RETINOL AND RETINYL ESTERS: BIOCHEMISTRY AND PHYSIOLOGY

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Running Title: Retinoid Storage and Metabolism

Key Words: vitamin A, retinoic acid, retro-retinoids, anhydro-retinoids, hepatocyte, hepatic stellate cell, adipocyte, enterocyte, lipid droplets, Stra6, DGAT1, and RBP4

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Abstract.

Since the identification of fat soluble A a century ago (1), retinoids (vitamin A and its natural and synthetic analogs) have been the most extensively studied of the fat soluble vitamins. This research has identified essential roles for retinoids in many different aspects of mammalian physiology including embryonic development, adult growth and development, maintenance of immunity, maintenance of epithelial barriers and vision (1-5). This review will focus on retinoid biochemistry in mammals, primarily on retinol and retinyl ester metabolism. However, we also will consider other retinol metabolites including retro- and anhydro-retinoids and retinoid-β-glucuronides.

Retinoid chemical forms.

The different retinoid forms present within the body (see Figure 1) are generated by and large through modifications to the terminal polar end group of the molecule. Retinol and retinyl esters are the most abundant retinoid forms present in the body. All-trans-retinol is by definition vitamin A. When a fatty acyl group is esterified to the hydroxyl terminus of retinol, a storage form of retinol, the retinyl ester, is formed. The most abundant retinyl esters present in the body are those of palmitic acid, oleic acid, stearic acid and linoleic acid (6, 7). Although retinyl acetate can be found in supplements to foods and vitamin formulations, only long chain acyl groups are esterified to retinol by animals. Retinyl esters have no known biological activity aside from retinol storage and for serving as the substrate for the formation of the visual chromophore 11-cis-retinal, which must be formed from all-trans-retinyl ester through linked hydrolysis and isomerization reactions catalyzed by the enzyme RPE65 (3, 4, 8,
9). Retinol is a transport form and a precursor form, which is enzymatically activated to retinoic acid via a two-step oxidation process. The primary role of retinal is in the eye where 11-cis-retinal is needed for visual pigment formation. In tissues, retinal serves as an intermediate in the synthesis of retinoic acid from retinol (6, 7). The literature also suggests a direct role for retinal in adipose tissue, where it was shown to inhibit adipogenesis and suppress peroxisome proliferator-activated receptor-gamma (PPARγ) and retinoid X receptor (RXR) responses in cell culture models and in mouse models fed high fat diets (10).

The all-trans- (tretinoin, Retin-A) and 9-cis- (alitretinoin) isomers of retinoic acid are transcriptionally active retinoids and are thought to account for the gene regulatory properties of retinoids within cells and tissues (11, 12). The concentration of retinoic acid within tissues is generally very low and usually 100- to 1000-times less than that of retinol (6, 7). All-trans-retinoic acid can be isomerized through a nonenzymatic process to form the 9-cis- or 13-cis-retinoic acid (isotretinoin, Accutane) isomers (6, 7). Although 13-cis-retinoic acid is a naturally occurring retinoid, it is less transcriptionally active than either the all-trans- or 9-cis-isomers (11, 12). The actions of retinoic acid in regulating transcription are considered in detail in this Thematic Series by Rochette-Egly and colleagues (13).

Various oxo- and hydroxy-forms of retinol and retinoic acid as well as glucuronides of both retinol and retinoic acid are present in the body, albeit at very low concentrations relative to retinol and retinyl esters (6, 7). Although some of these oxidized and conjugated retinoid forms may have biologic/transcriptional activity, it appears likely that most of these forms are catabolic in nature and destined for
elimination from the body. Since there are no known enzymes that can reduce retinoic acid to retinal, excessive or unneeded retinoic acid is not recycled back to retinol/retinyl ester and must be catabolized and eliminated from the body. This catabolism is catalyzed by one of several cytochrome P450 (CYP) enzymes (14-18) giving rise to more water soluble oxidized and conjugated retinoid forms that can be more easily excreted. These CYPs are discussed in depth by Kedishvili in a review in this Thematic Series (19). The metabolism of retinoids described above and the enzymes responsible for catalyzing this metabolism are summarized in Figure 2.

Retro- and anhydro-retinoids are also naturally occurring retinoid forms that can be synthesized by cells and tissues, which are present within the body (20, 21). Enzymes able to catalyze the formation of retro- and anhydro-retinoids have been identified (20, 21). It has been proposed that the retro- and anhydro-retinoids may have actions in regulating immune function but the biochemical mechanisms responsible for these actions have not yet been elucidated (5, 20, 21). The enzyme responsible for the saturation of the 13-14 double bond of all-trans-retinol to produce all-trans-13,14-dihydroretinol, termed retinoid saturase (RetSat), was described and cloned several years ago (21-23). Expression of RetSat is regulated by PPARγ, a key transcriptional regulator of adipogenesis. RetSat activity has been implicated in the regulation of adipocyte development and differentiation. Ablation of RetSat expression in a cell culture model of adipocyte differentiation inhibited adipogenesis, whereas ectopic expression of RetSat enhanced differentiation (24). This block in adipocyte differentiation could not be rescued by addition of 13,14-dihydroretinol to the cells, implying that this enzyme may have other as yet unknown substrates (24). Mice totally
lacking RetSat exhibit increased adiposity, which is associated with an up-regulation of PPARγ (25). Future studies may identify other novel metabolites of retinol, possibly generated by RetSat, which may explain these phenotypes.

Over the last four decades many retinoid metabolites have been identified (6, 7, 20, 21). Some of these are proposed but not proven to have important physiological roles whereas others are simply catabolic products destined for elimination from the body. It is possible that some of these metabolites may yet prove to be very important physiologically, but we have chosen to consider in our review only those associated with a relatively substantial literature. The reader should turn to earlier review articles (6, 7, 20), for more details regarding these other retinoid metabolites.

**Retinol esterification and retinyl ester hydrolysis.**

Although the liver and intestine are the major tissue sites of retinol esterification in the body, many tissues are able to esterify retinol and accumulate some retinyl ester stores, including the eye, lung, adipose tissue, testes, skin, spleen and others (6, 7). It is now understood that the enzyme responsible for the preponderance of retinyl ester formation in the body is lecithin:retinol acyltransferase (LRAT). This understanding was obtained from study of mutant mice where the gene encoding LRAT had been totally ablated (26-28). *Lrat*-deficient mice possess significant retinyl ester stores only in adipose tissue but not in liver, eyes, lungs, testes, skin or spleen (26-29). LRAT catalyzes a transesterification, transferring long chain fatty acyl moieties (primarily palmitic, stearic, oleic and linoleic acids) present at the sn-1 position of membrane bilayer phosphatidylcholine to retinol, forming retinyl esters (6, 7, 30-34). LRAT is one
of 6 distinct proteins/genes which compose the vertebrate members of the NlpC/P60 protein family (35). Members of this protein family share a common property of having conserved cysteine, histidine and polar amino acid residues that are required for catalytic activity (35). The other vertebrate member of this family that is relatively well studied is adipose-specific phospholipase A2 (AdPLA) (36, 37). AdPLA was described by Sul and colleagues as a phospholipase that is associated with the development of obesity induced by high fat feeding (37). The other vertebrate members of this protein family are less well studied and are reported to possess either phospholipase and/or phosphatidylcholine-dependent acyltransferase activities (38-42). Some show a sn-1 preference and others a preference for the sn-2 acyl moiety (38-42). It should be noted that LRAT is completely distinct from, and shares no relationship with, lecithin:cholesterol acyltransferase (LCAT).

The older literature suggests that another enzymatic activity may be physiologically important for catalyzing retinyl ester formation, acyl-CoA:retinol acyltransferase (ARAT). ARAT is proposed to esterify retinol using fatty acyl groups present in the acyl-CoA pool (33). ARAT also is reported to differ from LRAT with regards to its ability to acquire retinol within the cell. LRAT is capable of esterifying retinol when it is bound to one of the cellular retinol-binding proteins (CRBPI, CRBPII, or CRBPIII), whereas ARAT is not (34). Several published studies have established that one of the two enzymes responsible for the final step in triglyceride synthesis, diacylglycerol acyltransferase 1 (DGAT1), possesses ARAT activity in vitro (28, 43-46). DGAT1, when expressed in vitro, will catalyze retinyl ester formation using non-protein bound retinol as a substrate but is unable to catalyze retinyl ester formation when the
retinol is supplied bound to CRBPI or CRBPII (28). When \textit{Lrat}-deficient mice were challenged with an oral physiological dose (6 µg) of retinol provided in oil, some retinyl esters were still present in nascent chylomicrons (28). When this same oral challenge was given to mice totally lacking expression of both \textit{Lrat} and \textit{Dgat1}, no retinyl esters could be detected in newly synthesized chylomicrons (47). This observation was taken to indicate that DGAT1 acts \textit{in vivo} as an intestinal ARAT (47). Interestingly, when an oral pharmacological dose (1.0 mg) of retinol in oil was given to the mice lacking both \textit{Lrat} and \textit{Dgat1}, some retinyl esters were observed in the postprandial circulation (47). This observation suggests that a third enzyme is able to esterify retinol in the intestine when very high levels of retinol have been consumed. Studying \textit{Dgat1}-deficient mice, Farese and colleagues independently established that DGAT1 can influence retinoid homeostasis in the skin of mice (44). Thus, in at least two mouse tissues, intestine and skin, DGAT1 acts physiologically in catalyzing retinyl ester formation. As noted above, \textit{Lrat}-deficient mice possess significant retinyl ester stores in adipose tissue (28, 29). This is also true for mice totally lacking both \textit{Lrat} and \textit{Dgat1} (47). This observation implies that another as yet unidentified enzyme acts in adipose tissue to catalyze retinyl ester formation.

\textit{LRAT} in the liver is thought to be structurally identical to intestinal \textit{LRAT}, which synthesizes retinyl esters from dietary retinol for incorporation into nascent chylomicrons. Interestingly though, hepatic but not intestinal \textit{LRAT} expression is regulated by retinoid nutritional status (48). The regulation of expression of the \textit{Lrat} gene in the liver involves the presence of a retinoic acid response element present in the \textit{Lrat} gene, and probably the actions of retinoic acid receptors (RARs) and/or RXRs
This retinoic acid-responsive regulation is proposed to give rise to a positive feedback loop when cellular retinoic acid levels are high, turning on *Lrat* expression and increasing the synthesis of retinyl esters and preventing the synthesis of additional retinoic acid. Supporting this proposal is the observation that hepatic expression levels of the retinoic acid catabolic enzyme, *Cyp26A*, (which is also a retinoic acid responsive gene), are markedly upregulated in *Lrat*-deficient mice (27, 28). In addition to a role for retinoic acid in regulating *Lrat* gene expression, studies carried out in the PC-3 prostate cancer cell line indicate that GATA transcription factors also act importantly in regulating *Lrat* transcription (49).

Since retinyl esters represent a retinoid storage form, they must be hydrolyzed to retinol before conversion to retinoic acid. Unlike LRAT, which is accepted to be the major enzyme responsible for retinyl ester formation, there are many retinyl ester hydrolases (REHs) that are proposed to be responsible physiologically for the generation of free retinol from retinyl ester stores (50-52). One is a bile salt-dependent retinyl ester hydrolase. Most or all of this enzymatic activity in the liver probably arises from the actions of bile salt-activated carboxylester lipase (CEL) (53). However, since mice lacking CEL display no alterations in retinoid storage, metabolism or actions, so this enzyme cannot be the sole physiologically relevant REH (53). Another group of enzymes, collectively known as bile salt-independent REHs have been described. Based on their pH optima, there are two groups of bile-salt independent REHs, neutral REHs and acidic REHs. It has been reported that the activities of the neutral and acidic REHs are unaffected by retinoid nutritional status. There is evidence demonstrating that three known hepatic carboxylesterases (also known in the literature
as ES-2, ES-4 and ES-10) act as REHs in vitro (50-52). However, it is not yet established whether any or all of these REHs are physiologically important in retinyl ester/retinol metabolism. The reader is also referred to the review of Eroglu and Harrison (54) in this Thematic Series for further consideration of this point.

**Retinoid-binding proteins.**

In order to solubilize, protect and detoxify retinoids in the aqueous intracellular and extracellular environment, retinol, retinal and retinoic acid are bound to specific retinoid-binding proteins (6, 7, 55). These binding proteins can be classified using several different criteria. Some of these proteins, specifically retinol-binding protein (RBP4), interphotoreceptor matrix retinoid-binding protein (IRBP), epididymal retinoid-binding protein (ERBP), and β-trace are found in extracellular fluids, whereas the remaining are found only intracellularly (6, 7, 55-65). Of the intracellular binding proteins, some bind only retinoic acid (cellular retinoic acid-binding protein, type I (CRABPI) and cellular retinoic acid-binding protein, type II (CRABPII)) (55, 60); some preferentially bind both retinol and retinal (cellular retinol-binding protein, type I (CRBPI) (55, 61-63), cellular retinol-binding protein, type II (CRBPII) (55, 60, 61)); some preferentially bind retinol (cellular retinol-binding protein, type III (CRBPIII) (63) and cellular retinol-binding protein, type IV (CRBPIV) (64)); and one that preferentially binds retinal (cellular retinal-binding protein (CRALBP) (55, 60, 62, 65)). These proteins can also be grouped by the protein families to which they belong. RBP4, ERBP and β-trace are all members of the lipocalin protein family (55-59). CRBPI, -II, -III, and -IV as well as CRABPI and CRABPII are members of the fatty acid-binding protein family of proteins (55, 57, 60, 61, 63, 64).
CRALBP is a member of the CRAL-TRIO protein family that also contains the vitamin E-binding protein, α-tocopherol-transport protein (TTP) (65). For more details on TTP and vitamin E, the reader is referred to the review from Traber in this Thematic Series (66).

Each of the known retinoid-binding proteins has been proposed to have a role in facilitating retinoid transport and/or metabolism. However, most if not all of these proteins do not have essential roles in facilitating these processes as the genes for nearly all of these retinoid-binding proteins have been ablated in mouse models and none of the gene disruptions is lethal or associated with a severe phenotypes (67-73). It seems likely that these proteins are needed to facilitate optimal retinoid retention, transport and metabolism. When dietary retinoid is available, the actions of the binding proteins are not essential for maintaining retinoid status of the body or the health of the organism. However, in times of dietary retinoid-insufficiency, the binding proteins and the enhanced metabolic efficiency and retention that they afford, convey an advantage to the organism.

**Intestinal absorption.**

The two most abundant retinoid forms that are present in the diet are retinol and retinyl esters. Dietary retinol is taken up directly by mucosal cells. However, dietary retinyl esters are unable to enter the intestinal mucosa and must first be acted upon by a luminal REH to yield free retinol. Retinyl esters can be hydrolyzed within the intestinal lumen by nonspecific pancreatic enzymes like pancreatic triglyceride lipase and cholesteryl ester hydrolase or at the mucosal cell surface where a retinyl ester hydrolase is associated with the intestinal brush boarder (6, 7). The free retinol formed
upon hydrolysis of the retinyl ester or unesterified retinol arriving as such from the diet is taken up into the intestinal cells (6, 7). In contrast to dietary preformed retinoid, dietary proretinoid carotenoids like β-carotene can either be converted to retinal within the enterocyte or absorbed unmodified by these cells. The intestinal enzyme responsible for the cleavage of proretinoid carotenoids to retinal is β-carotene-15,15'-monooxygenase (BCMO1) (see Figure 2) (74). CRBPII is present at high concentrations in the enterocytes and binds both retinal and retinol (55, 75). Retinal, formed upon proretinoid carotenoid cleavage by BCMO1, binds to CRBPII and this is proposed to be the preferred substrate for reduction to retinol by an intestinal retinal reductase. Retinol bound to CRBPII is then re-esterified to long chain fatty acids predominantly through the action of LRAT, which utilizes retinol bound to CRBPII as a substrate for esterification (55, 60, 75). The resulting retinyl esters are then packaged along with the rest of the dietary lipids into nascent chylomicrons and secreted into the lymphatic system for uptake into the general circulation (6, 7). The molecules and molecular events responsible for the intestinal absorption of retinoids and carotenoids are considered in more detail elsewhere in this Thematic Series (54).

**Transport in the postprandial and fasting circulations.**

A number of different retinoids are found in the circulation and these differ in the fasting and postprandial states. These potential retinoid delivery pathways are summarized in Figure 3. They include: retinyl esters in chylomicrons, chylomicron remnants, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high density lipoprotein (HDL); retinol bound to retinol-binding protein (RBP4); retinoic acid
bound to albumin; and the water-soluble β-glucuronides of retinol and retinoic acid. In the postprandial circulation following consumption of a retinoid-rich meal, retinyl ester concentrations in the 5-10 µM range can be reached (76), although this will depend directly on the quantity of retinoid consumed in the meal. The liver secretes some retinyl ester bound to nascent VLDL, and, upon metabolism of the VLDL, some of this retinyl ester can be found in LDL or transferred to HDL in species which express cholesteryl ester transfer protein. Concentrations of retinyl esters in the fasting circulation can vary but are generally in the range of 100-200 nM (77). In the fasting circulation, retinol bound to RBP4 is the predominant retinoid species with normal concentrations ranging from 2-4 µM in humans and around 1 µM in mice (28, 56, 78). In the circulation, the retinol-RBP4 complex binds another plasma protein, transthyretin (TTR), and this stabilizes the complex, reducing renal filtration of the retinoid (56, 79).

Retinoic acid is present in both the fasting and postprandial circulations where it is found bound to albumin. Nau and colleagues have reported that immediately following consumption of a retinoid-rich meal consisting of 100 g of turkey liver, human blood levels of retinoic acid can reach 80-90 nM (76). However, this level is quickly restored by the body to fasting levels that range from 1-3 nM (76, 80). The half-life of plasma all-trans-retinoic acid in a fasted chow fed rat is very short, with the plasma pool completely turning over every 2 minutes (80). The studies of Nau and colleagues (76) suggest that the intestine contributes significantly to the retinoic acid that is present in the postprandial circulation. The tissues responsible for contributing retinoic acid to the fasting circulation remain to be established. At the present time, it is unclear whether only one or a few tissues contribute to the circulating retinoic acid pool
or whether retinoic acid is simply "leaking" into the fasting circulation from most or all tissues.

Plasma/serum concentrations of retinyl- and retinoyl-β-glucuronides were reported by Olson and colleagues to be in the range of 5-15 nM (81, 82). Although it has been proposed that retinyl- and retinoyl-β-glucuronides, which are known to be readily hydrolyzed by a number of β-glucuronidases (6, 7), may serve as sources of retinoids for tissues, it is generally believed that these fully water soluble metabolites are filtered in the kidney and eliminated quickly from the body.

In addition to preformed retinoid delivery through the circulation, proretinoid carotenoids like β-carotene are absorbed intact by the intestine and these too can be found in the blood bound to chylomicrons and their remnants, VLDL, LDL, and HDL (83, 84). Since many tissues, including liver, lungs, and testes, express BCMO1, intact carotenoid delivered to these tissues can be converted in situ to retinoids that may be needed for supporting retinoid-dependent functions. Fasting blood levels of the canonical proretinoid carotenoid β-carotene in humans, a species which absorbs carotenoids well, can be as great as 5-8 µM (84). Although the mouse, owing to its ability to be genetically manipulated, is presently being used to study β-carotene uptake from the diet, as well as β-carotene metabolism and physiologic actions, it should be noted that rodents are very poor absorbers of β-carotene. Consequently, studies involving the feeding of β-carotene to mice must employ very high concentrations of β-carotene in the diet, concentrations which are many times greater than those which would be found in any human diet.
As can be surmised from the text above, the delivery of retinoids to tissues is complex, involving many different retinoid forms and carriers. Quantitatively the two most important pathways are those involving retinol bound to RBP4 and the postprandial delivery pathway. However, the importance of retinoic acid delivery to tissues from the blood should not be discounted, since the accumulation of retinoic acid from the blood is tissue-dependent (80). In this regard, the delivery of retinoid to tissues, as either retinol or retinoic acid, is not different than the delivery of vitamin D or thyroid hormone, involving the presence of relatively large concentrations of the transcriptionally-inactive precursor (retinol, 25-hydroxy-vitamin D, or T₄) and relatively low concentrations of the transcriptionally active metabolite (retinoic acid, 1,25-dihydroxy-vitamin D, or T₃).

**Cellular uptake of retinoids.**

Retinyl esters in chylomicrons enter the circulation and are taken up by tissues as the chylomicron undergoes lipolysis and remodeling. Approximately 66-75% of chylomicron retinyl ester is cleared by the liver and the remainder is cleared by peripheral tissues (85). Prior to uptake by peripheral tissues, chylomicron retinyl ester must undergo hydrolysis. It has been proposed that the enzyme lipoprotein lipase (LpL) performs this function in peripheral tissues, facilitating retinol uptake (86). Postprandial unesterified retinol taken up by cells is thought to bind immediately to CRBPs that are present in tissues (55, 87). As noted above, it has been suggested that CRBPs facilitate/optimize retinol uptake process and facilitate its subsequent metabolism (55, 87). In the liver, retinyl ester hydrolysis occurs as the chylomicron remnant particle is
internalized by hepatocytes during the early stages of endosome formation (6, 7). However, it is not presently established what enzyme(s) is responsible for hydrolysis although one of several carboxyesterases may play a role in this process. Once retinol is formed upon retinyl ester hydrolysis within the hepatocyte, it is quickly bound by apo-CRBPI, which is in molar excess of retinol in these cells (88, 89).

How retinol is taken up by cells from the circulating retinol-RBP4 complex has been the subject of much research interest for many years. Studies of intestinal cells imply that retinol enters by diffusion and this is likely true for other cell types (90-93). However, in 2007, a cell surface receptor for RBP4 termed STRA6 (Stimulated by Retinoic Acid 6) was identified (94, 95). STRA6 is expressed in a number of tissues/cells which have a high demand for retinoid, especially the retinal pigmented epithelium (RPE) cells of the eye, but is not expressed in many others, including liver cell types (94-97). This receptor interacts with RBP4 and increases cellular uptake of retinol (95). In addition, STRA6 is reported to facilitate retinol efflux from cells (98-100). Both LRAT and CRBPI are proposed to couple with STRA6 within the cell (95, 100, 101). Von Lintig and colleagues have convincingly established that the functional coupling of LRAT with STRA6 increases cellular retinol uptake into tissues and have proposed that LRAT is a critical component of this process (101). In vitro experiments have established that STRA6 facilitates efficient retinol exchange between intercellular CRBPI and extracellular RBP4 (100). Recently, mice lacking STRA6 were generated and studied (102). These mice are viable and generally normal. The primary phenotype of the Stra6-deficient mice is a visual one, similar to the mice lacking Rbp4 (71, 102). Interestingly, the results of studies of Stra6-deficient mice suggest that there
are other pathways facilitating retinol uptake into the RPE, but that the one involving Stra6 is the most important one (102).

Over the last several years, a large number of published reports have suggested a link between RBP4 and obesity, diabetes and insulin signaling. The first of these studies looked at the impact of RBP4 levels in various mouse and human models (103-105). RBP4 levels appear to be increased in human obesity and this increase impairs insulin signaling, possibly contributing to the development of type 2 diabetes, although the published studies are sometimes not in agreement (103-109). There is also evidence now that STRA6 may be involved in mediating this effect of RBP4 on insulin signaling (110, 111). We will further consider this topic below in "Retinoids and Adipose Tissue."

Unlike retinol delivered bound to RBP4, which is thought to be taken up by cells through a process involving a cell surface receptor, RA uptake into tissues is not presently thought to involve a cell surface receptor. It is well established experimentally that RA can "flip-flop" across a phospholipid bilayer and it is generally assumed that it is taken up into cells from the circulation through this process (112, 113). This same "flip-flop" mechanism was earlier proposed to account for most unesterified fatty acid uptake by cells across the plasma membrane from the circulation (114). However, it is now widely accepted that several plasma membrane proteins are responsible for most unesterified fatty acid uptake by tissues (115). The possibility that RA also may be taken up into cells by one or more cell surface receptors has not been systematically explored and remains to be established.
Retinoids and proretinoid carotenoids present in VLDL and LDL are presumably taken up along with the lipoprotein particles by their cell surface receptors, but this too has not been systematically investigated. Retinyl- and retinoly-β-glucuronides are fully water soluble and have been proposed to serves as a source of retinoids for use by tissues but it is unclear whether and how these may be taken up by cells (81, 82).

**Hepatic storage.**

It is well established that hepatocytes are responsible for the uptake of chylomicron remnant retinoid into the liver (6, 7, 116-118). Hepatocytes not only take up postprandial retinoid into the liver but they also account for about 10-20% of all of the retinoid stored within the liver and are the sole cellular site of RBP4 synthesis in the liver (6, 7, 116, 119). Moreover, hepatocytes possess enzymatic activities needed for the hydrolysis of retinyl esters and the synthesis and catabolism of retinoic acid. After postprandial retinoid is taken up by the hepatocyte, this retinoid is either secreted back into the circulation bound to RBP4 (see above for more details) or is transferred to the hepatic stellate cells (HSCs) for storage (6, 7, 119). It has been estimated that for healthy well nourished individuals approximately 70% of the retinoid present in the body will be stored in the liver and approximately 70-90% of this is found in HSCs (6, 7, 116, 117, 119). Within HSCs, retinoid is stored as retinyl esters in the large lipid droplets that are characteristic of these cells (see **Figure 4**) (6, 7, 117-119). Nearly all of the retinoid present in HSCs is retinyl ester (primarily retinyl palmitate, with smaller amounts of retinyl stearate, retinyl oleate and retinyl linoleate) (116-119). Normally,
unesterified retinol accounts for less than 1% of the total retinoid present within these cells.

When the body senses a need for retinoid, hepatic retinyl esters are hydrolyzed by REHs to free retinol, which through some poorly characterized process, is mobilized from the liver bound to its plasma transport protein, RBP4. Mice lacking RBP4 are unable to mobilize their retinoid stores, which are effectively trapped within the liver (71, 120, 121). It remains to be established how a signal is conveyed from peripheral tissues to the liver regarding their need for retinoid, thus, stimulating retinoid mobilization from hepatic stores. Retinol-RBP4 is secreted from the liver into the circulation as a means of delivering retinol to peripheral tissues (56, 71). The liver is the major site of synthesis of RBP4 in the body and within the liver the hepatocyte is the sole cellular site of RBP4 synthesis (56, 116, 119). Other tissues including adipose tissue, kidney, lung, heart, skeletal muscle, spleen, eyes and testes also express RBP4 (56) and this may be important for recycling retinoid from peripheral tissues back to the liver (56, 117).

One of the key unanswered questions regarding hepatic retinoid storage and metabolism is how retinoid(s) is (are) transported between hepatocytes and HSCs. It is well established that postprandial retinoid is take up by hepatocytes and equally well established that HSCs are the major cellular storage site for retinoid in the liver (6, 7, 116, 117, 119). This immediately raises the question as to how dietary retinoid is transferred from hepatocytes to HSCs for storage. The early literature proposed that RBP4 was responsible for this intercellular movement and it was generally accepted that RBP4 was responsible for intercellular retinoid movement within the liver (117).
However, studies of Rbp4-deficient mice indicate that this cannot be correct since these mutant mice possess normal HSC lipid droplet retinyl ester stores (71, 120, 121). This implies that other proteins/factors must be responsible for retinoid transport between these two hepatic cell types. Moreover, this transport needs to be bidirectional. Since the hepatocyte is the site of RBP4 synthesis in the liver, when dietary retinoid is insufficient, retinol newly released from HSC retinyl ester stores must be transported back to hepatocyte where it binds nascent apo-RBP4 for secretion into the circulation and delivery to peripheral tissues. A few early investigators proposed that CRBPI, which is found in high abundance in both hepatocytes and HSCs, mediates retinol transfer possibly by moving through/across cell-to-cell contacts which exist between hepatocytes and HSCs (6, 7, 88). Other early authors had suggested that some unidentified protein was involved in facilitating this process (6, 7, 88). In late 2012, Alapatt et al. reported the identification of a novel retinol transporter that is expressed primarily in mouse liver and intestine and which is able to bind RBP4 (naming this receptor RBP4 receptor-2 or RBPR2) (122). It is possible that this protein has a role in intercellular retinol transfer within the liver. Interestingly, Alapatt et al. report that RBPR2 shares considerable structural similarity with STRA6 (122). However, at present, these and other possibilities for explaining how retinol is transported between hepatocytes and HSCs remain to be established.

The lipid droplets present within HSCs are specialized for retinoid storage (6, 7, 119, 123). The lipid composition of HSC lipid droplets isolated from control fed rats is unique and consists of approximately 40% retinyl ester, 32% triglyceride, 20% cholesteryl ester and cholesterol, 6% phospholipid, and 2% unesterified fatty acids.
The relative lipid composition of these lipid droplets is dependent on dietary retinoid intake, with greater retinyl ester accumulation seen in rats fed an excess-retinol diet and diminished accumulation seen for rats fed a retinol-restricted diet (123). Changes in the triglyceride composition of the diet do not affect either the relative or absolute lipid composition of rat liver HSC lipid droplets (123). A number of lipid droplet associated proteins, members of the perilipin family, are reported to be expressed by HSCs, including perilipin 2 and perilipin 3 (119). Given the central role of HSC lipid droplets in retinoid storage and metabolism, both LRAT and REHs must be able to associate with these lipid droplets but this possibility remains to be elucidated. Interestingly, as illustrated in Figure 4, the HSCs of Lrat-deficient mice lack lipid droplets. This observation was unexpected since the majority of lipid present in the HSC lipid droplets is not retinyl ester. This suggests that the synthesis of HSC lipid droplets is regulated in some manner that takes into account either LRAT presence and/or hepatic retinoid status.

One of the most intriguing questions that can be asked regarding hepatic retinoid storage concerns why more than 50% of all retinoid present in the body is stored in HSCs (for a healthy individual, 70% of what is present in the body is in the liver and 80-90% of this is in HSCs). Thus, although HSCs account for only approximately 8% of the total cells in the liver and only 1% of hepatic protein, they account for more than half of all retinoid present in the body (88, 119, 124, 125). What evolutionary factors or processes are responsible for this? HSCs, upon activation, are known to play a major causal role in the development of hepatic disease (88, 119, 124, 125). HSCs quickly lose their lipid droplets and retinoid stores during the process of activation (119, 124,
Possibly HSC lipid droplet retinoid stores are needed to buffer against disease development, but this hypothesis remains to be established. In this regard, recently published data indicate that HSC retinoid stores do not protect against CCl₄-induced fibrosis in mice (126) but they are required to ensure optimal liver regeneration in response to a 70% partial hepatectomy (127).

**Retinoids in adipose tissue.**

The major tissue storage site for retinoid in the body is the liver. Other tissues including the eyes, lungs, adipose tissue, skin, testes and spleen have capacity to store retinoid, albeit at a lesser concentration than liver. Adipose tissue is able to accumulate significant retinyl ester stores (6, 7, 128). The concentrations of retinol/retinyl esters in adipose tissue of rats and mice maintained on a control retinoid-sufficient diet are reported to be in the range of 6-7 µg total retinol (retinol + retinyl ester)/g tissue wet weight (128). On a per gram of tissue basis this is much less than in the liver. For this same study (128), hepatic total retinol levels were reported to average approximately 150 µg/g tissue wet weight. But considering that the liver contributes only 3-4% of the total mass of the body whereas adipose tissue can contribute a much greater percentage of total body mass, it was estimated that adipose tissue may account for as much as 15-20% of the total retinoid present in the body of a healthy well nourished rat maintained on a control chow diet (128). Moreover, adipose tissue expresses RBP4, CRBPI and CRBPIII (63, 128, 129). The adipocyte is the cellular site of retinyl ester accumulation within adipose tissue and adipocytes are able to synthesize and secrete
RBP4 (128, 129). Thus, the adipocyte must be considered to be an important cell type in the body for retinoid storage and mobilization.

As discussed above, mice totally lacking Lrat have no detectable retinyl esters in nearly all tissues, with the notable exception of adipose tissue (27, 28). The adipose tissue of Lrat-deficient mice has, in fact, an elevation in retinyl esters compared to chow fed wild type mice. This is probably due to the inability of other tissues to store retinol. Moreover, this finding implies the presence of another enzyme capable of synthesizing retinyl esters within adipose tissue (28). Although DGAT1 has been shown to be capable of synthesizing retinyl esters in intestine, mice lacking both Lrat and Dgat1 expression display the same elevation of retinyl esters in adipose tissue, implying that there is another as yet unidentified enzyme active in adipocytes (28, 47). Like in the liver, retinyl esters stored in adipose tissue can be mobilized and secreted back into the circulation bound to RBP4 synthesized in adipocytes (55, 128-130). These retinyl esters are first hydrolyzed by hormone-sensitive lipase (HSL) which acts as a physiologically relevant REH in adipocytes (130, 131). Mice lacking HSL expression are unable to hydrolyze adipocyte retinyl esters and this results in abnormal retinoid signaling leading to aberrant adipocyte differentiation (131).

One of the most exciting recent findings from research focused on adipose retinoid physiology has been the observation by Kahn and colleagues that RBP4 synthesized by adipocytes and secreted into the circulation may have a role in modulating tissue responsiveness to insulin (103-105). Since the first report by Kahn and colleagues of this relationship in 2005, there have been reports, involving studies in humans, animals and cell culture models, from many independent research
laboratories confirming and extending this work which links adipocyte RBP4, obesity, and impaired peripheral tissue insulin responsiveness (103-107, 109, 132). However, not all investigators have been able to confirm this linkage and this remains an active and somewhat controversial research area (108, 109, 133).

Our goal is not to summarize in a detailed manner the extensive literature concerning adipose-derived RBP4 and insulin responsiveness but rather simply to familiarize the reader with some of this literature, which in our view ultimately is biochemically linked to adipose retinoid storage. Since 2005, the literature has suggested linkages between serum RBP4 concentrations, obesity, impaired insulin responsiveness (103-107, 132), fatty liver development (134-140), vessel wall disease (141, 142), and heart disease (143). At the molecular level, the best studied of these effects is the action of excessive RBP4 acting as a cytokine activating a signaling cascade within cells, which can eventually lead to both direct and indirect alterations in insulin signaling. Kahn and colleagues have proposed that apo-RBP4 induces the expression of proinflammatory cytokines in mouse and human macrophages and thereby indirectly inhibits insulin signaling in co-cultured adipocyte macrophages via the activation of c-Jun N-terminal protein kinase (JNK) and Toll-like receptor 4 (TLR4) pathways (144). Noy and colleagues have reported data showing that high apo-RBP4 concentrations can cause apoptosis of cultured fibroblasts as well as of cultured kidney cells, induced via activation of the JAK/STAT pathway (110, 145). These investigators also showed that an interaction between RBP4 and the cell surface transporter of retinol, STRA6, plays a role in insulin signaling within muscle and fat cells. When STRA6 binds holo-RBP4, a JAK/STAT signaling cascade is triggered resulting in
impaired insulin signaling (110, 145). Moreover, another recent study by Noy and colleagues established that TTR can inhibit RBP4 signaling this STRA6-dependent pathway (146). Based on all of the evidence to date, it appears that these studies are opening up a new field of retinoid signaling that is mechanistically distinct from the well understood canonical effects that retinoids have in transcriptional regulation. Thus, the horizons and impact of retinoid metabolism continue to expand, encompassing whole body macronutrient and energy metabolism. Research in this area promises to reveal exciting new findings in coming years.
ACKNOWLEDGEMENTS

The work from the authors' laboratory that has been discussed in this review was supported by grants from the National Institutes of Health (RC2 AA019413, R01 DK68437, R01 DK079221 and R21 AA020561).
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FIGURE LEGENDS

Figure 1. Chemical structures of different retinoid chemical species. The chemical structure of the major proretinoid carotenoid, β-carotene, is shown at the top. The chemical structures for all-trans-retinol (which by definition is vitamin A), an all-trans-retinyl ester, 11-cis-retinal (the active retinoid in vision) and the all-trans-, 9-cis- and 13-cis-isomers of retinoic acid are shown.

Figure 2. The metabolism of β-carotene and retinoids. All retinoid is originally derived from proretinoid carotenoids such as β-carotene (1). Retinal (2) can be formed by the central cleavage of β-carotene by the enzyme β-carotene-15,15- monooxygenase 1 (BCMO1). Retinol (3) is formed by the reversible reduction of retinal (2) by one of the retinal reductase family members. The enzyme lecithin:retinol acyltransferase (LRAT) synthesizes retinyl esters (4) by transferring a fatty acyl moiety from the sn-1 position of membrane phosphatidyl choline to retinol. Unesterified retinol is liberated from retinyl ester stores through the action of a retinol ester hydrolase (REH). Retinol is oxidized to retinal (by one of several retinol dehydrogenases (RDH)), which is then irreversibly oxidized (by one of three retinal dehydrogenases (RALDHs)) to form transcriptionally active retinoic acid (5). Retinoic acid is oxidized/catabolized to a more water soluble hydroxy- and oxo-forms by one of several cytochrome P450 enzyme family members.

Figure 3. The delivery of retinoids and carotenoids through the circulation to cells. Retinoids and proretinoid carotenoids are delivered to cells and tissues through a
number of alternative delivery pathways. In the fasting circulation retinol delivered via RBP4 (1) accounts for most delivery to cells/tissues. However, in the postprandial state retinyl esters present in chylomicrons and their remnants (2) can contribute substantially to the retinoid taken up by cells. Similarly, proretinoid carotenoids like β-carotene are present in the postprandial circulation(4) and this can be taken up by cells/tissues and converted to retinoid. Retinyl esters and carotenoids (3) are also present in VLDL, LDL, and HDL in the fasting circulation. Retinoic acid is present in both the fasting and postprandial circulation (7), albeit at relatively low levels compared to retinol and retinyl esters. The water soluble retinyl- (5) and retinoyl-β-glucuronides (6) are also present at relatively low levels in the circulation.

Figure 4. The genetic ablation of Lrat results in the total absence of lipid droplets in hepatic stellate cells (HSC). Electron micrographs of liver sections obtained from age-, gender-, and genetic background matched wild type (left panel) and Lrat-deficient (right panel) mice maintained throughout life on a retinoid-sufficient chow diet. The large lipid droplets, indicated by the arrow in the left panel, are a characteristic morphological feature of HSCs, which are clearly present in the wild type liver, whereas but totally absent in liver from Lrat-deficient mice. Abbreviations: H, hepatocyte; and HSC, hepatic stellate cell.
Figure 1

**Chemical Structures of Vitamin A metabolites**

- **all-trans-β-Carotene**
- **all-trans-Retinol**
- **Retinyl Ester (R=Acyl Chain)**
- **11-cis-Retinal**
- **all-trans-Retinoic Acid**
- **9-cis-Retinoic Acid**
- **13-cis-Retinoic Acid**
Figure 2

**Metabolic Conversion of Retinoids**

1. **all-trans-β-Carotene**
   - β-Carotene 15, 15' monooxygenase 1 (BCMO1)

2. **all-trans-Retinal**
   - Retinal reductase (RALR)
   - Retinol dehydrogenase (RDH)

3. **all-trans-Retinol**
   - Lecithin: Retinol acyltransferase (LRAT)
   - Retinyl Ester hydrolase (REH)

4. **Retinyl Ester**
   - Storage Form (R = Acyl Chain)

5. **all-trans-Retinoic Acid**
   - Cytochrome P450s (CYP enzymes)
   - Retinoic acid metabolites

Retinal dehydrogenase (RALDH)
Figure 3

1. Retinol-RBP-TTR
2. Retinyl Esters in Chylomicrons or Their Remnants
3. Retinyl Esters in Lipoproteins
4. Carotenoid in Lipoproteins and Chylomicrons
5. Retinyl-β-glucuronides
6. Retinoyl-β-glucuronides
7. Retinoic Acid Bound to Albumin

Retinol $\rightarrow$ Retinal $\rightarrow$ Retinoic Acid

Retinol $\rightarrow$ Retinyl Esters
Retinyl Esters $\rightarrow$ Carotenoid

Transcriptional Activity

BOUND TO

REH
LRAT
CRBPI
CRABPI
CRABPII