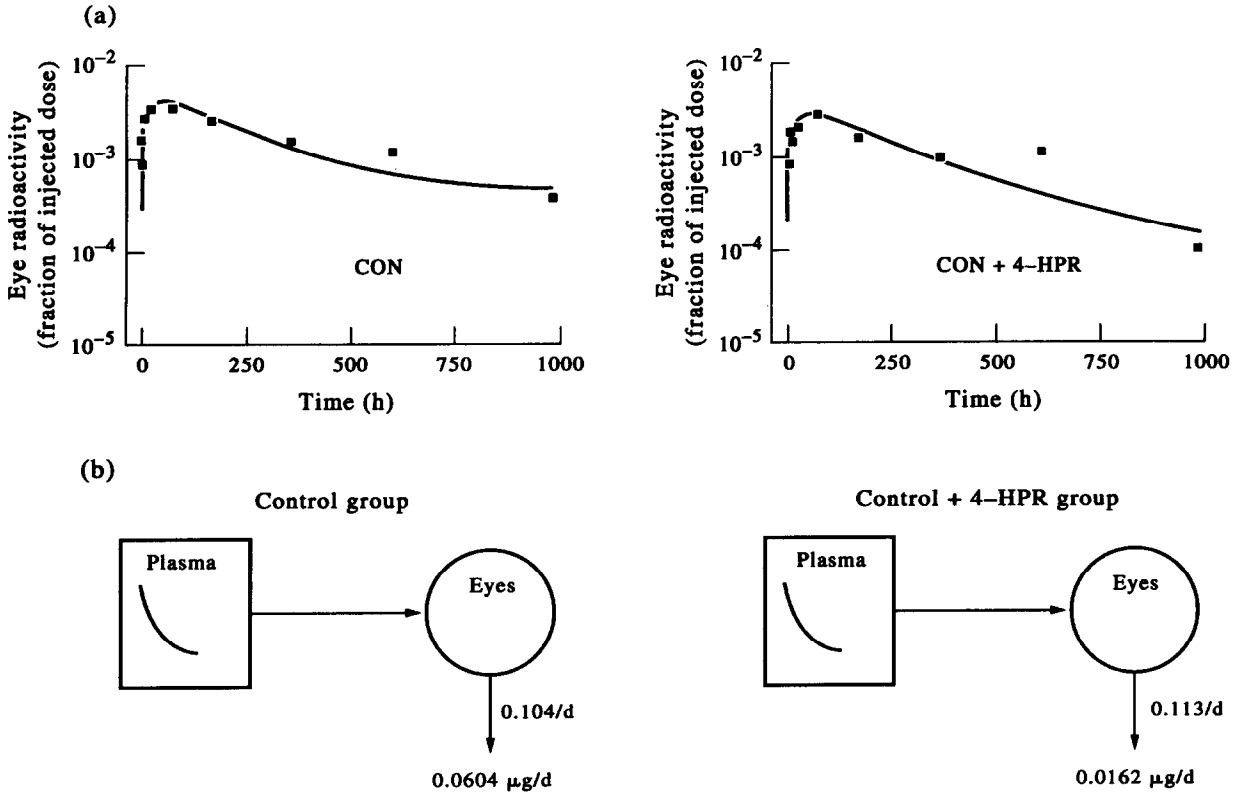

Figure 3. Tracer response curves in the eyes of CON versus CON + 4-HPR groups during the 41 day turnover study period (a) and proposed model of vitamin A kinetics in the eyes of CON versus CON + 4-HPR groups showing the fractional rate constants (per day) and flow of vitamin A mass out of the eyes (μg/d) (b).

DISCUSSION

Our laboratory has been investigating the basic mechanisms involved in the regulation of plasma and tissue levels of vitamin A. Since chemopreventive and chemotherapeutic approaches involving retinoids are increasingly being carried out on a long-term basis, information involving basic metabolism of vitamin A and how this is affected by extended administration of different retinoids is essential to help assure the efficacy and safety of such treatments. The present report focuses specifically on the effects of the synthetic retinoid 4-HPR on vitamin A metabolism in ocular tissue. The administration of 4-HPR in the present study was associated with a gradual depletion over time of vitamin A stores in the eyes such that by the final time point examined, these stores were approximately five times less than those of the control group. These findings in the eyes were in contrast to what was observed earlier for the livers and kidneys of these animals [19], the latter tissues remaining similar throughout the course of the turnover study period when the two groups were compared.

The kinetic parameters calculated to describe vitamin A turnover in the eyes of both groups indicated that, even though the ocular stores of vitamin A were steadily decreasing throughout most of the study, the fraction of these stores leaving (i.e. fractional rate constant) on a daily basis was unchanged in the 4-HPR treated group as compared to the control. This finding was of particular interest since, in an earlier study of a similar nature carried out with vitamin A deficient animals (data not shown), we had observed that, in addition to the eyes retaining a greater portion of their vitamin A stores relative to other tissues, the model-derived fractional rate constant for the eyes of these animals was significantly decreased (0.020/day as compared to 0.080/day for a corresponding control). This decrease in fractional rate constant helped to compensate for a diminishing store of ocular vitamin A (0.6 and 1.0 RE for the deficient group and corresponding control, respectively) and/or a decreased input of vitamin A to the eyes. This finding is in agreement with a more recent report which examined the effects of different states of vitamin A nutrition, including vitamin A deficiency, on vitamin A metabolism [25]. In this recent study [25], the mass of vitamin A in the eyes of the deficient animals was decreased as compared to a control group (0.5 and 1.0 RE, respectively). However, the fractional rate constant for vitamin A leaving the eyes of the deficient group was also significantly depressed compared with the control (0.0133 and 0.0798 per day, respectively) in what appeared to be an attempt to conserve ocular vitamin A stores. In the present study, for the 4-HPR treated group, a similar compensatory response to a decreased ocular store of vitamin A in the eye was not evident. Our model did suggest that the amount of vitamin A flowing through the eyes was decreased in the 4-HPR-treated animals compared to the controls. However, this alone may not necessarily account for the decreased vitamin A levels in the eyes of this group, since the vitamin A deficient groups mentioned earlier had plasma vitamin A levels that were as low if not lower than those of the 4-HPR-treated group in the present study. Yet they were apparently able to adapt to the lower circulating levels of vitamin A as well as to low levels of vitamin in the diet, and still maintain their ocular vitamin A

stores at near normal levels. This would suggest that the decreased levels of vitamin A in the eyes of the 4-HPR treated animals are not necessarily a function only of the low circulating levels of the vitamin, but rather are more likely a result of a combination of factors, including the possible interference by 4-HPR of normal uptake of retinol by the retinal pigment epithelium and/or subsequent metabolism of vitamin A by the eyes. It is also possible that of the vitamin A that was leaving the eyes, a greater portion was being utilised or at least not recycling to the eyes as compared to the control, which would contribute further to a decline in ocular vitamin A stores. The forcing function modelling approach that we have used does not provide an estimate of what portion of the vitamin moving through the eyes is actually utilised versus that which is recycled and might return to the tissue.

Another way of viewing the information presented in the model is to estimate and compare the residence times for vitamin A in the eyes of both groups. The residence time is the time, on average, that a molecule of vitamin A spends in a tissue or tissue subsystem prior to being lost from this tissue. The reciprocal of the fractional rate constant values for vitamin A leaving the eyes provides an estimate of the residence time for vitamin A in this tissue, and it can be calculated that vitamin A remained in the eyes of both of the groups in the present study for a similar period of time, averaging approximately 8.9 and 9.6 days for the CON and CON + 4HPR groups, respectively. In contrast, if one calculates a residence time for the vitamin A deficient group studied earlier [25] in a similar manner, it is found to be in the range of 75 days, approximately six times longer than the corresponding control group value of 12.5 days and eight times longer than that of either the CON or CON + 4-HPR groups in the present study. This would suggest the possibility that a normal compensatory response to decreased levels of ocular vitamin A might involve lowering the fractional rate constant for movement of vitamin A out of the eyes and lengthening the residence time for the vitamin in this tissue. Since evidence of such a response was not apparent in the 4-HPR-treated group despite the depressed vitamin A stores in their eyes, it would appear that 4-HPR is in some manner, interfering with normal compensatory responses that would function to conserve vitamin A stores.

Abnormalities of visual function associated with chronic administration of 4-HPR, which most often manifest themselves in an altered dark adaptation ability, would normally be thought to be associated with a vitamin A deficiency. Thus, it is of concern that despite an adequate vitamin A intake and otherwise normal diet, a number of subjects in clinical trials involving chronic administration of 4-HPR, experience visual problems usually indicative of vitamin A deficiency. These visual problems appear to be at least partially corrected by periodic withdrawals of the retinoid for prescribed periods (usually 3 days), a so-called drug holiday. Although plasma retinol levels usually do increase during this period, they rarely if ever return to original baseline values, but rather remain considerably below their pretreatment levels. The relatively high incidence of dark adaptation abnormalities found in a recent study by Decensi and colleages [18] is noteworthy. As measured by dark adaptometry testing, these investigators found that approximately half of the women they studied who were being treated with a daily dose of 200 mg of 4-HPR, had either a mild or moderate alteration of dark adaptation ability as compared with less than 10% of the untreated patients. It is also of interest that approximately half of the women in their study found to have altered dark adaptometry were asymptomatic. It would appear that, at least in regard to visual function, patients receiving 4-HPR on a long-term basis, are responding as if they are borderline deficient in vitamin A. The long-term effects of maintaining the visual tissue in a state of quasi-deficiency for extended periods should be carefully evaluated.

Since both in human trials as well as our own studies, diets were sufficient in vitamin A content, it would appear that 4-HPR is interfering in some way with normal uptake and or metabolism of vitamin A in the eye. In the case of our animal studies, even in the face of adequate vitamin A nutrition, 4-HPR administration was associated with what was essentially a localised deficiency of the vitamin in this tissue. The long-term consequences of lowering plasma vitamin A levels with 4-HPR for extended periods of time deserves closer examination. Besides the more apparent effects upon visual function, there is also the possibility that other tissue functions not easily identified or measured might also be affected. Our earlier work indicated that plasma and overall tissue vitamin A kinetics were profoundly affected by inclusion of 4-HPR in the diet [19].

We are presently using a compartmental analysis approach to study individual tissue and whole body metabolism of vitamin A in a variety of tissues including the eyes. This process involves development of a whole body kinetic model to describe various aspects of vitamin A metabolism. Within the context of such a whole body model, it will be possible to determine more precisely the overall effects of 4-HPR on ocular tissue vitamin A metabolism. In conjunction with our *in vivo* studies, we are also carrying out a series of *in vitro* tissue culture experiments examining the possible mechanisms that may be involved in the effects of 4-HPR upon visual function that have been observed. Until more detailed information is available, the results of our studies presented herein would suggest that, with regard to the long-term administration of 4-HPR, a prudent approach at this time might involve continuing to monitor closely patients for signs of potential visual problems, as well as a heightened awareness of the possibility that, although less apparent, other important tissue functions might be affected as well.

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Acknowledgements—The authors gratefully acknowledge the excellent technical assistance of Lisa Riffle and Dr Paul Horng in carrying out portions of the animal experiments and chemical analyses, respectively.