Effects of Pharmacological Retinoids on Several Vitamin A-metabolizing Enzymes

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ABSTRACT

Fenretinide (HPR), 13-cis-retinoic acid, and all-trans-retinoic acid are vitamin A derivatives used in the treatment of cancer and severe acne. Patients taking these drugs often show side effects resembling the symptoms of hypovitaminosis A, namely, night blindness and decreased plasma retinol levels. A dietary vitamin A deficiency is not suspected in these patients; therefore, interference with normal vitamin A metabolism seems likely. The effect of these drugs on two enzymes involved in vitamin A metabolism was investigated. At micromolar concentrations, all three derivatives were found to inhibit intestinal lecithin-retinol acetyltransferase (LRAT) and to a lesser extent liver LRAT and intestinal retinal reductase. Inhibition of intestinal LRAT by HPR and 13-cis-retinoic acid was enhanced by preincubation prior to assay, whereas inhibition of the other activities was not. The K_i for the inhibition of intestinal LRAT by HPR was determined to be 24.1 ± 5.6 μM. The ability of these drugs to inhibit retinal reduction and retinol esterification in vitro suggests an ability to interfere with normal vitamin A metabolism in vivo, particularly during absorption. This may be most significant for HPR, which is known to accumulate in the liver and intestine after chronic dosing.

INTRODUCTION

Several vitamin A derivatives are now being used for the treatment of diseases such as cancer and severe acne. Unfortunately, these drugs have side effects ranging from night blindness to teratogenic defects in fetuses. Some of these effects may be due to interference with normal vitamin A function or metabolism. For example, recent clinical trials have utilized fenretinide or HPR as a chemopreventative agent against recurrence of breast cancer (1, 2). Patients taking this drug sometimes show effects similar to those seen in patients with hypovitaminosis A, specifically night blindness (3) and decreased plasma retinol levels (4-6). Pharmacokinetic studies indicate that chronic dosing with HPR leads to levels in the small intestine at least 4-fold higher than in any other tissue examined. The liver, while not retaining HPR to the same extent as the small intestine and stomach, still contains levels of HPR 14-fold higher than in the blood plasma (7). This suggests the possibility of interference with normal vitamin A processing in the intestine and liver during the absorptive process.

One widely prescribed retinoid is 13-cis-retinoic acid or isotretinoin (Accutane), which is effective in the treatment of severe acne. Recent evidence indicates that it may also be of use in cancer therapy (8, 9). Similarly, all-trans-retinoic acid is used both as a topical ointment and in the treatment of acute promyelocytic leukemia (10). Both of these retinoids also have several side effects, primarily dermatological, although night blindness has been reported (11). Neither accumulates in the small intestine or the liver, nor do they have any effect on plasma retinol levels (9, 12-14).

Since inadequate dietary intake is not suspected in those patients exhibiting deficiency symptoms such as night blindness, interference in normal vitamin A metabolism appears to be the more likely cause. Previous work on the effects of HPR and 13-cis-retinoic acid on vitamin A metabolism has focused on ARAT, an enzyme that will catalyze esterification of retinol in vitro (15, 16). Recent work, however, indicates that LRAT, not ARAT, is the primary vitamin A-esterifying enzyme in the intestine and the liver (17, 18). This has prompted us to investigate the effects of HPR, 13-cis-retinoic acid, and all-trans-retinoic acid on both liver and intestinal LRAT and also on the intestinal retinal reductase believed to be involved in the conversion of β-carotene to retinol (19); for a review of retinol metabolism, see Ref. 20. We report here that all three drugs inhibited intestinal LRAT and, to a lesser degree, liver LRAT and intestinal retinal reductase, indicating the potential for interference with normal vitamin A metabolism. The greatest effects were seen with HPR.

MATERIALS AND METHODS

Preparation of Retinoids. All work with retinoids was done under subdued yellow light. All-trans- and 13-cis-retinoic acids were from Sigma Chemical Co. (St. Louis, MO). HPR was a generous gift from McNeil Pharmaceuticals (Spring House, PA). Dilutions were made in DMSO (Sigma), and the concentrations were determined spectrophotometrically. Retinol was labeled at the C-15 position as previously described (21). The specific activity was between 11 and 15 Ci/mmol. [1H]Retinal was prepared from [1H]retinol by oxidation with MnO2 as described by Bridges and Alvarez (22).

Preparation of Retinoid-binding Protein. Apo-CRBP was purified from an Escherichia coli expression system as described previously (23). Apo-CRBP(II) was purified from E. coli strain JM103 transfected with the plasmid pMON-CRBP1 (24). The cells were grown in Luria broth with 100 μg/ml ampicillin at 37°C overnight and then diluted 1:50 into the same medium. These cultures were incubated at 37°C until an A600 of 0.5-0.6 was obtained, at which time nalidixic acid (10 mg/ml in 0.1 N NaOH) was added to a final concentration of 50 μM. The cells were incubated 3 h and then harvested by centrifugation (5000 × g; 30 min) and resuspended in 10 mM Tris-1 mM EDTA-100 mM KCl, pH 8.3. The cells were sonicated and centrifuged at 32,000 × g for 30 min. The resulting supernatant liquid was filtered (Whatman no. 4) and concentrated by ultrafiltration on an Amicon YM3 membrane to approximately 40 ml. This material was then applied to an 87- × 5-cm Sephadex G-75 column equilibrated in 0.008 M imidazole acetate, pH 6.6, with 1 mM β-mercaptoethanol at a flow rate of 1.5 ml/min. The fractions containing CRBP(II) were identified by their ability to bind retinol, combined, and concentrated by ultrafiltration to approximately 4 ml. This material was applied to a DEAE 5 PW column (15 × 20 cm) with guard column (Tosohaas, Philadelphia, PA), equilibrated in 0.008 M imidazole acetate, pH 6.6, with 1 mM β-mercaptoethanol, using an LKB high-performance liquid chromatography system. CRBP(II) was eluted by a gradient from 0.008 M imidazole acetate to 0.054 M imidazole acetate and 1 mM β-mercaptoethanol at 3 ml/min, which resolved two forms, differing only in their NH2-terminal processing. The first form retained the initial NH2-terminal methionine, while the second had been cleaved to NH2-terminal threonine. The second form is equivalent to the natural form previously called CRBP(II)A and was used for these studies.

Preparation of Microsomes. Liver microsomes were prepared as previously described (18), except that the microsomal pellet was dispersed with 0.1 M potassium pyrophosphate containing 1 mM DTT before a second centrifugation at 100,000 × g for 60 min. The resulting pellet was then suspended in 0.01 M Tris-acetate, pH 8.3, with 20% glycerol and 1 mM DTT before quick freezing and storage at −70°C until use.

Intestinal microsomes for LRAT assay were prepared as before (17). Intestinal microsomes for the assay of retinal reductase were prepared essentially as for the assay of LRAT, except that the buffer contained 50 mM potassium
Solubilization of LRAT. Both intestinal and liver LRAT were solubilized by dropwise addition of 10% Brij 35 (Sigma) to microsomes (5 mg/ml) in 0.2 M phosphate buffer, pH 7.2, and 1 mM DTT to achieve a final concentration of 1% Brij 35. The mixture was slowly stirred for 1 h at 4°C, followed by centrifugation at 200,000 x g for 30 min to remove insoluble matter. The resulting supernatant was diluted 1:1 with phosphate buffer containing 40% glycerol and stored at 4°C.

Assay of Retinol Esterification. LRAT was assayed essentially as previously described (17). All incubations were carried out in duplicate, under subbed yellow light, in disposable borosilicate culture tubes in a shaking water bath at 37°C. Briefly, for intestinal LRAT, 5 μg of microsomal protein were assayed in 100 μl of 0.2 mM phosphate buffer (pH 7.5) with 1 mM DTT, 40 μM dilauroyl phosphatidylcholine, and 12% DMSO. The inhibitor was added in 2 μl of DMSO immediately before assaying, except in the preincubation time courses when it was added immediately before beginning the incubation. The reaction was started by the addition of [3H]retinol bound to CRBP(II). Liver LRAT was assayed in the same way except that 3 μg of protein were assayed, the dilauroyl phosphatidylcholine concentration was 10 μM, and the [3H]retinol was bound to CRBP. For solubilized intestinal and liver LRAT, the solubilized material was diluted 5-fold into the assay buffer and then assayed as above using 5 μl of this dilution.

Assay of Retinol Reduction. All incubations were carried out in duplicate, under subbed yellow light, in disposable borosilicate culture tubes in a shaking water bath at 37°C. [3H]Retinol-CRBP(II) was prepared beforehand by the addition of [3H]retinol in DMSO to a 20% molar excess of apo-CRBP(II). Briefly, 5 μg of microsomal protein were assayed for 10 min in 100 μl of 0.1 mM imidazole acetate buffer (pH 6.0) with 60 μM NADPH. The inhibitor was added in 2 μl of DMSO immediately before assaying, except in the preincubation time courses when it was added immediately before beginning the incubation. The reaction was initiated by the addition of 3 μM [3H]retinal-CRBP(II). The reaction was terminated by the addition of 400 μl of ice-cold ethanol containing 100 μg/ml butylated hydroxytoluene. After the addition of 300 μl of H2O, 2.0 ml of hexane containing 100 μg/ml of butylated hydroxytoluene were added and vigorously mixed. The hexane layer (1.0 ml) was applied to a column of 1.2 g of aluminum oxide deactivated with H2O (10% of weight). The [3H]retinol was eluted with hexane containing 15% ethyl ether and discarded. The [3H]retinol was eluted with 50:50 hexane:ether directly into scintillation vials, dried under nitrogen, and quantitated by scintillation counting.

RESULTS

Effects of HPR, 13-cis-Retinoic Acid, and All-trans-Retinoic Acid on Intestinal LRAT. All three of the retinoids investigated showed a concentration-dependent ability to inhibit esterification of retinol by LRAT in intestinal microsomes (Fig. 1). At retinoid concentrations of 200 μM, activity retained was 35% for 13-cis-retinoic acid, 50% for HPR, and 60% for all-trans-retinoic acid relative to controls. Interestingly, the inhibition by HPR and 13-cis-retinoic acid was noticeably enhanced when either compound was preincubated with the microsomes at 37°C before initiation of the assay (Fig. 2). After 25 min of preincubation, the activity measured was reduced to 16% of control incubations for HPR and 25% of control incubations for 13-cis-retinoic acid. In contrast, preincubation with all-trans-retinoic acid did not increase its inhibitory effect, a result similar to that reported previously for ARAT (16).

The Kᵢ for HPR was determined at various concentrations of HPR (Fig. 3). The calculated Kᵢ was 24.1 ± 5.6 μM, considerably lower than its Kᵢ for ARAT, previously determined to be 150 μM (15). The pattern of inhibition shown is characterized as mixed, noncompetitive, but a detailed understanding of this mechanism was not possible for this complex system. Similar results were obtained when the Kᵢ was determined after a 10-min preincubation at 37°C before assaying (data not shown). This Kᵢ is substantially greater than the Kᵢ for retinol, previously determined to be 0.24 μM (23). The Kᵢ was not determined for the other retinoids.

Effects of HPR, 13-cis-Retinoic Acid, and All-trans-Retinoic Acid on Liver LRAT. Microsomal liver LRAT was more resistant to inhibition by these retinoids than was its intestinal counterpart. HPR reduced the activity to 60% of controls at 200 μM (Fig. 4), while all-trans-retinoic acid reduced activity to 80% of controls, independently of concentration (data not shown). In contrast, the presence of 13-cis-retinoic acid actually led to increased ester formation (Fig. 5). This result was unexpected but may be due to detergent-like action by the retinoid, since small amounts of some detergents have been found...
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Fig. 3. Determination of $K_i$ for HPR and intestinal LRAT. HPR was added to the reaction mixture in 2 μl of DMSO immediately before assaying. Controls contained only DMSO. Final HPR concentrations were 0 (○), 20 (●), 30 (△), 40 (▲), and 60 μM (▼). The Lineweaver-Burke plot yielded lines with $r^2$ values >0.98 when analyzed using SigmaPlot 4.0 (Jandel Scientific, Corte Madera, CA). Points (bars), means (±SEM) of two experiments, each done in duplicate.

Fig. 4. Effects of HPR on liver LRAT. Immediately before HPR was assayed, increasing concentrations of HPR in 2 μl of DMSO were added to the reaction mixtures containing either microsomal LRAT (○) or solubilized LRAT (●). Comparison was to controls containing only DMSO. Points (bars), means (±SEM) of two separate assays, each done in duplicate.

Effects of HPR and 13-cis-Retinoic Acid on Solubilized LRAT. The differences in behavior exhibited by intestinal and liver LRAT could be due to membrane effects rather than to enzymatic differences. To examine this possibility, we studied the effects of HPR and 13-cis-retinoic acid on liver and intestinal LRAT after solubilization. For intestinal LRAT, the activity remaining in the presence of 200 μM HPR decreased to 25% with solubilization. In contrast, solubilization had no effect on the inhibition by 13-cis-retinoic acid (data not shown).

Liver LRAT exhibited a marked increase in inhibition when solubilized. When the solubilized enzyme was treated with HPR, only 16% of the original activity remained at 50 μM HPR, compared to 75% when microsomal LRAT was treated with the same concentration of inhibitor (Fig. 4). Treatment with 13-cis-retinoic acid demonstrated similar differences. The membrane-bound enzyme had increased activity in the presence of 13-cis-retinoic acid, while the solubilized form was inhibited, retaining only 40% of its original activity at 200 μM 13-cis-retinoic acid (Fig. 5).

Effect of HPR, 13-cis-Retinoic Acid, and All-trans-Retinoic Acid on Intestinal Retinal Reductase. Both HPR and all-trans-retinoic acid showed a concentration-dependent inhibition of the intestinal retinal reductase (Fig. 6). In contrast, 13-cis-retinoic acid reduced activity to 75% at the lowest concentration examined (25 μM) but showed no further increase in inhibition with higher concentrations (data not shown). Preincubation at 37°C did not increase the inhibition by HPR or all-trans-retinoic acid (data not shown). The enzyme activity is heat labile in the absence of substrate. Interestingly, the presence of 13-cis-retinoic acid actually protected the enzyme from the loss of activity caused by extended incubation (Fig. 7), indicating a potential interaction with the binding site of the enzyme.

DISCUSSION

These studies indicate that all three clinically useful retinoids have some potential to affect adversely both intestinal and liver metabolism of vitamin A, based on their ability to inhibit both retinal reduction and retinol esterification in vitro. Of course, the effects observed in vitro might well be modulated by other factors in vivo, particularly the pharmacodynamics of the various drugs. However, the relatively small reductions in activity observed here with all-trans-retinoic acid, the natural retinoid, suggest it would not markedly alter intestinal metabolism or liver storage of vitamin A.

When microsomal intestinal LRAT was assayed with the retinoids, both HPR and 13-cis-retinoic acid inhibited at least 50% of the enzyme activity in vitro when compared to controls. Both solubilization to stimulate LRAT activity. After 15 min of preincubation at 37°C, such stimulation was not evident, and 13-cis-retinoic acid then inhibited the enzyme to the same extent as HPR, whose inhibition, like that of all-trans-retinoic acid, did not increase with preincubation (data not shown), in contrast to the situation with intestinal LRAT.

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of the enzyme and preincubation with the inhibiting retinoid further reduced measured activity compared to controls. Such reductions may well be sufficient to significantly interfere with intestinal processing of vitamin A. These results are particularly interesting in view of pharmacokinetic studies in rats demonstrating that after chronic oral dosing HPR accumulates in the small intestine to levels of 177 nmol/g tissue 4 h after dosing, a level at least 4-fold higher than in any other tissue examined and 118-fold higher than in the blood plasma (7). If we assume that 1 g of tissue approximates 1 ml in volume, this is a concentration of 177 µM, which is within the range of the HPR concentrations we examined. This value is for the entire small intestine. Levels in the proximal intestine, where the retinol-metabolizing enzymes are at the highest levels (25), could conceivably be higher. In contrast, neither 13-cis- nor all-trans-retinoic acid accumulates significantly in the intestine or liver when compared to the serum levels (26, 14). Interestingly, HPR depresses both the level of serum retinol and the level of RBP, the serum vitamin A transport protein (4, 5, 6), whereas neither 13-cis- nor all-trans-retinoic acid has any effect on serum retinol concentrations (12, 13). Previous reports indicate that night blindness induced by intake of all-trans-retinoic acid may be due to its interference with the 11-cis-retinol dehydrogenase in the eye (27). These observations suggest that the side effects of the retinoic acids may result in part from interference with aspects of vitamin A utilization rather than acquisition. In contrast, the side effects from HPR may be due in part to its interference with the intestinal vitamin A-metabolizing enzymes, particularly LRAT. Recent work by Berni and Formelli (28) has demonstrated that HPR can form a tight complex with RBP, the serum retinol-binding protein, and that this complex will not bind to transthyretin. Furthermore, administration of HPR to rats lowers concentrations of retinol and RBP, suggesting that alteration in release or metabolism of RBP does occur in vivo (29). We suggest that the effects we have demonstrated may work in concert with the effects of HPR on RBP, although the RBP effects are perhaps the more important. However, dietary supplementation with vitamin A may be of particular use for patients undergoing HPR therapy.

Since HPR contains the basic retinoid structure, it presumably inhibits LRAT via interaction with the binding site of the enzyme, although membrane effects are clearly demonstrated in a comparison of microsomal and solubilized enzymes. Attempts to clearly define an inhibitory mechanism yielded mixed results, probably due in part to complex partitioning of the HPR into the microsomal membrane. Since we were interested in determining a Kf in the most physiological system possible, we did not investigate further using the solubilized enzyme. The high level of accumulation of HPR in the intestine could, therefore, cause some decrease in rate of esterification of retinol.

Our initial studies revealed several differences in the interaction of intestinal and liver LRAT and the retinoids, particularly 13-cis-retinoic acid. Whereas 13-cis-retinoic acid inhibited the intestinal enzyme quite well, it actually enhanced the activity of the liver enzyme. This could be due to differences in the microsomal membranes resulting in differing enzyme-drug interactions. However, subsequent studies in which solubilized enzyme were used to eliminate the microsomal membrane continued to demonstrate distinct differences in the behavior of the enzymes. Solubilized liver LRAT was almost completely inhibited by 100 µM HPR (10% activity), while solubilized intestinal LRAT retained 45% of the original activity. Both of these studies and previous work (18, 30) suggest the possible existence of isozymes of the enzyme. The possibility should be considered, given the different roles of esterification in the two organs. In the liver, LRAT functions to help store vitamin A because the retinyl esters are retained. In contrast, the intestinal enzyme produces retinyl esters from newly absorbed vitamin A for packaging into chylomicrons, and consequently, the retinyl esters are for export rather than retention (20). Since 13-cis-retinoic acid is not retained in the liver (14), the results seen here suggest that it would not markedly alter liver metabolism of vitamin A.

All three retinoids reduced the activity of the retinal reductase of the intestine. However, only HPR was as effective an inhibitor of the reductase as it was of LRAT, and preincubation did not increase inhibition. Therefore, although there is the potential for some interference with retinal reduction, the inhibition observed may be insufficient to significantly reduce intestinal β-carotene metabolism to retinol. The more important effect would appear to be on LRAT.

In addition to LRAT and the retinal reductase, we have also investigated the influence of these retinoids on a third intestinal enzyme, the retinyl ester hydrolase of the brush border (31), but no effect was
observed when single concentrations of 100 μM were tested for each retinoid, and this area was not pursued further.

The inhibitory effects of the three retinoids, particularly the potent effect of HPR on intestinal LRAT, suggest the possibility of interference with additional vitamin A-utilizing enzymes, such as those maintaining the visual cycle in the eye. For example, isomerization to 11-cis-retinal requires esterification of all-trans-retinol by an LRAT activity coupled with the isomerase (32). In light of these studies, investigation of the interactions of these retinoids with additional vitamin A-metabolizing enzymes would seem relevant.

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