Regulation of Hepatic Retinol Metabolism: Perspectives from Studies on Vitamin A Status¹,²

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ABSTRACT Liver vitamin A (retinol) is obtained from several sources and is subject to multiple fates. Lecithin:retinol acyltransferase (LRAT), a microsomal enzyme present in liver and several other retinol-metabolizing tissues, esterifies retinol that is associated with a cellular retinol-binding protein, CRBP or CRBP-II. Recent research has shown that LRAT mRNA expression and enzyme activity are regulated in a tissue-specific manner. In vitamin A-deficient liver, both LRAT mRNA and activity are significantly down-regulated as well as rapidly induced after the administration of vitamin A or its principal hormonal metabolite, retinoic acid (RA). In long-term feeding studies and the metabolic steady state, liver LRAT is expressed dose-dependently across a wide range of dietary vitamin A. Additionally, an RA-inducible cytochrome P450, P450RAI or CYP26, is down-regulated in liver during vitamin A deficiency and up-regulated dose-dependently by dietary vitamin A and exogenous RA. Based on these results, we propose that LRAT and CYP26 serve as two molecular mechanisms, coordinately regulated by all-trans-RA, to control the availability of retinol and RA, respectively. The LRAT reaction, besides providing a readily retrievable storage form of vitamin A, may regulate the availability of retinol to other pathways, while the CYP26 reaction may serve to prevent a detrimental “overshoot” of RA concentration. Moreover, retinoid metabolism in the liver is likely to be closely integrated with that in peripheral tissues through the rapid interorgan transfer and recycling of retinoids, affecting the whole-body economy of vitamin A.


KEY WORDS: • retinol esterification • retinoic acid • cytochrome P450 • gene expression • liver • mouse • rat

Vitamin A (retinol) serves as an essential dietary precursor for the biosynthesis of at least two critical metabolites, retinal, which is required for rhodopsin formation and vision, and retinoic acid (RA)⁴, a potent hormone capable of altering the expression of numerous genes. Not surprisingly, both a deficiency of retinol and an excess of RA adversely affect human and animal health. For most humans, however, vitamin A status falls somewhere within a broad “middle range,” spanning what may be categorized as marginal vitamin A deficiency, vitamin A adequacy, and vitamin A excess without overt toxicity. It is estimated that 250 million persons are marginally deficient in vitamin A (1). While the number of persons who consume vitamin A well in excess of physiological needs is unknown, it also could be high, especially in economically-developed countries in which foods, fortified foods and nutritional supplements containing preformed vitamin A are widely available. Surprisingly little is known concerning the regulation of retinoid metabolism in this middle range of vitamin A status.

In this review, we first discuss the regulation of the enzyme lecithin:retinol acyltransferase (LRAT), a microsomal protein which catalyzes the transfer of the sn-1 fatty acid from membrane-associated phosphatidyl choline to retinol bound to a cellular retinol-binding protein (CRBP or CRBP-II), thus forming esterified retinol. Recent work has shown that the level of LRAT enzyme activity and gene expression in the liver differs significantly with long-term vitamin A status, including in the broad middle range of vitamin A status discussed above, and is highly responsive to exogenous RA. Other studies of LRAT protein structure have revealed certain features that are essential for its catalytic activity and physiological function. Secondly, we turn attention to a newly recognized cytochrome P450 (termed either cytochrome P450RAI or CYP26), present in liver, brain and some other tissues, which catalyzes the oxidation of all-trans-RA to polar...
metabolites. CYP26 is unusual among members of the cytochrome P450 family in that its expression is strongly induced by all-trans-RA. CYP26 expression also varies significantly with dietary vitamin A intake, and with liver total retinol concentration, considered the "gold standard" indicator of whole-body vitamin A status. Thirdly, based on these observations we suggest an updated, integrated model of whole-body retinol metabolism in which the regulated expression of LRAT and CYP26 in the liver is closely linked to the production of activated retinoids in peripheral tissues.

**Retinol: a molecule at a metabolic crossroads**

Within many tissues, and especially in the liver, retinol is situated at a metabolic crossroads from which it can undergo several biochemical reactions (Fig. 1) (2). Dietary retinol is taken up by liver parenchymal cells during chylomicron metabolism, while circulating retinol, mostly bound to retinol-binding protein (RBP), has been shown to enter and leave the liver several times a day in the process known as retinol recycling (3). Once taken up, retinol may be esterified and stored as retinyl esters, mostly in stellate cells; bioactivated in a two step process which forms RA; or secreted into plasma as holo-RBP (4,5). Retinol, as well as RA, may be metabolized to more polar forms through oxidation on its β-ionone ring and conjugation to form the water-soluble retinoyl glucuronides found in liver and bile (6–8). By some means, the liver seems able to "interpret" its vitamin A status and make significant adjustments in how retinol entering the liver is partitioned therein. For example, in vitamin A-deficient animals chylomicron vitamin A is transferred within just a few hours from liver parenchymal cells, the site of initial uptake, to stellate cells where it is stored as retinyl esters. In contrast, in vitamin A-deficient animals chylomicron vitamin A is rapidly mobilized from the liver into plasma as unesterified retinol, presumably for distribution to peripheral tissues (9). Given these potentially different fates, one must wonder what factors determine the metabolic partitioning of retinol among the various pathways available to it. And moreover, is metabolic partitioning responsive to changes in the host's vitamin A status? It is well known that dietary vitamin A may be consumed in seasonally varying amounts, or sporadically. It therefore seems reasonable to speculate that the metabolism of retinol should be different when vitamin A is scarce compared with when it is in present in excess of the body's needs. Indeed, various lines of evidence indicate that the concentration of unesterified "free" retinol is closely regulated. In animals fed diets containing a wide range of vitamin A, liver unesterified retinol was almost constant, while retinyl esters increased nearly dose-dependently with dietary vitamin A (10). Consistent with this, the concentration of human plasma retinol was shown to remain nearly constant (1.7–2 μmol/L) even as liver total retinol varied by >15-fold (between ~20 and 300 μg/g tissue) (11). Since unesterified retinol appears to regulate the secretion of holo-RBP (12–14), the constancy of plasma retinol concentration over a wide range of liver total vitamin A could imply that retinol itself is held within a narrow concentration range. If so, by what mechanism(s) might this occur?

**LRAT activity: regulation by vitamin A status and exogenous retinoids**

Two enzymes, LRAT and acyl-CoA:retinol acyltransferase (ARAT), have been shown to be capable of esterifying retinol in various tissues. These enzymes differ in reaction mechanism, as LRAT catalyzes the transfer of the sn-1 fatty acid from membrane-associated phosphatidyl choline to retinol, while ARAT transfers the fatty acyl moiety of fatty acyl-CoA to retinol [reviewed in (5,15)]. They also differ in the form of retinol they metabolize because LRAT, but not ARAT, is capable of esterifying retinol bound to CRBP or CRBP-II. Because the majority of retinol in tissue cytosol apparently is bound to one of these proteins (10), LRAT is thought to be the physiologically significant enzyme for retinol esterification in the small intestine, retinal pigment epithelium, liver, and possibly other tissues. In earlier studies, we (16) observed that LRAT activity is negligible in the liver of vitamin A-deficient rats. However, when vitamin A-deficient rats were treated with an oral dose of retinol, LRAT activity reappeared in liver within ~6 h and reached normal levels within about 20 h. In these in vitro assays, the concentration of CRBP-bound retinol was never the limiting factor, and thus these observations indicated that LRAT activity per se is highly regulated. In the same vitamin-A deficient animals, there was no reduction in liver ARAT activity nor in LRAT activity in the small intestine (16). Follow-up studies showed that, in retinol-depleted rats treated with all-trans-RA, liver LRAT activity increased more rapidly than it did after treatment with retinol, and very low doses of RA, as little as 2 μg per rat, were effective (17). Moreover, the increase in LRAT enzyme activity after retinoid treatment measured in vitro correlated well with the liver's capacity to esterify retinol in vivo (17,18). Besides being up-regulated by all-trans-RA, liver LRAT activity was also markedly increased by several synthetic retinoids that bind selectively to the RAR family of nuclear retinoid receptors, but not by RXR-selective ligands (19). The increase in LRAT activity caused by RA was blocked entirely when vitamin A-deficient rats were pretreated with either actinomycin D or cycloheximide, inhibitors of RNA and protein synthesis, respectively (17).

**LRAT cDNA cloning, expression and partial characterization**

While the data above suggested that RA might induce the expression of the LRAT gene, it was not possible at the time to test this hypothesis because necessary reagents were not yet available (LRAT protein had resisted purification and LRAT cDNA had not been cloned). This impasse was resolved when LRAT cDNA was cloned from bovine and human retinal.

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**FIGURE 1** Diagram of the multiple biochemical fates, and physiological processes affected, of retinol in liver. Some of these processes may also take place in other tissues, but uptake of dietary vitamin A, storage of retinyl esters, and secretion of retinol-binding protein (RBP) are functions in which liver plays a predominant role.
pigment epithelium (RPE) (20,21), and from mouse and rat liver (22). The LRAT cDNA reported for human and bovine RPE (20) and rodent liver (22) are ~2.5 kb in length, although these cDNA hybridized to several tissue mRNA species ranging in size from 2.5 to 5 kb. When these LRAT cDNA were expressed in cells by transfection, they were expressed and functional retinol esterification was demonstrated (20,22). LRAT mRNA has a relatively short open reading frame (ORF) and a long 3′ untranslated region. The predicted protein sequence for both human and bovine RPE LRAT consists of 230 amino acid residues (20,21), which are perfectly aligned with the residues in rodent liver LRAT (22), each of which has 231 amino acid residues due to an extra methionine residue at position 229. Human and bovine LRAT proteins share about 88% amino acid identity with each other and each is about 80% similar with mouse and rat LRAT, while the latter are 94% similar and 91% identical to each. The human LRAT gene has been identified but its 5′- and 3′-end boundaries have not yet been defined; it is about 7 kb in length, consisting of three exons and two introns, and is located on chromosome 4q31.2 (21).

LRAT is a membrane-bound protein located in the endoplasmic reticulum (ER) compartment. It is predicted to contain two membrane-spanning regions located near the N-terminal and C-terminal region of the protein, respectively, and a long region that is exposed to the cytoplasmic compartment (Fig. 2) (23,24). Both N-terminal and C-terminal region residues are predicted to be present in the lumen of the ER. The cytoplasmic region is predicted to contain several conserved amino acid residues which have been shown by site-directed mutagenesis and expression of the mutated human LRAT cDNA to be essential for LRAT activity. These residues include two conserved cysteines (C161 and C168) (23), and two catalytically essential histidines, H57 and H163, among a total of 5 conserved histidine residues (23). The cytoplasmic region which consists of 165 amino acid residues apparently contains all the amino acid residues necessary for LRAT activity. These positions are well conserved among the four species for which LRAT cDNA sequences are known. However, expression of the cytoplasmic region of rodent LRAT cDNA by transfection in HEK293T cells did not support any esterification of CRBP-bound retinol in vitro, implying that the integrity of the protein or its targeted localization may be essential for expression of LRAT activity (Zolfaghari and Ross, unpublished observations).

Recently, two different mutations in the LRAT gene have been shown to be associated with severe, early-onset diseases (25). Two individual women were shown to carry a homozygous T to A transversion at nucleotide 525 in the ORF of LRAT mRNA, which resulted in changing the serine 175 to an arginine residue; this genotype cosegregated with a known phenotype of early severe retinal degeneration. When COS-7 cells were transfected with the human LRAT nucleotide sequence containing the ORF with serine 175 mutated to arginine (S175R), no LRAT enzymatic activity was expressed (25). It is not known at this time whether it is the absence of this serine residue or the presence of the arginine residue which results in the mutant phenotype. A second LRAT mutation was identified in a male patient with a heterozygous 2-bp deletion (396delAA) that shifts the reading frame following codon 133 to encode 11 unrelated amino acid residues followed by a premature stop codon. This patient was reported to have no central vision and was diagnosed with retinal degeneration at age 3 (25).

Based on a lack of strong homology with other known proteins, LRAT is considered as a novel enzyme which is not related to any other protein with similar catalytic function. However, comparison of the LRAT protein sequence with those of other proteins present in the Genbank database has revealed several small proteins which have 25 to >50% similarity in the region corresponding mostly to the cytoplasmatic region of LRAT protein as described above. These proteins include human HRAS-like suppressor (accession # XP 002991), mouse HRAS suppressor (accession # AAF13304), human retinoid inducible gene 1 (AAF22294), etc. Interestingly, the predicted LRAT proteins described above contain a conserved 12-amino-acid domain (160NCEHFVTYCRYG171, Fig. 2), which includes 2 essential cysteine residues and one essential histidine residue and is considered to be the active site for LRAT. This domain is also apparently conserved in these small proteins. Further studies will be required to clarify the structure-function relationships, if any, between LRAT and these other partially homologous proteins.

**Regulation of LRAT gene expression in liver**

Using liver LRAT cDNA as a probe, we set out to determine the regulation of LRAT mRNA and to compare LRAT mRNA and LRAT enzyme activity using animals from experiments of two different designs. First, we compared vitamin A-deficient and vitamin A-sufficient animals (mice and rats), before and after treatment with all-trans-RA, since this design is likely to reveal the most regulatory information. The results for LRAT mRNA (data not shown) agreed nearly exactly with previous results for LRAT activity, showing that there is little if any LRAT mRNA in vitamin A-deficient liver, and a nearly normal level of expression in the liver of vitamin A-deficient rats or mice after treatment with RA (22). Second, we used liver tissue from rats that were part of a long-term study in which three nonextreme levels of dietary vitamin A (referred to as marginal, control, and supplemented) from weaning until they were 2–3, 8–10 and 18–20 mo old. With this design (26), liver total retinol concentrations spanned a very wide range (<1 to >19,000 nmol/g liver) by the end of the study (27). Liver LRAT mRNA and enzyme activity were well correlated (r = 0.799, P < 0.0001) (22). In rats fed the control diet, LRAT mRNA expression (Fig. 3A) and enzyme activity (27) were maintained during aging. In the
vitamin A-marginal group, LRAT mRNA expression was very low in young rats and declined to nearly undetectable levels as rats aged, during which time their liver retinol levels declined. Conversely, LRAT mRNA was elevated in the vitamin A-supplemented group at all ages. These results provided evidence that LRAT gene expression and enzyme activity are well regulated under steady-state nutritional conditions, with nonextreme diets, across the life span. To better understand the relationship between LRAT regulation and liver total retinol as an index of vitamin A status, we plotted these data for all of the diet-age groups versus liver total retinol, as shown in Figure 3B. When liver total retinol was <100 nmol/g, LRAT expression was very low or negligible. Above ~200 nmol/g and extending to the highest liver total retinol values in the study, LRAT mRNA was fairly constant, with a slight increase at the highest liver vitamin A levels. The lack of a significant increase in LRAT mRNA in rats fed the vitamin A-supplemented diet agrees with previous calculations that the total LRAT enzyme activity in normal rat liver is sufficient to esterify all of the retinol entering the liver from the absorption of a standard or moderately vitamin A-supplemented diet. Thus a further increase in LRAT gene expression would seem unnecessary, and it was not observed experimentally.

From these studies conducted over a wide range of vitamin A status, LRAT mRNA varies progressively: vitamin A deficient < marginal < control ≤ supplemented = RA-treated. A model of the regulation of LRAT expression can be proposed in which RA, the most active end-product of retinol metabolism, functions directly as a signal of the body’s vitamin A adequacy (Fig. 4). As long as the concentration of RA is adequate, RA provides a “feed forward” signal to positively regulate LRAT in the “on” position. When RA falls, as in the case of marginal vitamin A deficiency, LRAT expression is no longer fully on, and it continues to fall as vitamin A deficiency becomes overt. Physiologically, the maintenance of liver LRAT expression by RA appears to subserve a useful function by conserving unneeded retinol in a readily retrievable storage form. Coincidentally, retinol may be effectively diverted away from one or more of the other biochemical fates available to it, as indicated in Figure 1, thereby affecting these processes as well.

Precisely how RA regulates LRAT expression is still to be determined. While the rapid increase in LRAT mRNA expression caused by RA and other RAR-selective retinoids in retinoid-depleted animals and the inhibition of this increase by actinomycin D suggest regulation at the level of LRAT gene transcription, other mechanisms are also possible; thus an understanding of the regulatory process(es) involved requires additional experiments.

**CYP26 regulation: relationship to liver vitamin A**

The ability of RA to induce its own catabolism has been demonstrated experimentally (28) and observed in patients treated with RA (29). In the 1970–80s, the existence of one or more cytochrome P450-dependent pathways was inferred from biochemical studies (30) and inhibitor studies in which ketoconazole and related drugs blocked the oxidation of RA in cultured cells (31) and in vivo (32). In 1996–97, several groups reported the presence of a specific RA-inducible cytochrome P450-related RA hydroxylase, CYP26, also known as P450RA1 (33–36). CYP26 is an unusual member of the cytochrome P450 family in having <30% amino acid identity with other known mammalian CYP family members (33,36) and greater homology with some bacterial and plant P450s. A second subfamily termed CYP26B1, cloned from a human retinal library (34), is 44% identical to that of the previously cloned CYP26 (A1), but its function has not been...
reported and it is yet not known whether CYP26B1 responds to RA. An analysis of the CYP26 promoter region (38) showed that it contains a nearly perfect RARE of the direct repeat (DR)-5 type, and a G-rich element necessary for induction by RA. The expression of CYP26 cDNA in transfected F9, P19 or HeLa cells (34,35,39) resulted in the enzymatic oxidation of all-trans-RA and formation of products identified as 4-hydroxy- and 4-oxo-retinoids, plus unidentified metabolites. Interestingly, expressed CYP26 catalyzes the hydroxylation of all-trans-RA, but not 9-cis-RA, retinol, retinal or \( \beta \)-carotene (35,40). The implication of this for retinoid signaling is discussed below. Because CYP26-transfected cells exhibited reduced sensitivity to RA-induced differentiation and gene transactivation, it was inferred that RA was being inactivated (35). However, it is noteworthy that C-4 retinoid oxidation also has been associated with bioactivation (41,42), suggesting context-dependent functions for these metabolites.

These studies, however, provided scant information on the regulation of CYP26 in the adult. While retinoid 4-hydroxylase activity has been reported in adult human liver microsomes (43,44), its induction by retinoids has not been studied. Ray et al. (36) reported that CYP26 mRNA is induced by all-trans-RA in adult mouse liver but, in contrast, not in brain. This result suggests there may be tissue- and perhaps even liver-specific regulation, but the only dose of RA used was extremely high (0.0001 g/g body weight) and physiological studies were not conducted. To determine whether CYP26 in adult liver is regulated by dietary vitamin A within a range that is nutritionally relevant, we investigated CYP26 expression in the liver of the same rats used for the study of LRAT expression described above. By Northern blot analysis of pooled samples, CYP26 mRNA was higher in the liver of vitamin A-supplemented rats compared to control rats, but it also increased with age in rats fed either of these diets [data not shown, see Fig. 1 of (45)]. These results suggested that CYP26 expression may be regulated according to the liver’s cumulative exposure to vitamin A (total retinol content) rather than the amount of retinol in the diet (daily influx). To test this further, CYP26 mRNA levels were quantified in livers of young, middle-aged and old rats fed diets that were marginal, adequate or supplemented in vitamin A (45), such that the final liver total retinol concentrations spanned a 4-log range. By two-factor ANOVA, both diet (\( P < 0.0001 \)) and age (\( P < 0.0003 \)) were significant main effects for CYP26 mRNA expression. Similar to the analysis shown for LRAT in Figure 3B, CYP26 mRNA was plotted versus liver total retinol as an indicator of vitamin A status. As shown in Figure 5, CYP26 mRNA was down-regulated, compared to the young control group, when liver retinol was less than \( \approx 70 \) nmol/g (20 \( \mu \)g/g). And it was significantly up-regulated, in a concentration-dependent manner, when liver vitamin A exceeded \( \approx 1000 \) nmol/g (286 \( \mu \)g/g). Based on studies in models of acute and chronic retinoid exposure, hepatic CYP26 expression is regulated dynamically in the order: vitamin A-deficient < marginal < control < vitamin A-supplemented (45), and in a dose-dependent manner by exogenous all-trans-RA (46). The increase in CYP26 expression above \( \approx 70 \) nmol retinol/g liver compares well with a previously described increase in the biliary excretion of \( ^3 \)H-RA and its metabolites when liver retinol concentration exceeded 20 \( \mu \)g/g (70 nmol/g) (47). Perhaps retinoid-regulated CYP26 activity provides a molecular mechanism for this physiological result. Many questions still remain about CYP26 and its regulation, including the kinetics of its response to diet, exogenous RA and receptor-selective retinoids; its cellular and subcellular localization; and whether the modulation of CYP26 expression by diet and RA alters the metabolic partitioning of retinol and RA in vivo.

It is interesting that CYP26 did not metabolize 9-cis-RA, an RXR ligand, or 13-cis-RA (40,48), nor was RA metabolism induced by these isomers (48). The selectivity for all-trans-RA as inducing agent and substrate for CYP26 suggests that the metabolism of all-trans-RA is segregated from that of 9-cis-retinoids. If this is the case, the implications for gene expression are likely to be significant. Essentially, the regulation of gene promoters to which ligand-activated RAR/RXR heterodimers bind could be controlled separately from those of promoters that bind the numerous other nuclear receptors for which RXR also is a partner. Specificity for retinoids in the trans or cis conformations has also been demonstrated for several enzymes involved in RA biosynthesis (see Blaner et al. in this symposium). Together, these data suggest the possibility of two parallel but independently regulated metabolic systems, one for trans and another for cis retinoids. However, considerable additional research is needed to test this hypothesis.

**Other mechanisms**

Our focus on LRAT and CYP26 is based on their strong regulation by vitamin A and RA. Other factors may also be important. The level of CRBP, a “coprotein” for LRAT as well as for a microsomal retinol dehydrogenase (49,50), has been reported to have regulatory functions. When CRBP is deleted genetically, vitamin A deficiency ensues very rapidly, suggesting a role for metabolic processes controlled by CRBP in vitamin A homeostasis (51). However, in genetically normal animals the level of CRBP in most tissues is not dramatically regulated due to vitamin A deficiency or retinoid treatment (52–54), and it thus seems unlikely that its expression is rate-limiting for vitamin A metabolism. The ratio of apo, or
Is the whole-body economy of vitamin A an integrated, centrally regulated system?

Kinetic studies have shown that each molecule of retinol circulates several times between plasma and liver before undergoing irreversible disposal (3). In humans, ~50 μmol/d (14.3 mg/d) of retinol passes through plasma, compared to an estimated disposal rate of 4 μmol/d (1.14 mg/d) (56). Conceptually, recycling seems to provide an ideal means for the liver to constantly sample and adjust the concentration of retinol available in plasma for peripheral tissues. Although recycling has been less thoroughly studied for RA, the same type of interorgan flux is likely to exist, as RA is readily diffusible across plasma membranes (57). Moreover, kinetic studies indicate that the preponderance of liver RA is obtained by uptake from plasma (57). A model (Fig. 6) illustrates the possible “cross-talk” between the liver and peripheral tissues in the export of retinol, peripheral metabolism of retinol to RA, return of excess RA to the liver, and regulation by RA of liver retinoid metabolism. Further research is needed to test whether peripheral RA production and hepatic retinoid partitioning are, indeed, closely linked.

From the viewpoint of evolution and adaptation, the hepatic regulation of both LRAT and CYP26 would provide a dual means to adapt to a diversity of diets in which vitamin A may be available only seasonally, or sporadically, or in excess. In this framework, an economic analogy may be appropriate. Long-term vitamin A status, indicated by the body’s total of stored retinyl esters, may be viewed as “money in the bank.”  If RA is readily deposited and withdrawn on demand, the liver’s role as central bank and banker seems undisputed, as vitamin A in liver may be deposited in long-term storage or withdrawn and “wired” to other accounts (exported to peripheral tissues) as needed. But vitamin A status may also have a short-term component, analogous to cash on hand. It seems plausible and indeed likely that RA functions both physiologically as an indicator of the body’s short-term vitamin A status, and biochemically as a direct molecular signal to which genes including LRAT and CYP26 respond. In liver, their dose-responsive expressions appear to be significant mechanisms for adjusting retinoid partitioning and maintaining whole-body retinoid homeostasis.

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FIGURE 6 Model of inter-relationship (cross-talk) between liver and peripheral tissue retinoid metabolism. Tissues listed as loci of retinoic acid (RA) biosynthesis are ones in which less than half of tissue RA was derived by uptake from plasma [see reference (57)].


