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ALL-*TRANS*-RETINOIC ACID REGULATES VITAMIN A HOMEOSTASIS

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ABSTRACT

All-*trans*-retinoic acid (RA) is the active metabolite of vitamin A (VA) that mediates the majority of VA-dependent functions, such as embryonic development, cellular proliferation and differentiation, testicular function, and immune function. RA and its isomers and metabolites have been extensively studied for their ability to treat or prevent numerous disease conditions. For instance, isomers of RA have been used pharmacologically to treat diseases of the skin and to treat certain cancers. Because of the clinical impact of RA on different disease conditions, it is important to delineate the affects of acute and chronic RA administration on both whole-body and tissue specific retinol and RA utilization and kinetics. Therefore, the overall research hypothesis in the present study is that RA regulates retinol and RA homeostasis in rats. This hypothesis was addressed in four separate studies

First, the effects of chronic dietary RA administration on retinol utilization and kinetics were determined in rats with low VA stores using compartmental analysis. The results showed that RA supplementation lowered the disposal rate and system fractional catabolic rate of VA, resulting in a positive VA balance. Moreover, the model-predicted traced mass and residence times of the liver, lung, small intestine, and kidney were higher in the LA + RA rats. In contrast, there was no major difference in VA kinetics in the eyes, testes, adrenal glands, or remaining carcass between the groups. Thus, we conclude that chronic administration of RA to rats with low VA status affects the recycling, uptake, and mass of VA at both the whole-body level and in specific organs.

Second, the effect of VA-status on the distribution and metabolism of a dose of [^{3}H]RA was examined in VA-deficient (VAD), VA-marginal (VAM), VA-sufficient

(VAS), and VA-repleted (VAD + retinyl palmitate) rats. The results of our study demonstrate that VA status affects both the organ distribution and metabolism of RA in rats. Specifically, VA-deficiency increased the total amount of radioactivity recovered from the liver, lung, and small intestine, while significantly reducing the amount of [^{3}H]-metabolites of RA recovered in liver, lung, and small intestine. In contrast, the ratio of metabolized to unmetabolized RA was highest in the VAS rats. Additionally, the administration of retinyl palmitate to VAD rats, in a 20-h period resulted in increased hepatic stores of retinol, which allowed them to metabolize the [^{3}H]RA in a manner similar to the VAS rats. Overall, our results indicate that the uptake, recycling, and utilization of RA are altered in a tissue-specific manner in response to decreasing stores of vitamin A.

Third, the effects of exogenous RA on hepatic CYP26A1 expression was examined in naïve and RA-pretreated (RA-primed) rats. Our results showed that the acute administration of an intravenous dose (10 μg / 100 g body weight) significantly increased CYP26A1 mRNA expression in a time-dependent manner. In addition, the mRNA expression of CYP26A1 was higher in the RA-primed rats than in the naïve rats. Similarly, hepatic CYP26A1 protein levels were significantly increased within 30 minutes in both the naïve and RA-primed rats. However, CYP26A1 protein levels returned to (RA-primed) or near (naïve) levels within 90 minutes. Additionally, CYP26A1 mRNA and CYP26A1-related protein levels were directly correlated in both the naïve and RA-primed rats between 0 and 60 minutes. Finally, the average formation of RA-metabolites was greater in the RA-primed rats than in the naïve rats and the amount of metabolites of RA generated by rat liver microsomes increased over time.

In conclusion, the results of our study demonstrate that CYP26A1 mRNA expression, CYP26A1-related protein expression, and hepatic RA-metabolizing activity all rapidly increased in response to i.v. RA in both naïve and RA-primed rats.

Fourth, we investigated the effects of RA and lipopolysaccharide (LPS), a bacterial cell-wall component capable of stimulating an inflammatory response in rats, alone and in combination on the distribution and metabolism of a dose of [^{3}H]RA in VA-marginal rats. The results showed that LPS significantly reduced the total amount of ^{3}H recovered in the liver 10 and 30 minutes post-injection. Whereas LPS did not affect the total amount of [^{3}H]-metabolites present in the liver, lung, or small intestine, RA pretreatment significantly increased the amount of [^{3}H]-metabolites in each tissue at both 10 and 30 minutes. Moreover, this induction in RA metabolism occurred irrespective of LPS administration. Thus, the results of this study demonstrate that acute inflammation alters systemic RA homeostasis by disrupting the normal distribution of RA, while the conversion of [^{3}H]RA to [^{3}H]-labeled metabolites was significantly increased by RA alone indicating that LPS and RA independently affect RA metabolism in VA-marginal rats.

Overall, the results presented here suggest that VA (retinol and retinoic acid) homeostasis is maintained through an autoregulatory process, with RA acting as the feedback messenger. In addition, we propose that tissue and plasma RA levels are homeostatically maintained within a narrow range despite nutritional and physiological stress, which allows the body to respond to minor alterations in whole-body VA status.

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LIST OF ABBREVIATIONS

ANOVA:	Analysis of variance
ARAT:	Acyl-coenzyme A:retinol acyltransferase
BW:	Body weight
CRABP:	Cellular retinoic acid-binding protein
CRBP:	Cellular retinol-binding protein
CYP:	Cytochrome P450
FABP:	Fatty acid-binding protein
FATP:	Fatty acid-transport protein
HPLC:	High performance liquid chromatography
4-HPR	N-(4-hydroxyphenyl)retinamide
IRAK:	Interlukin-1 receptor activated kinase
LBP:	Lipopolysaccharide-binding protein
LPS:	Lipopolysaccharide
LRAT:	Lecithin:retinol acyltransferase
NF-kB:	Nuclear factor kappa beta
PBS:	Phosphate-buffered saline
RA:	all-*trans*-Retinoic acid
RAR:	Retinoic acid receptor
RARE:	Retinoic acid response element
RBP:	Plasma retinol binding protein

RP:	Retinyl palmitate
RT-PCR:	Real-time polymerase chain reaction
RXR:	Retinoid X receptor
SEM:	Standard error of the mean
SPE:	Solid phase extraction
TLR:	Toll-like receptor
TTR:	Transthyretin
VA:	Vitamin A
VAD:	Vitamin A-deficient
VAD + RP:	Vitamin A-deficient + retinyl palmitate
VAM:	Vitamin A-marginal
VAS:	Vitamin A-sufficient
WinSAAM:	Windows version of the simulation, analysis, and modeling computer program

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INTRODUCTION AND HYPOTHESES

Retinoids, including all-*trans*-retinoic acid (RA), have been extensively studied for their ability to treat or prevent numerous disease conditions. For instance, isomers of RA have been used pharmacologically to treat diseases of the skin, such as acne and hyperkeratotic disorders (1). Similarly, results obtained from epidemiological and experimental studies show that retinoids can both reduce the risk for and treat certain cancers (2). Specifically, RA has been clinically used to treat a specific form of leukemia, acute promyelocytic leukemia, with a high rate of complete remission (3,4). Furthermore, recent work has shown that RA could possess adjuvant properties, suggesting that it could be administered in conjunction with vaccines to prevent or reduce the severity of different viral infections (5-7). Because of the clinical impact of RA on different disease conditions, it is important to delineate the affects of acute and chronic RA administration on both whole-body and tissue specific vitamin A (VA; retinol) and RA utilization and kinetics. Our long-term goal is describe the underlying mechanisms that regulate retinol and RA metabolism *in vivo* so that retinoids can be effectively utilized to treat or prevent different disease conditions without serious consequences. Therefore, through the use of rodent models, we hope to elucidate the effects of RA treatment on aspects of VA kinetics and RA metabolism. Our overall research hypothesis is that **RA levels regulate VA (retinol and RA) homeostasis in rats**. Specifically, we hypothesize that: **1) RA administration will increase the recycling, uptake, and mass of VA in specific organs and the whole body of rats with low VA stores**; and **2) the distribution and catabolism of RA will be affected by the VA status of the rat, RA pretreatment, and inflammation in specific organs.**

The above hypotheses were addressed in four separate studies. **First**, before the discovery of the molecular mechanisms of RA action, it was shown that dietary RA could substitute for VA in most physiological functions (8-10). Subsequently, it was shown that feeding RA to rats with low VA status influenced increased liver VA stores and spared whole-body VA levels (11). More recently, work performed in this laboratory demonstrated that the key enzyme involved in the hepatic storage of VA in rats is regulated by RA independent of the VA status of the rat (12,13). Taken together, these studies suggest that RA administration will alter the kinetics of VA in rats. Using compartmental analysis, we showed that RA supplementation of rats with low VA status affected VA metabolism at both the whole-body level and in specific organs. **Second**, VA utilization is affected by the VA status of the animal. For instance, kinetic parameters associated with VA uptake and utilization were significantly different in the organs and plasma of rats with low, marginal, or high liver VA stores (14). In view of this observation, we were interested in determining how VA status affects RA distribution and utilization in rats. Our results show that vitamin A status affects both the organ distribution and metabolism of RA in rats, indicating that the uptake, recycling, and utilization of RA are altered in a tissue-specific manner in response to decreasing stores of vitamin A. **Third**, exogenously administered RA is able to induce its own metabolism *in vivo* resulting in decreased plasma RA levels (15). Studies performed in this lab have established that CYP26A1, a RA-inducible enzyme capable of oxidizing RA, is induced by RA in a time and dose dependent manner (16,17). To expand upon these findings, we determined the rapidity of the response of hepatic CYP26A1 to an intravenous dose of RA. Our results demonstrate that exogenous RA can increase hepatic CYP26A1 mRNA

and protein expression, resulting in an increase in amount of RA metabolites generated by the liver in both naïve and RA-primed rats. These results suggest that CYP26A1 can respond to exogenous RA rapidly in order to regulate RA concentrations in vivo. **Fourth**, previous studies have established that the detoxification capacity of the liver for endogenous and exogenous compounds is diminished during inflammation (18,19). Specifically, lipopolysaccharide (LPS)-induced inflammation has been show to reduce both the expression and activity of various members of the cytochrome P450 family (20). Work performed in our lab has demonstrated that CYP26A1 expression is significantly decreased following LPS administration in THP-1 cells (21). Additionally, we have shown that hyporetinemia accompanies infection-induced inflammation in humans and rats, which could alter retinoid utilization since plasma retinol levels are the major determinant of VA utilization in rats (22-25). Therefore, we designed a 2 x 2 factorial study to examine the effects of RA and LPS, both alone and in combination, on RA distribution and metabolism in rats. The results of this study show that RA and LPS independently affect the distribution and metabolism of RA in a tissue-specific manner.

Overall, our results presented here show that VA status affects the distribution and metabolism of both retinol and RA in rats. Moreover, we show that exogenous RA can rapidly up-regulate hepatic CYP26A1 directly resulting in the rapid metabolism of RA in vivo. Finally, we show that LPS-induced inflammation alters the organ distribution of RA, but it did not affect the metabolism of RA in rats. Taken together, this work illustrates the dynamic interaction that exists between hepatic VA stores and exogenous RA in terms of regulating retinol and retinoic acid kinetics in vivo.

CHAPTER 1:

LITERATURE REVIEW

INTRODUCTION

Since the discovery of VA nearly a century ago, our understanding of its physiological functions has expanded exponentially. Early studies demonstrated that dietary VA was essential for the maintenance of epithelial tissue and that individuals with VA deficiency displayed an increase in the severity and frequency of infections (26,27). Further studies established that VA is part of the visual pigment, which is required for normal vision (28-30). Later, it was shown that the VA-metabolite RA could not support vision and reproduction, suggesting that there was more than one active physiological form of the vitamin (9,10). Sommer et al. (31) demonstrated that children displaying signs of VA deficiency had a 3-6 fold greater mortality rate than those who had adequate amounts of VA. More recently, a nuclear receptor capable of binding RA and acting as a transcriptional regulator was discovered, thus identifying the molecular mechanism necessary for VA to affect development, proliferation, differentiation, immunity, and more.

VITAMIN A METABOLISM

Digestion and Absorption

Humans obtain VA (retinol) from two distinct dietary sources: preformed VA from animals and pro-vitamin A carotenoids from plants. Because of the lipophilic nature of retinol, retinyl esters, and carotenoids, normal fat digestion and absorption is required for proper absorption of the vitamin. Intestinal retinol, derived from the hydrolysis of retinyl esters or synthesized from β-carotene, is packaged into

chylomicrons along with triglycerides, cholesterol, phospholipids, and other fat-soluble vitamins (32-35). Prior to packaging into chylomicrons and secretion into the lymphatics, the majority of newly absorbed retinol is re-esterified with long-chain fatty acids, such as palmitate, stearate, and oleate (32,36). Hydrolysis of 70-80% of chylomicron triglycerides (37) by lipoprotein lipase on the surface of extrahepatic tissues results in the formation of chylomicron remnants. Although some remnants may be removed by extrahepatic tissues, most of the remnants, which still contain virtually all of the dietary retinyl esters, are taken up by liver hepatocytes via the apo E receptor. These retinyl esters are rapidly hydrolyzed to unesterified retinol which can be *1)* transferred to stellate cells for re-esterification and storage, *2)* complexed with retinol-binding protein (RBP) for whole-body distribution, or *3)* converted into the active metabolite RA. Each of these pathways will be discussed in further detail below.

Cellular Retinoid-Binding Proteins

Because of retinol's hydrophobic nature and susceptibility to oxidation, specific carrier proteins for vitamin A exist in cells. To date, four retinoid-binding proteins that are not involved in the visual cycle have been identified. Each is a member of a superfamily of carrier proteins that transport hydrophobic compounds. These proteins are important to overall retinoid homeostasis because they impose enzymatic specificity for different reactions, influence the direction of flow of a retinoid through various pathways, and protect retinoids from non-enzymatic reactions (38).

Unesterified retinol is sequestered by cellular retinol-binding proteins (CRBPs) that were first described by Bashor et al. (39) by using sucrose gradient

ultracentrifugation. To date, two CRBPs, CRBP-I and CRBP-II, have been identified. CRBP-I and II share considerable structural and biochemical properties, including the ability to bind all-*trans*-retinol, 13-*cis*-retinol, and 3,4,-didehydroretinol with high affinity (40). Despite being nearly identical in size and structure, in terms of amino acid sequence, CRBP-I and CRBP-II are only 56% identical (38). Because of their structural homology and their binding specificities, CRBP-I and II possess a central role in retinol trafficking and homeostasis. For instance, CRBPs bind unesterified retinol in order to keep its concentration low in cells, the binding proteins shuttle retinol within the cell (e.g., from the basolateral to apical side), and they present retinol to and interact with different enzymes involved in retinol metabolism (38). However, despite their considerable similarities, several differences between the two proteins exist. CRBP-I is a 14.5 kD polypeptide that is widely distributed with expression in the liver, kidney, lung, and testes (41,42). In contrast, CRBP-II is a 16 kD polypeptide that is expressed mainly in the absorptive cells of the small intestine (43). In fact, Ong and Page (43) showed that CRBP-II accounts for about 1% of the total soluble protein recovered from human jejunal mucosal extracts. The importance of CRBP-II in the absorption of VA is further highlighted by its response to dietary VA manipulation. Rajan et al. (44) showed that CRBP-II mRNA levels significantly increased in the small intestine of VA-deficient rats.

Cellular retinoic acid-binding proteins (CRABPs) are a second class of intracellular retinoid-binding proteins; they are able to bind retinoic acid with high affinity (45). Similar to the CRBPs, there are two distinct retinoic acid-binding proteins: CRABP-I and CRABP-II. Sani and Hill (46) were the first to observe CRABP and its subsequent purification showed that it is highly conserved between species (47,48). The

two CRABPs are comprised of a single polypeptide chain and they share 78% amino acid identity. They are differentially expressed in a wide range of cells and both are expressed in different developmental stages. For example, CRABP-I is expressed almost ubiquitously while CRABP-II is only expressed in skin, uterus, ovary, and the choroid plexus (49). At present, the exact function of CRAPBs *in vivo* is not fully understood, but several studies have investigated their potential role in retinoid homeostasis. Boylan and Gudas (50) reported that the rate of retinoic acid degradation was markedly increased when the expression of CRABP-I was enhanced. Dong et al. (51) showed that CRABP-II directly interacts with and facilitates the delivery of retinoic acid to its nuclear receptor (see below). Hence, it is currently hypothesized that CRABPs facilitate the catabolism of retinoic acid, sequester retinoic acid, and assist in the formation of the retinoic acid-retinoic acid receptor complex in the nucleus.

Retinol Esterification and Storage

In tissues, two retinol esterification reactions have been identified. The first is an acyl-CoA-dependent reaction that was first identified in microsomes from lactating rat mammary tissue. This reaction is catalyzed by the enzyme acyl-coenzyme A:retinol acyltransferase (ARAT). The second is an acyl-CoA-independent reaction that is catalyzed by the enzyme lecithin:retinol acyltransferase (LRAT).

ARAT is a microsomal protein that has been shown to esterify retinol in mammary tissue (52), intestine (53), and liver (54). It is similar to other microsomal acyl-CoA transferases, including acyl-CoA:cholesterol acyltransferase. Ross (54) showed that retinyl esters were consistently generated following retinol incubation with

liver microsomes. Furthermore, the rate of esterification was increased by the addition of exogenous fatty acids or by a fatty acyl CoA generating system (54). Helgerud et al. (53) showed that the microsomal fraction of intestinal cells could also generate retinyl esters in an acyl-CoA-dependent manner and that the maximal rate of retinol esterification was in the jejunum. Rasmussen et al. (55) showed that intestinal ARAT is physiologically important to VA homeostasis. These authors showed that large dietary doses of VA significantly increased ARAT levels in intestinal cells (55). Thus, it is currently believed that ARAT activity is involved in esterifying retinol when high concentrations are present in cells, such as following supplementation with the preformed vitamin.

LRAT is a 25 kD microsomal enzyme that is capable of esterifying retinol bound to either cellular retinol binding protein (CRBP) I or II. Ong et al. (56) observed that the reaction catalyzed by LRAT was acyl-CoA-independent, utilizing an endogenous acyl donor. MacDonald and Ong (57) showed that the endogenous acyl donor for the reaction is the sn-1-acyl moiety of phosphatidylcholine (lecithin). This mechanism of action of LRAT is similar to that of lecithin:cholesterol acyltransferase, which esterifies cholesterol by transferring the 2-acyl moiety of lecithin to cholesterol. Kinetic analyses determined that the K_m for retinol esterification with endogenous acyl donors when retinol is bound to CRBP-I in liver or CRBP-II in intestine is 4.0×10^{-6} M and 2.4×10^{-7} M, respectively (43,56,58). Thus, CRBP-I and II enhance the affinity of retinol for LRAT *in vivo*.

LRAT contributes to retinol homeostasis by responding to the retinoid status of the animal or individual. Randolph and Ross (59) showed LRAT activity varies directly with the VA status of the animal. They observed that hepatic LRAT activity was

undetectable in VA-deficient rats, but repletion with retinol restored the activity within 24 hours. Dawson et al. (60) expanded upon the above findings by showing that rat hepatic LRAT activity is regulated across a physiological range of dietary VA and it is regulated throughout life. Subsequent studies expanded upon the above findings by showing that LRAT mRNA expression and enzymatic activity in liver and lung were modulated by exogenous retinoic acid. Matsuura and Ross (12) showed that LRAT activity was significantly increased in VA-deficient rats following a single dose of RA. Zolfaghari et al. (61) later showed that the increase in LRAT mRNA expression and activity in response to retinoic acid was time- and dose dependent (13,62). Finally, Shimada et al. (63) demonstrated that different natural (retinol, 9-*cis*-retinoic acid, and all-*trans*-retinoic acid) and synthetic retinoids (RAR pan-agonist and RARα-selective agonist) increased LRAT activity in VA-deficient rats. Taken together, the studies conducted on LRAT have shown that it is nutritionally regulated; its expression and activity increase or decrease in response to VA supplementation or deficiency, respectively. Thus, because it is VA sensitive, it helps regulate the balance between retinol storage and retinol oxidation throughout the vitamin A continuum.

Fifty to 85% of total body retinol is stored in the liver when VA levels are sufficient; the majority of this VA is present as retinyl esters (64). Under normal conditions, it is estimated that 80-90% of stored liver VA is found in stellate cells (65). Stellate cells (also known as Ito cells, interstitial cells, or pericytes) are one of four main types of nonparenchymal liver cells (65). They store retinol in the form of retinyl esters in cytoplasmic lipid droplets. In fact, Moriwaki et al. (66) found that between 12 and

65% of total lipid mass in stellate cell lipid droplets was comprised of retinyl esters and that the amount of esters present was dependent on the VA status of the animal.

Retinol Mobilization and Retinol-Binding Protein

Even though retinyl esters are found in a variety of tissues, liver stores represent the major endogenous source of VA for peripheral tissues (67). Retinol mobilization begins with the hydrolysis of stellate cell retinyl esters by retinyl ester hydrolases. Two different hydrolases have been described, a bile salt-dependent (68) and a bile salt-independent retinyl ester hydrolase (69,70). The bile salt-dependent hydrolase is a microsomal enzyme that requires specific bile salts for maximal activity (68,71) and it is functionally and structurally similar to rat pancreatic cholesterol ester hydrolase (69). Despite significant individual variation in activity levels (72), bile salt-dependent retinyl ester hydrolases are present in both hepatic parenchymal and stellate cells, suggesting a role in the hydrolysis of retinyl esters stored in the liver (73,74). The second type of retinyl ester hydrolase is the bile salt-independent hydrolase, which can function in the absence of bile salts. Unlike the bile salt-dependent type, the bile salt-independent retinyl ester hydrolase does not display marked variation among individual rats and is localized to mainly to the plasma membrane. Thus, it has been hypothesized that the bile salt-independent retinyl ester hydrolase functions in the breakdown of dietary retinyl esters delivered to the liver by chylomicron remnants. Both retinyl ester hydrolases appear capable of responding to low intracellular retinol levels *in vivo* (75), leading to an increased mobilization of liver stores to help maintain retinoid homeostasis in both the liver and extrahepatic tissues.

Because of retinol's hydrophobic nature and susceptibility to oxidation, a specific carrier protein for retinol exists in plasma. Plasma retinol-binding protein (RBP) was first isolated by Kanai and colleagues (76). Human RBP is a single polypeptide chain comprised of 182 amino acids with three disulfide bonds and a molecular weight of about 21 kD, while rat RBP contains 182 amino acids and has an 86% sequence homology with human RBP (77-79). Studies by various labs have shown that mature RBP contains no added lipids or carbohydrates and it has only one retinol-binding site (76,77).

RBP mRNA and protein are synthesized in both the liver and many extrahepatic tissues, including the intestine, kidney, and adipose tissue. However, because the liver is the main vitamin A storage site, its ability to produce and secrete adequate amounts of RBP is vital to whole-body retinol homeostasis, since plasma vitamin A concentration and RBP levels are highly correlated (80,81). Liver parenchymal cells contain nearly 98% of total liver RBP, and they are able to synthesize and secrete large amounts of RBP. In blood, RBP circulates in a 1:1 molar ratio with a larger protein, transthyretin (TTR). The generation of the RBP-TTR complex has two important functions: *1)* to reduce the glomerular filtration of RBP and *2)* to help stabilize the interaction of retinol with RBP (77,82,83). In humans, the normal serum levels of RBP range from 2-3 μM, remaining constant except during disease or vitamin A deficiency or toxicity (84,85).

The physiological transport of retinol is a kinetic process that involves extensive recycling. One of the first experiments to provide direct evidence for retinol recycling was performed by Lewis et al. (86). Through the use of an intravenous injection of [^{3}H]retinol bound to the RBP/TTR complex, the authors were able to demonstrate that retinol recycled to both the plasma and the liver. Subsequently, Green et al. (87)

analyzed data from a long-term kinetic study in rats with marginally adequate liver VA reserves using model-based compartmental analysis in order to provide a quantitative estimate of plasma retinol recycling. They showed that plasma retinol recycled 12 times before it was irreversibly utilized, with a system residence time of 21 days. Furthermore, the authors determined that the turnover rate of retinol was 13 times greater than its disposal rate suggesting that an average plasma retinol molecule recycles multiple times prior to irreversible loss (87).

Similar studies were carried out in VA-deficient and sufficient rats in to determine how VA status affects retinol kinetics and dynamics. By comparing the long-term plasma disappearance curves for rats of differing VA status, it was apparent that certain aspects of retinol kinetics were similar between the groups whereas others were different. For instance, VA status does not appear to affect the transit time, recycling number, or plasma factional catabolic rate of retinol in rats (87-89). In contrast, VA status directly affected both retinol residence time and disposal rate, with both parameters increasing in response to increased VA intake and status. Moreover, it was observed that the rats with low VA reserves utilize a larger fraction of their VA per day as compared to rats with greater stores, indicating a greater system fractional catabolic rate. Thus, these studies illustrate some of the complex physiological mechanisms involved in maintenance of retinol homeostasis across a broad range of VA intake.

In addition to VA status, numerous endogenous and exogenous compounds have been shown to alter plasma retinol kinetics. For example, protein and calorie malnutrition, different hormonal states, renal disease, and the availability of RBP all alter plasma retinol homeostasis (81,90,91). Furthermore, several retinoids, including RA and

N-(4-hydroxyphenyl)retinamide (4-HPR), have been shown to influence VA metabolism *in vivo*. Similarly, Underwood et al. (92) showed that the administration of RA to a VA-deficient diet caused a rapid and consistent reduction in the plasma retinol pool size in young rats. Likewise, treatment with 4-HPR, a chemopreventive agent that has been used pharmacologically (93), causes a rapid and drastic reduction in plasma retinol levels, an increase in hepatic VA levels, and a decrease in VA utilization (94,95). Thus, it has been hypothesized that dietary retinoids, such as RA, can "spare" retinol by utilizing the exogenous retinoids for the physiological processes that require it, which could result in alterations to VA kinetics.

Retinoic Acid

RA is an active metabolite of vitamin A that regulates numerous physiological processes, including proliferation, differentiation, immune response, and embryonic development (96,97). The importance of RA in mediating the functions of vitamin A was illustrated by Lamb et al. (8) who showed that dietary RA could substitute for vitamin A in most physiological functions. Further studies expanded upon this finding by showing that RA influenced liver VA stores and spared whole-body levels in rats with low VA status (11).

The majority of RA present in the body is obtained from the cellular conversion of retinol to RA. Alternatively, Smith et al. (98) demonstrated that rats efficiently absorb RA from the intestine, and nearly two-thirds of the newly digested RA is distributed throughout the body. Overall, however, the diet contributes little, if any, RA to total-

body RA pools is minimal. That is, the endogenous conversion of retinol to RA represents the primary mechanism for generating adequate amounts of retinoic acid.

The enzymatic conversion of retinol to RA requires two separate reactions. First, the alcohol form of VA, retinol, is converted to its aldehyde form, retinal, in a reversible reaction. Zachman and Olson (99) showed that the nonspecific liver enzyme, alcohol dehydrogenase, could catalyze the conversion of retinol to retinal. However, inhibitors of alcohol metabolism do not inhibit the production of RA in kidney cells, suggesting that other retinoid-specific metabolizing enzymes exist (100). Since the initial discovery by Zachman and Olson, three retinol dehydrogenases have been identified. Each enzyme belongs to the multifunctional short-chain dehydrogenase/reductase family, and they are all capable of metabolizing both free and CRBP-bound retinol (101-103). Following the dehydrogenation of retinol to retinal, the retinal is enzymatically converted to RA. That reaction is catalyzed by cytosolic retinal dehydrogenases that are members of the aldehyde dehydrogenase family (104). Four retinal dehydrogenase isozymes have been characterized, and each is able to synthesize RA from free or CRBP-bound retinal (101,105,106). Most cells and tissues appear to possess the binding proteins and enzymes necessary for RA synthesis, enabling them to be dependent only on circulating retinol levels to meet their RA needs (107).

Under normal physiological and dietary conditions, RA is present in the circulation at very low levels. The fasting plasma level of RA is 4-14 nmol/L in humans, which is approximately 0.2-0.7% of plasma retinol levels, and 7.3-9 nmol/L in rats (100,108,109). In plasma, RA circulates bound to albumin (98). Kurlandsky et al. (110) studied the contribution of plasma RA to tissue pools of the retinoid in rats. They

showed that RA uptake and accumulation from plasma were tissue- and cell type-specific, with most of the tissues examined taking up significant amounts of the retinoid. For instance, the brain and liver derived more than 75% of their RA from plasma, whereas the testes derived less than 1% from the circulation. Therefore, the authors concluded that plasma delivery of RA to tissues appears to be an important process that is necessary for maintaining tissue RA pools.

Retinoic Acid and Retinoid X Receptors

Retinoic acid is essential for the control of normal cell differentiation and proliferation, for the regulation of embryogenesis and limb formation, and for the proper functioning of cells and tissues. The ability of retinoic acid to modulate diverse physiological functions occurs because retinoic acid is a ligand for specific nuclear receptors that control gene transcription. In 1987, Giguere et al. (111) and Petkovitch et al. (112) independently isolated a human orphan nuclear receptor that was activated by physiological levels of all-*trans*-retinoic acid. Retinoic acid receptor alpha (RAR-α) contains both a DNA-binding domain and a ligand-binding domain that is structurally and functionally similar to members of the nuclear-receptor superfamily (112,113). Subsequently, two additional RARs were isolated and characterized, RAR-β (114) and RAR-γ (115). The ligand-binding domains of the three forms of RAR are highly conserved with greater than 75% amino acid identity among them (116).

A second class of retinoid receptor, named the retinoid X receptor (RXR), was discovered by Mangelsdorf et al. (117) and found to contain a dissimilar sequence than the RARs. Two additional RXRs were isolated (118) and it was later shown that an

isomer of all-*trans*-retinoic acid, 9-*cis*-retinoic acid, was the high-affinity ligand for RXR (119,120). Both the RAR and RXR isoforms are differentially expressed during development and in the mature organism in a cell- and tissue-specific manner. Within the nucleus, RAR forms heterodimers with RXR (121), whereas RXR can form a homodimer with a second RXR or participate as an active or silent heterodimeric partner with RAR (121), the thyroid receptor (122), the vitamin D receptor (123,124), the liver X receptor (125), and the peroxisome proliferator activator receptor (126). Thus, because of the expression patterns of RAR-α,-β, and -γ and RXR-α,-β, and -γ, along with the promiscuity of RXR, retinoids are capable of controlling a wide range of physiological processes.

It is now known that ligation of RAR and/or RXR triggers a cascade of dynamic events ultimately resulting in gene transcription. In the absence of ligand, retinoid receptors are found primarily in the nucleus bound to specific DNA sequences termed retinoic acid response elements (RAREs) (116,127). RAREs, which are typically composed of direct repeats (116), have been identified in the promoter region of a larger number of genes, encompassing a wide variety of functions. Unliganded retinoid receptors repress transcription by associating with the various corepressors (128), such as NcoR (129) and SMRT (130). Upon ligand binding, the retinoid receptors recruit coactivators and chromatin remodelers to the nuclear receptor complex, causing the DNA to open and allowing transcription to begin (128). In conjunction, various kinases catalyze the phosphorylation of retinoid receptors, which helps stabilize both the interaction between the receptors and the coregulators and the binding of RAR and/or RXR to DNA (131-135). Together, these cellular events result in the formation of an

active transcriptional site that causes an increase in the mRNA expression of the target gene. Termination of retinoid-induced transcription occurs through an ubiquitin/proteosome-mediated process that is able to degrade the receptors and stop transcription (136,137).

Retinoic Acid Catabolism

Cellular levels of RA are tightly regulated *in vivo*. Homeostatic concentrations are maintained by regulating either the synthesis or the degradation of RA. Early work demonstrated that exogenously administered RA was rapidly excreted as either RA or water-soluble conjugates of RA in bile (138-141). Subsequent studies showed that the C4 position of RA could be sequentially oxidized to 4-hydroxy- and 4-oxo-RA in the liver (142). Furthermore, Roberts et al. (143) reported that the conversion of RA to more polar metabolites occurred in the microsomal fraction of intestine and liver, and it required NADPH and oxygen. Expanding upon the above findings, it was discovered that the general cytochrome P450 inhibitors ketoconazole and liarozole blocked the production of the 4-hydroxy- and 4-oxo-metabolites of RA (144-146). Thus, it was hypothesized that the cytochrome P450 system was the primary mechanism involved in the catabolism of RA.

Cytochrome P450 enzymes are heme iron containing, membrane-bound proteins that are most prominent in liver. Cytochromes were initially discovered in the late 1940s. The majority of their reactions involve the addition of one oxygen atom to the substrate, while another is used to generate a molecule of water (147-149). In general, the reaction occurs in the following manner. First, the substrate binds to the ferric iron form of the

enzyme. Next, an electron is transferred from the substrate to the iron atom, resulting in its reduction. Finally, ferrous iron binds O_2, directly oxidizes the substrate, and generates one molecule of water (147). In this manner, the reactions catalyzed by the cytochrome P450 enzymes increase the polarity of small, non-polar molecules by generating metabolically active sites, such as the formation of hydroxyl groups.

White et al. (150,151) identified a retinoic acid inducible RA 4-hydroxylase cDNA (Cytochrome P450RAI; CYP26) in epithelial cells of the regenerating caudal fin of zebrafish. Through the use of transfection studies, the authors showed that cytochrome P450RAI rapidly catabolized RA to both lipid- and aqueous-soluble metabolites (151) in COS-1 cells. Analysis of the full-length cDNA revealed sequence homology between cytochrome P450RAI and numerous members of the cytochrome P450 superfamily, but it exhibited less than 30% identity with other cytochromes (White et al, 1997). Furthermore, the cDNA for cytochrome P450RAI contains a heme binding motif in the C-terminal region, a hydrophobic region at the N-terminus region, and 5 conserved regions with >80% identity to other members of the cytochrome P450 superfamily, which together suggest that P450RAI is a microsomally located cytochrome P450 (152). Therefore, cytochrome P450RAI encodes for a novel member of the cytochrome P450 family designated CYP26 (152).

Since its initial discovery, CYP26A1 (cytochrome P450RAI) has been identified in several species, including mouse, rat, and human. Human, rat, and mouse CYP26A1 is a 497 amino acid polypeptide (17) that is usually associated with the endoplasmic reticulum. Its sequence is highly conserved across species, with the rat sequence sharing 95% and 91% identity with mouse and human CYP26A1, respectively (17). CYP26A1 is

expressed mainly in the liver, duodenum, colon, and placenta (152,153). Thus, CYP26A1 appears to be involved in both hepatic and extrahepatic catabolism of all-trans-RA. CYP26A1 displays substrate specificity because it dose not hydroxylate 9-*cis*- or 13-*cis*-RA, nor does it catabolize retinol (154). Specifically, CYP26A1 is responsible for the phase I metabolism of RA by converting RA to the polar metabolites all-*trans*-4-hydroxy-RA, all-*trans*-4-oxo-RA, (150) 4-hydroxy-retinyl acetate, 5,6-epoxy-RA (155), and 13-*cis*-4-hydroxy-RA (153), with an apparent K_M of 52 nM (156).

To date, three additional isoforms of CYP26, CYP26B1, CYP26C1, CYP26D1 have been identified and characterized (157-161). Nelson (157) and White et al. (156) independently identified and characterized a second member of the CYP26 family, CYP26B1. Similar to CYP26A1, CYP26B1 is expressed in several species, including zebrafish (156), mouse (157), and human (156,157). They showed that the amino acid sequence of CYP26B1 was 42% identical to CYP26A1 (156,157) and that CYP26B1 was able to rapidly catabolized RA with efficiency and specificity comparable to that of CYP26A1 (156). Unlike CYP26A1, the highest expression of CYP26B1 was in the brain, especially in the pons and cerebellum (156), suggesting that CYP26B1 is required for normal brain functioning. More recently, Bowles et al. (162) showed that CYP26B1 is expressed in Sertoli cells and is responsible for preventing oogenesis and supporting spermatogenesis in mice. In addition to CYP26A1 and CYP26B1, CYP26C1 (161) and CYP26D1 (158,159) were identified and characterized. Both CYP26C1 and CYP26D1 share significant amino acid identity with both each other and with CYP26A1 and CYP26B1. Unlike the other members of the CYP26 family, CYP26C1 is able to metabolize both all- *trans*- and 9-*cic*-RA with equal efficiency to more polar metabolites.

However, to date, the exact roles that CYP26C1 and D1 play in RA metabolism have yet to be clearly delineated.

The reactions catalyzed by the cytochrome P450 enzymes, including all the members of the CYP26 family, generate reactive sites that can be further acted upon to make the substrate more water-soluble. In the case of RA, two distinct conjugates can be formed. First, a molecule of glucuronic acid can be conjugated to the carboxyl end of the molecule to form retinoyl β-glucuronide. The synthesis of retinoyl β-glucuronide is catalyzed by glucuronyl transferases in the liver, intestine, and kidney (163-165), such as the human recombinant UDP-glucuronosyltransferase, UGT2B7. Studies by several groups have shown that retinoyl β-glucuronide was secreted into bile following an oral dose of RA (138,139,166-169). Second, various studies demonstrated that the glucuronyl transferase activity in liver was also capable of conjugating several metabolites of RA, including 4-hydroxy- and 4-oxo-RA, to form the 4-O-β-glucuronide of 4-hydroxy-RA (155,170,171). Thus, the generation of both oxidized derivatives (phase I metabolism) and glucuronidated conjugates of retinoic acid (phase II metabolism) maintain optimal cell and tissue levels of RA by hastening its elimination via urine and bile.

The importance of CYP26 in maintaining homeostatic levels of RA is clearly illustrated during embryonic development. CYP26A1 is expressed throughout the murine developmental process (150) and can be detected as early as embryonic day 8.5 in mouse embryos (152). For instance, MacLean et al. (172) showed that CYP26A1 is expressed in the tail bud to prevent the neuroepithelial tissues from being exposed to RA prematurely and later in the developmental process it is expressed in the hindbrain and retina to ensure that these tissue develop properly. Furthermore, genetic ablation of

CYP26A1 is embryonic lethal and results in many of the defects observed when embryos are exposed to teratogenic levels of RA (173). Similarly, murine CYP26B1 is differentially expressed in the developing hindbrain, forebrain, spinal cord, lung, kidney, heart, eye, and limb bud (174,175) and its expression is essential for normal development. In addition, CYP26C1 is expressed in the developing hindbrain, inner ear, and branchial arch of mice (161), while CYP26D1 is expressed during zebrafish development (158). Together, it is hypothesized that the members of the CYP26 family regulate RA levels during development, thus protecting the developing tissues from overexposure to RA.

In certain tissues and cell lines, CYP26A1 expression is induced by exogenous RA. For example, exogenous RA increases CYP26A1 expression in human breast and colon carcinoma cells (176) and in both the epidermis and keratinocytes of the skin (177). However, the most dramatic effects of exogenous RA on CYP26A1 expression occur in the liver. Yamamoto et al. (16) showed that an acute, intraperitoneal (i.p.) dose of RA significantly increased both hepatic CYP26 mRNA expression and RA metabolism by liver microsomes in control and VA-deficient rats. Furthermore, the authors demonstrated that CYP26A1 mRNA expression increased progressively with dietary VA intake and that CYP26A1 mRNA was directly correlated with liver retinol levels (16). Wang et al. (17) expanded upon these findings by demonstrating that the induction of CYP26A1 mRNA expression by RA was both time- and dose-dependent *in vivo*. Specifically, Wang and colleagues showed CYP26A1 mRNA levels were virtually undetectable in VA-deficient rats; however, a single dose of RA resulted in a significant increase in CYP26A1 mRNA levels by as early as 3 hours post-treatment. Additionally,

the authors observed that CYP26A1 mRNA increased linearly in response to increased exogenous RA doses (17). Taken together, the above studies suggest that the down-regulation of CYP26A1 during VA-deficiency and the acute up-regulation of CYP26A1 following exogenous RA administration in VA-deficient and sufficient rats are essential for the maintenance of homeostatic levels of RA in tissues and plasma.

The molecular mechanism governing the ability of RA to induce CYP26A1 was elucidated by Loudig et al. (178), who examined the proximal upstream region of the CYP26A1 promoter of zebrafish, mouse, and human. They observed a highly conserved region in the mouse, human, and zebrafish promoter that contains a canonical RARE (5'-ACTTCA-$(n)_5$-AGTTCA-3'), which is capable of binding RAR and RXR proteins (178). In addition, the authors showed that two conserved 5'-TAAT-3' motifs flank that RARE, along with an upstream Sp1/Sp3 binding site, enhance the activity of the promoter. Subsequently, Lampen et al. (179) showed that the RAR_-specific ligand Am580 induced CYP26A1 mRNA expression greater than the RAR_ and RAR_ specific ligands. Furthermore, the authors showed that RXR ligands alone were not able to induce CYP26A1 expression. However, when the RXR ligands were administered in conjunction with Am580, the induction in CYP26 expression was greater than when Am580 was administered alone (179). Together, the above studies demonstrate that CYP26A1 gene expression is regulated through the binding of an RAR and RXR heterodimer to an upstream RARE.

In addition to the CYP26 family, several other cytochrome P450 isoforms have been identified that are capable of converting RA into 4-hydroxy- and 4-oxo-RA, including CYP2C8, CYP2A4, CYP1A2, CYP2C3, and CYP3A6 (180,181). Together,

the various members of the cytochrome P450 superfamily, specifically members of the CYP26 family, along with the glucuronyl transferases work in concert to regulate RA concentrations in vivo.

VITAMIN A AND IMMUNITY

Soon after the discovery of VA, studies demonstrated that adequate amounts of VA were required for the maintenance of epithelial tissues and for normal immune function (26,27). In fact, Green and Mellanby (182) described VA as an "anti-infective" agent because rats with VA deficiency usually died with evidence of infection. Since these initial findings, our understanding of the relationship between VA and immune function has continued to expand.

Overview of the Immune System

The maintenance of homeostasis includes the ability to respond to challenges such as tissue injury or infection. Vertebrates have developed a dynamic system, termed the immune system, which is able to recognize and respond to foreign pathogens in a coordinated manner. It involves a complex network of cells, tissues, and mediator molecules, which function in concert to restore homeostasis and remove the cause of the disturbance.

The immune system has both a specific and non-specific component that work together to eliminate harmful pathogens. Innate immunity, which is the non-specific component of the immune system, is present at all times and does not require antigenic

exposure for activation. It is composed of four types of defensive barriers: anatomic, physiologic, phagocytic, and inflammatory barriers. Anatomic and physiologic barriers, such as the skin, low stomach pH, fever response, and mucous membranes, work together to keep microorganisms from entering the body and to prevent their growth inside the body. Phagocytic cells, including macrophages, neutrophils, natural killer cells, and blood monocytes, ingest and destroy microbes. Inflammation allows serum proteins with antibacterial activity and immune cells to rapidly enter the injured or infected area by increasing the diameter and permeability of the capillaries at the target site. Collectively, the above non-specific host defenses serve as the first line of defense against invading microorganisms.

The second component of the immune system is able to recognize and selectively eliminate foreign microorganisms. Unlike innate immunity, adaptive immunity displays antigenic specificity, diversity in its recognition of foreign antigens, immunologic memory, and self versus nonself recognition. The effectors of adaptive immunity are B cells, T cells (T helper and T cytotoxic cell), and antigen presenting cells. B cells are able to recognize unbound foreign antigens, become activated, clonally expand, and secrete antibodies that are specific for that antigen. In contrast, T helper (T_H) cells which recognize and interact with antigens bound to cell-membrane proteins called major histocompatibility complex molecules. This interaction activates the T_H cell, resulting in the production of cytokines that stimulate other aspects of the immune system (see below). In contrast, T cytotoxic (T_C) cells eliminate cells expressing a given antigen following activation. Finally, antigen presenting cells, such as B cells, macrophages, and dendritic cells are capable of processing and presenting specific antigens to T cells.

Furthermore, they deliver a co-stimulatory signal that is required for T_H cell activation. Together, the adaptive and innate immune systems interact, cooperate, and support each other in order to elicit an effective response to an infection. For review of innate and adaptive immunity, please see (183,184).

Inflammation, Lipopolysaccharide, and the Toll-like Receptors

One of the hallmarks of tissues damage and infection is the inflammatory response. Inflammation is a dynamic response that is characterized by pain, redness, heat, and swelling. It involves the complex interaction of various immune cells and mediators which work in concert to increase blood flow to the injured area, allow for the influx of immune and phagocytic cells, remove invading pathogens, and repair tissue damage. Studies examining inflammation and its physiological effects have employed numerous antigens capable of inducing an inflammatory response. However, one of the most extensively used and best-characterized inflammatory-inducing compounds is lipopolysaccharide (LPS).

The innate immune system is able to recognize structures present in a large group of microorganisms that are distinct from self. LPS, which is a cell wall component of Gram-negative bacteria, contains a conserved motif that can trigger an immune response *in vivo* (185). LPS is comprised of an O-side chain, a core polysaccharide, and a lipid A moiety, with the lipid A portion possessing the antigenic motifs (185). During bacterial invasion, LPS will be released into plasma when the bacteria replicates and divides. Free LPS is bound by the soluble protein LPS-binding protein (LBP), which helps transport the LPS to the proper immune cells (186,187). On the cell surface, the LPS:LBP

complex interacts with CD14, a 55 kD phosphoinositol glycolipid-linked membrane protein expressed on the surface of monocytes, macrophages, neutrophils, and dendritic cells (186,188,189). Binding of the LPS:LBP complex with CD14 activates the transmembrane protein Toll-like receptor-4 (TLR4) (190), which, along with MD-2 (191,192), induces intracellular signals that ultimately result in enhanced transcription of genes involved in host defense (193,194).

The first member of the Toll-family was discovered when mutations in *Drosophila melanogaster* embryos disrupted the establishment of dorso-ventral polarity (195). Lemaitre and colleagues (196) expanded upon the above findings by demonstrating that Toll participates in innate immunity in adult *Drosophila*. Subsequently, Medzhitov et al. (197) showed that the human homologue of the *Drosophila* Toll protein is able to initiate an immune response by increasing the expression of different cytokines. Furthermore, they demonstrated that transfected cells expressing a constitutively activated construct of Toll displayed increased nuclear factor kappa beta (NF-kB) activation and increased expression of genes controlled by NF-kB (197). To date, 10 mammalian TLR have been discovered that are all structurally similar but capable of recognizing different antigens (198). For instance, TLR2 recognizes components of Gram-positive bacteria (199), TLR3 recognizes double-stranded RNA (200), and TLR5 responds to flagellin. The best characterized of all the receptors is TLR4, which was first identified independently by Poltorak et al. (201) and Qureshi et al. (202). TLR4 is found on the cell surface of monocytes, PMNs, and dendritic cells, and it is responsible for LPS recognition *in vivo* (203). In fact, the target disruption of the TLR4 gene in mice renders them unresponsive to LPS (204).

Naturally, TLR4 has a low affinity for LPS, thus LBP and CD14 are required for normal signal transduction. Mice lacking LBP and/or CD14 display impaired and diminished responsiveness to LPS (187,205,206). Similarly, the protein MD-2 physically associates with TLR4 and is required for LPS recognition and signal transduction (191,193). Upon binding of the LPS:LBP complex to TLR4, the cytoplasmic portion of the receptor forms an active signaling complex with adapter protein MyD88 and the interlukin-1 receptor activated kinase (IRAK). IRAK, a member of the serine/threonine kinases, autophosphorylates and binds to TRAF6, a protein necessary for NF-kB activation (207). This leads to the activation of the IkB kinase complex and the phosphorylation of IkB. Phosphorylated IkB is degraded, liberating the transcription factor NF-kB. NF-kB translocates to the nucleus where it can induce the expression of various proinflammatory cytokines. In addition, stimulation of TLR4 by LPS leads to the activation of p38 and AP-1, which can both regulate cytokine production transcriptionally (185). Thus, the Toll-like receptors play an essential role in the detection of invading pathogens and the generation of an initial immune response in mammals.

Cytokines and the Hepatic Acute Phase Response

Activation of innate immune cells results in second messenger pathways that increase cytokine production through transcription modulation. Cytokines are small, hormone-like glycoproteins that are synthesized and secreted from monocytes, lymphocytes, neutrophils, endothelial cells, and hepatocytes. They act in picomolar concentrations through high-affinity cell surface receptors, mainly in a paracrine and autocrine manner. In general, cytokines represent the signaling molecules produced in

response to injury or infection that mediate the complex interaction among lymphoid cells, inflammatory cells, hematopoeitic cells, and tissues. They are required for a complete acquired immune response by stimulating lymphocyte activation, proliferation, and/or differentiation. Finally, cytokines are required for the induction of inflammation, and for stimulating the acute phase response (183,184).

The acute phase response is the coordinated sequence of systemic and metabolic changes that occur in response to tissue damage and infection. The systemic acute phase response is characterized by fever, neutrophilia, hypoferremia, hypozincemia (208), increase protein catabolism in muscle (209), increase gluconeogenesis, altered lipid metabolism, and the activation of the complement and coagulation pathways (210). Furthermore, during the acute phase response, the synthesis and secretion of certain plasma proteins is dramatically induced, including C-reactive protein and serum amyloid A (211). Conversely, the levels of certain proteins are reduced during the acute phase response, such as RBP and albumin (212-214). Thus, the acute phase response alters certain physiological conditions in order to facilitate the clearance of pathogens, tissue repair, and the restoration of homeostasis.

In conjunction with the above systemic and metabolic alterations, the detoxification capacity of the liver is diminished during the acute phase response. As mentioned previously, the cytochrome P450 enzymes are responsible for the bulk of the metabolic inactivation of compounds in the liver. Infection or inflammation alters both the expression and enzymatic activity of various members of the cytochrome P450 family, with the majority of the activities being suppressed (18). For instance, Ghezzi et al. (19) showed that certain pro-inflammatory cytokines inhibited the activity of liver

cytochrome P450s. Similarly, Siewert et al. (215) reported that IL-6 was required for the LPS-induced secretion of the cytochrome P450s. Sewer et al. (20) showed that Sprague-Dawley rats treated with LPS had less CYP2C11, CYP3A2, and CYP2E1 as compared to control animals. Subsequently, it was shown that the rates of transcription of the CYP2C11, CYP3A2, and CYP2E1 genes were reduced to approximately 20% of control levels (216). Furthermore, specific transporters involved in the elimination of metabolized compounds, the multidrug-resistance proteins, and hepatic bile flow are diminished by endotoxin and inflammatory cytokines (217-219). Therefore, while the acute phase response is an important aspect of disease resistance, prolonged or excessive cytokine production could have drastic effects on the liver's ability to eliminate endogenous and exogenous compounds.

Vitamin A and Immune Function

The past quarter century has witnessed a renewed interest in the relationship between vitamin A and immune function. This interest can be at least partly attributed to the important work of Sommer and his colleagues. His community-based, epidemiological studies demonstrated the critical role of vitamin A in reducing the morbidity and morality that accompanied infectious diseases in developing countries. Specifically, Sommer et al. (31,220) showed that the mortality rate among children with mild xerophthalmia, such as night blindness, was 4-12 times the rate among children without the condition. Furthermore, the authors showed that mild xerophthalmia was directly associated with approximately 16% of all deaths in children between the ages of 1 and 6. Their findings were important because they demonstrated that even "mild"

forms of vitamin A deficiency had significant effects on morbidity and mortality in children (220). Subsequent studies by Sommer and colleagues showed that children with mild vitamin A deficiency had a 2-3 times greater risk of developing respiratory disease and/or diarrhea (221) and that supplementation with vitamin A decreased disease-related mortality by 34% (222). Sommer's work demonstrated that vitamin A is vital to host immunity by showing that sub-clinical vitamin A deficiency is associated with increased morbidity and mortality in developing countries.

It is now known that vitamin A affects aspects of both innate and acquired immunity and disturbances in vitamin A homeostasis can compromise immune function and overall health (223). While a large body of work has concentrated on the effects of vitamin A deficiency and supplementation on the severity of infections and on the immune system itself, relatively little work has examined how infection-induced inflammation affects vitamin A homeostasis. Certain pathological conditions, such as protein-energy malnutrition (224), liver disease, and insulin-dependent diabetes mellitus (225), are accompanied by decreased plasma concentrations of vitamin A as a symptom of the disease. Similarly, hyporetinemia has been observed during the acute phase response to viruses (22), measles (23), and diarrhea (24). Rosales et al. (214) described the possible molecular mechanism underlying this hyporetinemia. They demonstrated that hepatic synthesis of RBP and secretion of the RBP:retinol complex was reduced during acute LPS-induced inflammation in rats. Moreover, they showed that hyporetinemia occurred independent of the vitamin A status of the animal (214). Recently, Gieng et al. (226) showed that hepatic retinol levels increased 68% while RBP levels decreased 41% following continuous IL-6 administration. These studies suggest

that the hyporetinemia observed during acute or chronic inflammation is due to a decrease in hepatic RBP availability.

In addition to affecting circulating retinol levels, the acute phase response could alter RA homeostasis. First, LPS-induced inflammation results in a substantial decrease in the expression and activity of numerous cytochrome P450 enzymes (18,20,216,227,228). In fact, an *in vitro* study using THP-1 cells showed that mRNA expression of CYP26A1 was reduced following LPS administration even in the presence of retinoic acid (21). Second, previous work has shown that LPS-induced inflammation causes a down-regulation of hepatic transporters involved in the movement of substances into bile, while enhancing the expression of transporters involved in the movement of substances from liver to blood (229). Third, LPS-induced inflammation has been shown to increase vascular permeability, which could alter blood flow to the liver, thus influencing the uptake and catabolism of RA *in vivo* (230,231). Fourth, acute inflammation reduces the expression of the fatty acid transport protein and the fatty acid translocase, which are involved in the uptake of long-chain fatty acids from plasma into tissue (232-234). While it is currently hypothesized that RA can readily diffuse across plasma membranes, alterations in the expression of proteins involved in the uptake of long-chain fatty acids could affect RA homeostasis as well. Taken together, experimental evidence suggests that RA uptake by tissue, recycling back to plasma, oxidation by cytochrome P450 enzymes, and excretion into bile will be altered during inflammation resulting in dramatic changes to RA metabolism.

CONCLUSION

Although our understanding of the underlying mechanisms that control VA and RA homeostasis has expanded considerably, many questions remain to be answered, including how RA affects VA kinetics and how different physiological conditions affect RA metabolism. Thus, four experiments were conducted to delineate: *(1)* how chronic RA administration affects VA turnover and mass, *(2)* how VA status affects the distribution and catabolism of RA, *(3)* how a pharmacological dose of RA affects the mRNA and protein expression of CYP26A1 *in vivo*, and *(4)* how the administration of RA, LPS, or their combination affect CYP26A1 levels and retinoic acid metabolism in rats.

CHAPTER 2:

RA affects whole-body and organ-specific VA kinetics in VA-marginal rats.*

* The results presented in this chapter were published in the Journal of Nutrition:
Cifelli CJ, Green JB, and Green MH. Dietary retinoic acid alters vitamin A kinetics in both the whole body and in specific organs of rats with low vitamin A status. *J Nutr*. 2005 Apr; 135(4):746-52.

INTRODUCTION

Because of the serious consequences of VA deficiency and in view of the role of retinoids in the prevention and treatment of numerous diseases, factors that influence VA utilization have been studied extensively. Previous work established that both VA stores and utilization are directly related to VA intake (235,236). For instance, Varma and Beaton (235) showed that as VA intake and liver stores increased, there was a parallel increase in VA utilization. More recently, work performed in this laboratory demonstrated that VA utilization is also influenced by the plasma pool size of retinol, especially in rats with low-to-moderate VA intake (25,88) Taken together, these studies have delineated some of the underlying mechanisms that control VA homeostasis, but many questions remain to be answered, including the effect of other retinoids on VA utilization and kinetics.

One retinoid that has been studied extensively in recent years is RA, an active metabolite of VA. RA regulates numerous physiological processes in normal cells, including proliferation, differentiation, immune response, and embryonic development (96,97). Before the discovery of the molecular mechanisms of RA action, it was shown that dietary RA could substitute for VA in some but not all physiological functions (8). Subsequent studies showed that feeding RA influenced liver VA levels and spared whole-body VA stores in rats with low VA status (11). In view of these observations, we were interested in determining how individual organs responded to chronic RA treatment and how their response affected VA turnover and mass. Thus, as part of a larger study on VA kinetics in rats with VA stats (237), data were also collected from rats with low VA status during chronic RA supplementation (237). We used model-based compartmental

analysis to analyze the kinetic data from the RA-supplemented rats; results are compared with new and previously published information on rats with low VA status that were studied at the same time (14,238).

OVERVIEW OF COMPARTMENTAL ANALYSIS

Mathematical models are mathematically formalized representations of a system that allow for the study of complex processes that occur simultaneously. In different disciplines of biology, mathematical modeling has been used to gain a deeper understanding of physiological processes and systems. For instance, mathematical modeling has been used to examine the rate of uptake of exogenous and endogenous compounds, to determine enzyme kinetics, to determine the pharmacological response to different drugs, and to calculate the digestion and absorption of nutrients. For review, please see Wastney et al. (239) and Novotny et al. (240).

Compartmental analysis (89,239,241), and in particular, model-based compartmental analysis, is the form of mathematical modeling that will be the focus of this chapter. In nutrition, model-based compartmental analysis has been used to model mineral uptake, turnover, and utilization (242-244), lipoprotein metabolism (245), glucose homeostasis (246), and vitamin kinetics (14).

Compartmental modeling is defined as the representation of a system by a finite number of homogenous states and processes, called compartments, which interact by means of material exchange (89,247) (**Figure 1**). This means, that the retinol within a specific compartment should be: (*1*) homogenous (i.e., having the same concentration throughout the compartment at all times), (*2*) be well mixed, and (*3*) kinetically and/or physically distinct from other retinol in the system. Therefore, the "system" is regarded as a finite number of compartments that are interconnected, which allows retinol to move into and out of a given compartment and exchange with other compartments (241). Model-based compartmental analysis assumes that the system under investigation

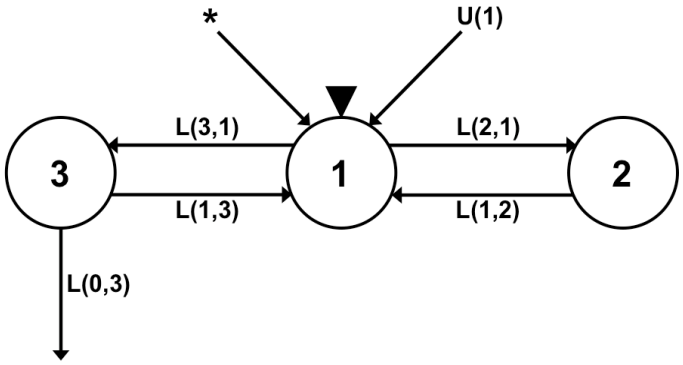

Figure 1. Three Compartment Model. Compartments are shown as circles and movement between compartments is represented by arrows and quantified by fractional transfer coefficients (see below). U(1) represents input of newly absorbed dietary retinol, the asterisk represents the site of tracer introduction, and the triangle (compartment 1) indicates that this compartment is a site of sampling.

exhibits deterministic behavior. This means that the future state of the system may be predicted based on its current state and future input and no probabilistic effects are included (248). Compartmental modeling provides both quantitative and predictive information about the system of interest. Furthermore, compartmental modeling provides unique insights into the underlying mechanisms and metabolic processes that govern the system's kinetic behavior. This approach is unique in that it allows the research investigate certain aspects of a system that might be difficult to study experimentally, which may provide unexpected insights into the metabolism of the compound of interest that may lead to the generation of new hypotheses (14,89).

The overall goal of model-based compartmental analysis is to describe and quantify the kinetics and dynamics of the system of interest. The initial model was formulated by conceptualizing the structural aspects of the metabolic system, which includes the relevant metabolic processes involved and their interconnections. In terms of retinol turnover and kinetics, the development of the conceptual model was based on both what is known (88,238) and theorized about our system. This conceptual model includes both the compartmental structure and estimates of the fractional transfer coefficients (see below) that represent the movement of tracer between compartments and out of the system. Next, the appropriate in vivo experiment is designed and data are collected as specified times. In this instance, radiolabeled retinol, as part of its plasma transport complex, is administered intravenously and the tracer concentration in plasma is followed over time. Plasma tracer data, along with other relevant information, such as the initial conditions, tracee mass, and site of input, are analyzed using the appropriate computer software.

Model identification involved translating the conceptual structure of the system into a mathematical model, which is comprised of a set of differential equations that describe the structure and contain various kinetic parameters that describe retinol transfer within our system. In this experiment, the differential equations were set up and solved using the Windows version of the Simulation, Analysis, and Modeling (WinSAAM) computer program. Specifically, WinSAAM mathematically compares the proposed model to the data collected and provides statistical information about model solutions (239). Solutions results are evaluated by comparing the observed and calculated data both graphically and numerically. If the model solution is not compatible with the experimental data, the model was adjusted by changing its structure and/or parameter values in order to improve compatibility. Compatibility was judged by minimizing both the error associated with the model solution and any inconsistencies that exist between the experimental data and the model simulation. During this process, the known or suspected physiology and biochemistry of the system are kept in mind so that a physiological reasonable model is developed (89).

To determine the simplest model that will provide an adequate fit to the data, multiple models are tested, with model complexity (e.g., the addition of compartments and fractional transfer coefficients) being increased only when it results in a significant improvement in the weighted sum of squares (249). Once a satisfactory fit is obtained, weighted nonlinear regression analysis is applied using WinSAAM to obtain best fit values for the fractional transfer coefficients that describe the movement of tracer between compartments as well as their statistical uncertainty. Based on these results, other kinetic parameters (see "Research Design and Methods" section) can be calculated.

Overall, the structure of the compartmental model provides a visual picture of how the compartments are linked while the model-based parameters provide information about transfer between compartments, the extent of recycling within the system, movement out of the system, and compartmental masses.

In this lab, model-based compartmental analysis has been used to delineate the underlying mechanisms that control VA homeostasis. Specifically, we have used modeling to study VA kinetics in plasma and tissues under different conditions, which include various levels of VA status (86-88,238) and after treatment with different exogenous factors (95), to better understand VA utilization and whole-body VA metabolism. The remainder of this chapter will provide information on the effects of chronic administration of RA to rats with low VA status on the recycling, uptake, and mass of VA in specific organs and the whole-body.

RESEARCH DESIGN AND METHODS

Animals and Diet:

Weanling male Sprague-Dawley rats (50-60 g; Hilltop Lab Animals) were housed in environmentally controlled quarters. Food and water were available ad libitum. All procedures for animal care and use complied with guidelines approved by the Pennsylvania State University.

The rats were provided a VA-free purified diet for 50 days to deplete liver VA stores and then a diet that provided a low amount of VA (LA group) in the form of retinyl palmitate (350 nmol retinol/kg diet or ~7 nmol/d). After consuming the LA diet for 19 days, rats were randomly assigned to either remain in this group or to consume the LA diet supplemented with RA (LA+RA; 40 μmol RA/kg diet or ~800 nmol/d) for 25-26 days. Supplementation continued during the subsequent *in vivo* kinetic studies.

Retinol Kinetic Studies

Plasma labeled with [^{3}H]retinol in its physiological transport complex was prepared as previously described (89) by administering a dispersion of [^{3}H]retinyl acetate (~11.1 MBq and 61 nmol/donor rat) in Tween 40 to VA-deficient donor rats (238). Blood plasma containing the [^{3}H]retinol in its normal physiologic transport complex was harvested from the donor rats and stored at 4°C under nitrogen and injected into recipients over the next 3 days.

Retinol kinetic studies were conducted as previously described (238). The [^{3}H]retinol-labeled plasma (~0.9 g containing ~0.11 MBq and ~1.4 nmol retinol) administered to the LA+RA and LA recipient rats caused an ~20% perturbation of the

plasma retinol pool. Serial blood samples were collected from a caudal vein from 9 minutes after dose administration until rats were killed at 12 minutes (n = 3/dietary group), 2 hours (n = 3/group), 10 hours (n = 3/group), 4 days (n = 3/group), 28 days (n =3/group), and 35 days (n =5 for LA and n = 3 for LA+RA); aliquots of plasma were frozen under nitrogen. At the time of killing, the whole body was perfused; organs (liver, kidneys, small intestine rinsed of contents, lungs, testes, adrenal glands, eyes, and remaining carcass) were removed, weighed, and frozen at -16°C for subsequent analysis.

Analytical Procedures

Aliquots of plasma and the [^{3}H]retinol-labeled dose were extracted using a modification of the method of Thompson et al. (238,250). When retinol mass was determined, an internal standard (retinyl acetate in absolute ethanol) was added prior to extraction. Appropriate aliquots of the lipid extracts were taken for analysis by HPLC and/or liquid scintillation spectrometry. Adrenals, eyes, aliquots of homogenized carcass, and aliquots of freeze-dried liver, kidneys, small intestine, lungs, and testes were extracted using a modification of the method of Hara and Radin (238,251). For quantification of retinol mass, the internal standard was added to aliquots of liver before extraction. Aliquots of the extracts were removed for HPLC analysis (liver samples) and/or liquid scintillation spectrometry.

Retinoids masses were quantified by reverse-phase HPLC as previous described (252), except that retinol and retinyl acetate in extracts of liver were eluted with methanol:water, 90:10, v:v (1 mL/min), and retinyl esters were eluted with 100%

methanol (2 mL/min). Solvent-free extracts of plasma and tissues were solubilized in scintillation solution and analyzed for radioactivity as previously described (238).

Kinetic Analysis and Parameters

Data on the geometric mean fraction of the administered dose in plasma, organs, and irreversibly lost [1 – (fraction of dose in plasma + organs + carcass)] at each time, as well as the standard error of the mean (SEM), were obtained from Lewis (237). Because results for the RA-supplemented rats were our main focus, data for the LA+RA group were analyzed first and then the LA group data were modeled similarly for comparison. To begin, group mean plasma data vs. time for rats in the 28- and 35-day groups were fit to a multiexponential equation using the Windows version of the Simulation, Analysis, and Modeling computer program (239) on a Dell OptiPlex GX1P computer. Then the "forcing function" option in WinSAAM (14,239) was used to develop a compartmental model for each organ subsystem based on the mathematical description of the tracer response profiles in plasma and tracer data from that organ. LA+RA data were analyzed first, with parallel models developed for the LA data. The forcing function approach makes use of the fact that plasma is the sole source of input of VA to individual organs or tissues. Thus, because each organ exchanges VA directly with plasma but not with other organs, we can uncouple individual organs from the whole system and model each one individually. This approach allows the modeler to develop initial models for each organ before working with all organs simultaneously. For each organ, we first tried to fit the data to a single compartment; when that was not sufficient, a second, or sometimes third, more slowly turning-over compartment was introduced, which exchanged VA with the

first, or second, compartment. Once a satisfactory fit was obtained, weighted nonlinear regression analysis using WinSAAM was applied to obtain the best fit value for the fractional transfer coefficients [L(I,J)s; see below]. Once all organs were satisfactorily fit for each group, the forcing functions were removed and the entire data set for each group, including irreversible loss, was modeled together. The LA+RA model was developed first and then a parallel model was developed for the LA group.

The following kinetic parameters were determined; please refer to Green and Green (89) for more details. The fractional transfer coefficients [L(I,J); d^{-1}] are defined as the portion of retinol in compartment J that is transferred to compartment I each day. The mean residence time [T(I,J); d] is the average of the distribution of times that a molecule of retinol spends in compartment I before irreversibly leaving that compartment after entering the system via compartment J. The system mean residence time [T_{SYS}] is the average time that a molecule of retinol spends in the system before irreversible loss and is calculated as the sum of the T(I,J)s. Plasma retinol pool sizes (nmol) for the LA+RA and LA groups were estimated from the average plasma retinol concentration during the kinetic study and the estimated plasma volume. Then, plasma retinol pool sizes were used in a steady-state solution in WinSAAM to estimate retinol pool sizes or traced mass [M(I); nmol] in other compartments so that transfer rates [R(I,J); nmol/d] could be calculated. Transfer rates, including the system disposal rate, are defined as the amount of retinol (nmol) transferred from compartment J to compartment I each day.

Statistics

Descriptive data are presented as group means ± SD. Data were compared statistically using a Student's unpaired *t* test in Microsoft Excel with $P \leq 0.05$ considered significant. Because compartmental modeling was done using group mean data at each time, it is not possible to compare kinetic parameters statistically between groups.

RESULTS AND DISCUSSION

Status of rats

In spite of normal growth, measurements of plasma and liver VA levels confirmed the low VA status of the LA+RA and LA rats. Compared with VA-adequate rats studied at the same time (plasma retinol, ~1.57 μmol/L; (88)), plasma retinol concentrations at the beginning and end of the kinetic studies were 0.308 ± 0.037 and 0.347 ± 0.065 μmol/L in the LA+RA group and 0.381 ± 0.036 and 0.344 ± 0.026 μmol/L in the LA group; the concentrations were statistically different at the beginning of the study. Hepatic VA content at the time of initiation of the kinetic studies was 6.03 ± 1.74 nmol in the LA+RA group and 7.64 ± 0.81 nmol in the LA group (NS). In rats killed 35 days later, liver VA content was 17.4 ± 11.4 nmol and 1.75 ± .035 nmol in the LA+RA and LA groups, respectively ($P < 0.05$). Thus, LA+RA rats appeared to be in a slight positive VA balance during the kinetic study (0.325 nmol/d), whereas LA group rats were in a slight negative VA balance (-0.168 nmol/d).

Kinetic Data

Plasma [^{3}H]retinol kinetics differed between LA+RA and LA group rats (**Figure 2**). Initially, there was a rapid disappearance of tracer from plasma in both groups. By 6 hours, however, the curve for the LA group began to level off and it continued to decline gradually throughout the remainder of the study. In contrast, a more rapid disappearance of tracer from plasma persisted in the LA+RA group until ~2.5 days after dose administration; then the decline became more gradual until ~4.5 days after the dose when the curve begins to flatten. Extensive recycling of retinol to plasma, as was described

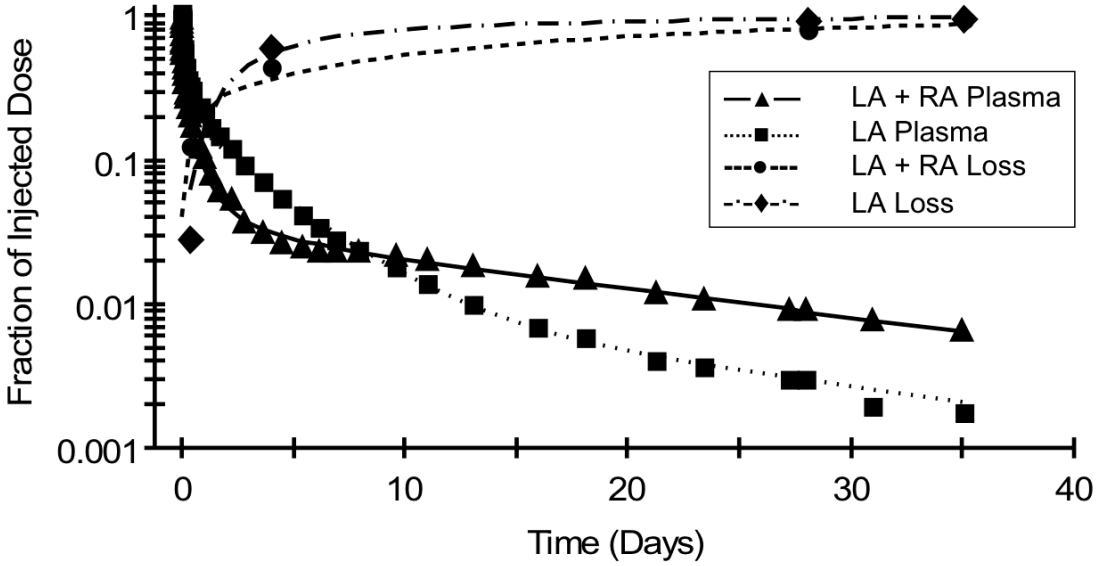

Figure 2. Mean observed (symbols) and model-predicted (lines) fraction of injected dose in plasma and irreversibly lost for LA+RA and LA vs. time (d) after administration of [^{3}H]retinol-labeled plasma to rats fed a diet low in vitamin A with (LA+RA) or without (LA) retinoic acid supplementation. Each point represents 3-5 rats.

previously for rats with moderate VA status (87), occurred in both groups as indicated by the decrease in the slope of both curves before 8 days. By 8 days postinjection, the curve for LA+RA rats crossed over that for the LA rats, and it remained above the LA curve for the remainder of the study. The plasma response profiles indicated that the total traced mass was larger in the LA+RA rats and that the system fractional catabolic rate was higher in the LA rats. The pattern of irreversible loss of [^{3}H]retinol (**Figure 2**) also differed between LA+RA and LA rats.

VA kinetics were visually different in several organs of LA+RA and LA rats (**Figure 3**). For liver, kidneys, small intestine, and lungs, differences between groups were evident by as early as 2 hours postinjection. For those four organs, the fraction of the dose remaining in the tissue was higher at all times in the LA+RA group. VA kinetics in other organs/tissues (eyes, testes, adrenals, and carcass) did not differ between the groups.

Model Development and Proposed Model

When plasma tracer data were fit by multiexponential equations using WinSAAM, a 4-component equation provided the best fit for both groups as judged by an *F* statistic (249). Using these equations to describe the plasma tracer response, the forcing function option in WinSAAM was then applied to uncouple the system so that tracer data for each organ could be modeled separately. The same approach was used previously to compare VA kinetics in the organs of LA rats with those in rats at two higher levels of VA status (14). Here, models were first developed for the LA+RA group

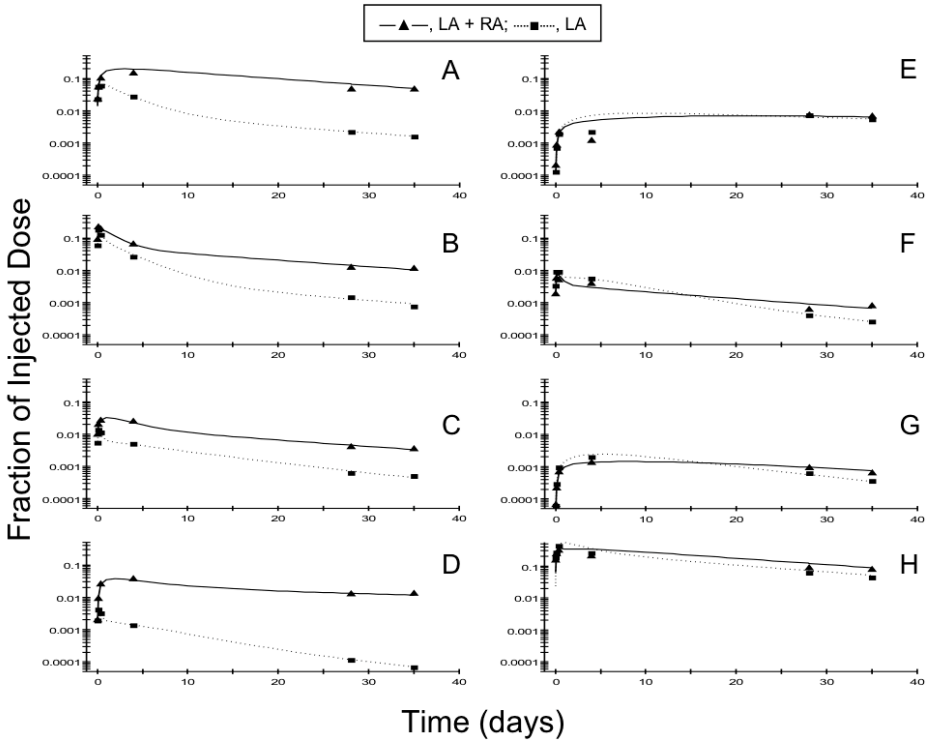

Figure 3. Mean observed and model-predicted fraction of injected dose in liver (A), kidneys (B), small intestine (C), lungs (D), eyes (E), testes (F), adrenals (G), and carcass (H) vs. time (d) after administration of [^{3}H]retinol-labeled plasma. Each point represents 3-5 rats. For eyes, one datum at 4 d in each group was unweighted for nonlinear regression analysis.

and then those parameters were applied to the LA group data. For three organs (eyes, testes, and adrenals) and the carcass, the weighted residual sums-of-squares did not differ between groups; however, for liver, kidneys, small intestine, and lungs, comparison of the sum-of-squares indicated, as suggested by visual comparison of the tracer response curves (**Figure 3**), that substantial changes would be required to fit the LA data. Thus, using the LA+RA models as a starting point, parallel models were developed for the LA group. When possible (i.e., when the addition or removal of a compartment for the LA group did not significantly alter the weighted residual sum-of-squares for the model) the same model structure was used for both groups. In two cases (liver and carcass), different structures were required in each group.

Two compartments were sufficient to fit the data for kidneys, lungs, small intestine, adrenal glands, eyes, and testes in both groups, and for LA group liver and LA+RA group carcass. That is, retinol kinetics in these organs could be described using one compartment that rapidly exchanged retinol with plasma as well as with a second, more slowly turning-over compartment. One compartment was adequate to fit the liver tracer data for the LA+RA group, and three compartments were required to fit the carcass data for the LA group. We speculate that these differences between groups occurred because there was more VA in liver and (presumably) carcass of LA+RA rats, and this VA dominated the kinetics, resulting in "lumping" of the more rapidly turning-over VA with the more slowly turning-over pool. For the LA group, these compartmental structures are comparable to those previously presented (14,238), except for eyes. Here, two compartments were required to fit the LA+RA group data; in our previous work, one compartment was used to fit the LA group data (14). In the current analysis, there was no

difference in the sum-of-squares using 1 vs. 2 compartments for LA group eyes; thus, two compartments were used for both groups.

Once organs were modeled individually, the plasma forcing function was released, and all data including irreversible loss were modeled simultaneously for each group. Our working hypothesis model includes the 7 sampled organs and remaining carcass around the central plasma compartment (**Figure 4**). The model includes recycling of tracer from the more quickly turning-over compartment to plasma in each tissue, and it shows output (irreversible loss) from carcass compartment 12. A good fit to tracer data for plasma, organ/tissues, and irreversible loss was obtained only when the output was from compartment 12. Although not normally considered a major site of VA loss, previous compartmental models, including one developed for a larger group of rats fed the LA diet (238) and another developed for rats with moderate levels of liver VA (87), predicted that the carcass was the site of VA output. The fit of the proposed model to the data can be seen by comparing the observed data with the model-predicted values (**Figures 2 and 3**); in addition, the weighted residual sums-of-squares (not shown) indicated that the models were highly compatible with the data.

Model-derived Kinetic Parameters

Model-predicted kinetic parameters are reported in **Tables 1-3** (presented at the end of this section). Predictions are compatible with the conclusions drawn from visual examination of the tracer response curves for individual organs (**Figure 3**) in that parameters for liver, kidneys, small intestine, and lungs differed between groups. For liver, the models predicted that both an increased fractional input and a decreased

fractional output of VA [L(2,1) and L(1,2); Table 1] in the LA+RA group compared with the LA group. Consequently, the model predicted that the VA traced mass in liver was 11 times greater in the LA+RA group than in the LA group (**Table 2**). This prediction is compatible with the difference measured in liver VA levels between the two groups. Because of the indicated kinetic changes, the residence time for liver [^{3}H]retinol was nearly 14 times greater in the LA+RA rats than in the LA rats (**Table 3**). However, the residence time for liver [^{3}H]retinol was still approximately 30 and 40 times lower than in VAS control rats in a different study (253). Similar kinetic differences were observed between groups for kidneys and small intestine. The transfer [^{3}H]retinol from the rapidly to the more slowly turning-over compartment [**Figure 4** and R(5,4) and R(7,6) in **Table 1**] was 4.5 (kidneys) and 33 times higher (small intestine) for the LA+RA than LA rats. The increased rate of transfer of retinol in the more slowly turning-over compartment contributed directly to increases in both the tissue residence time and traced mass in the kidneys and small intestine of the RA-supplemented rats. The residence time for [^{3}H]retinol in kidneys of LA+RA rats was 3.5 times higher that that in the LA rats (**Table 3**), and the traced mass of VA in the kidneys was 3 times higher with chronic RA treatment. Similarly, in the small intestine, both the residence time and traced mass were 5 times larger in the LA+RA group than in the LA group.

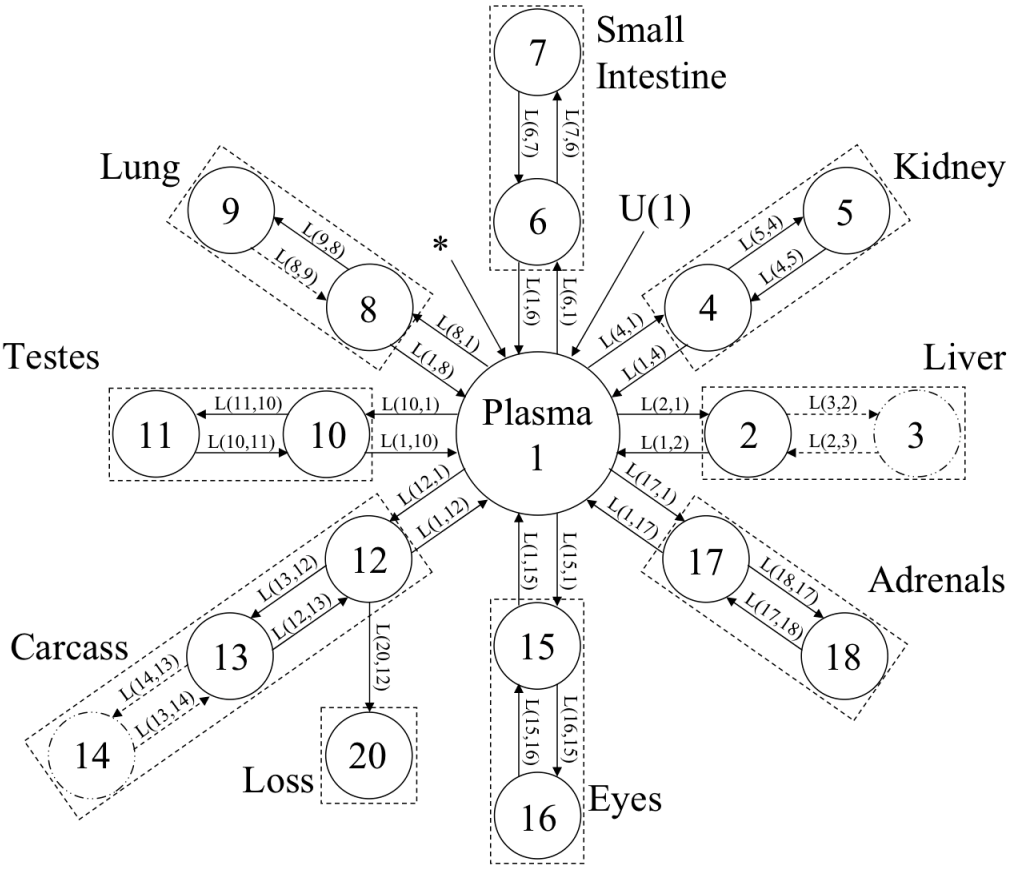

Figure 4. Proposed models for vitamin A metabolism in rats with low vitamin A status with (LA+RA) or without (LA) retinoic acid supplementation. Compartments are represented as circles; organ compartments are grouped inside dashed rectangles. The model for the LA group required an additional compartment in the liver (compartment 3) and carcass (compartment 14). Those are shown as dashed circles within the organs. Interconnectivities between compartments correspond to fractional transfer coefficients or L(I,J)s (the fraction of retinol in compartment J that is transferred to compartment I each day; Table 1. The asterisk represents the site of input of [^{3}H]retinol-labeled plasma; U(1) is input of dietary vitamin A

In this study, the lungs were the tissue most responsive to chronic RA treatment. In LA+RA rats, the more slowly turning-over compartment in the lungs (**compartment 9, Figure 4**) acted as a sink for [^{3}H]retinol. That is, during chronic RA treatment, our model predicted that retinol could be transferred from the first compartment (**compartment 8, Figure 4**) to the more slowly turning-over compartment of the lungs (**compartment 9**); however, its return to the first compartment was at a undetectable rate. Thus, [^{3}H]retinol was efficiently retained by the lungs in the LA+RA group as evidenced by the prediction that the residence time for [^{3}H]retinol was 75 times higher than that of the LA group (**Table 3**). This dramatic increase in tissue residence time is also reflected in the predicted amount of VA traced mass in lungs, which was 31 times greater in the LA+RA than in the LA group (**Table 2**). The model-predicted values for these parameters in the LA+RA group are minimum estimates because compartment 9 acts as a sink.

In contrast to the liver, kidneys, small intestine, and lungs, observed and model-predicted fractions of the injected dose (**Figure 3**), tissue residence times (**Table 3**), and traced mass (**Table 2**) did not differ in the LA+RA and LA groups for the eyes, testes, adrenals, and carcass, despite small differences in fractional transfer coefficients and transfer rates (**Table 1**). That is, in spite of some differences between the groups in the movement of retinol into and out of specific compartments in these organs, overall organ kinetics were not affected by RA supplementation. These results are not surprising for eyes and testes because dietary RA cannot fulfill the VA needs of those tissues (8). In addition, Kurlandsky et al. (110) found that very little of the infused [^{3}H]RA was

recovered in the testes of rats, indicating that little RA was taken up from plasma by this tissue. In contrast, infused RA contributed almost 80% of the RA pool in liver.

Using the model in Figure 3 and the R(I,J)s in Table 1, we predicted the contribution of different organs to plasma retinol output. LA+RA rats had a higher plasma retinol turnover rate (88.6 nmol/d) than the LA group (54.6 nmol/d). In both groups, <5% of plasma retinol turnover went to liver, 54-56% to the kidneys, 30-33% to carcass, 5-6% to the small intestine, and <5% went to the other 4 organs sampled. For example, only ≤ 0.1% of the plasma retinol turnover went to the eyes. In addition, <10% of plasma retinol output was irreversibly lost. Because VA disposal rate [R(20,12)] is determined by the difference between plasma retinol output [R(I,1)] and plasma retinol input [R(1,J), where I is the output to all organs and J is the input to plasma from all organs] plus liver VA balance (i.e., the change in liver VA during the kinetic study), we can calculate that diet provided 4.3 (LA+RA) or 4.8 nmol/d (LA) to plasma retinol input. If rats were consuming ~7 nmol VA/d, then absorption efficiency was ~62% (LA+RA) or 69% (LA), similar to what was measured in lymph duct-cannulated rats.

In addition to kinetic differences between groups at the organ level, our models also predicted differences in whole-body VA kinetics between the LA+RA and LA rats. The model-predicted VA disposal rate was 20% lower in the LA+RA rats [R(20,12); Table 1] and the system factional catabolic rate was half that of the LA group (0.0548 vs. 0.110 d^{-1}). In a preliminary comparison of these groups using input-output analysis (254), the same trends were observed. Together, the lower disposal rate and system fractional catabolic rate in the LA+RA rats contributed to a system VA residence time that was 2 times higher than that in the LA rats (Table 3).

Integrating out results on the effects of dietary RA on VA kinetics in specific organs (liver, kidneys, small intestine, and lungs) as well as the whole body, we hypothesize that model-predicted differences in residence times (**Table 3**) are related to the larger body pools of VA with which [^{3}H]retinol could equilibrate in LA+RA rats and a lower disposal rate. In examining body VA pools, liver VA levels were higher in LA+RA than in LA rats; in addition, the model predicts larger VA levels in LA+RA liver, kidneys, small intestine, and lungs (**Table 2**). They are also predicted by the relative geometries of the tracer response curves between groups (**Figure 3**). Interestingly, Nagy et al. (67) identified VA-storing stellate cells in all four of these organs in both control and VA-supplemented rats. Also, Zolfaghari et al. showed that although the mRNA and activity of LRAT was not detectable in liver (61) and lungs (62) of rats with low VA status, administration of RA increased both variables. These authors reported that RA must be present continuously to keep LRAT activated.

At the whole-body level, RA-supplemented rats more efficiently utilize dietary retinol, thus resulting in a slight positive VA balance (0.325 nmol/d vs. a negative balance of -0.168 nmol/d in the LA group). That is, the VA activity supplied by the LA+RA (retinyl palmitate plus RA) diet was sufficient to meet VA needs; however, without the supplemental RA (LA group), VA intake was insufficient to meet needs for the vitamin. We hypothesize that the difference in VA balance was the result of two independent mechanisms working in parallel. First, it is likely that dietary RA may have been available for some of the numerous physiologic and molecular functions that require RA in such tissues as liver and kidneys that can derive RA from plasma (110). This would spare the irreversible conversion of retinol to RA and allow VA to accumulate.

Our model predicts that both VA residence time and traced mass for the system were significantly higher in the RA-supplemented rats (**Table 3**).

The second factor contributing to the positive VA balance observed in the RA-supplemented rats was the change in the amount of [^{3}H]retinol that was irreversible lost. Our model predicted that the VA disposal rate was reduced by 20% in RA-supplemented rats. Kelley and Green (25) showed that VA utilization in rats with low VA intake depended more on plasma retinol levels than on VA stores or intake. Indeed, the results presented here further support this relation. The plasma retinol pool was slightly lower in the LA+RA group rats than in the LA rats (4.55 vs. 4.90 nmol), as was the model-predicted system fractional catabolic rate (0.0548 vs. 0.110 d^{-1}). That is, after the initial uptake of retinol by tissues, the amount of retinol present to provide the mass action for irreversible utilization and disposal was lower in the LA+RA than in the LA rats. Together, the proposed increased in tissue RA in the LA+RA rats and their lower plasma retinol levels contributed directly to a decrease in VA utilization, resulting in a slight positive VA balance.

In conclusion, the present analysis demonstrated that chronic administration of RA to rats with low VA status affects the recycling, uptake, and mass of VA in a tissue-specific manner and results in positive VA balance. We propose that this positive balance is related to the ability of some organs such as liver and kidneys to take up RA from plasma, thus sparing retinol, and allowing VA to accumulate.

TABLE 1

Model-predicted fractional transfer coefficients and transfer rates in rats with low vitamin A status with (LA+RA) or without (LA) retinoic acid supplementation[1]

(I,J)	L(I,J)		R(I,J)	
	LA+RA	LA	LA+RA	LA
	d^{-1}		nmol/d	
(2,1)	0.8074	0.3768	4.065	1.846
(1,2)	0.1531	1.320	4.065	1.846
(3,2)	NA	0.0153	NA	0.0214
(2,3)	NA	0.0542	NA	0.0214
(4,1)	10.56	6.306	48.06	30.90
(1,4)	23.76	21.66	48.06	30.90
(5,4)	2.364	0.9863	4.782	1.407
(4,5)	0.9601	2.106	4.782	1.407
(6,1)	1.240	0.5311	5.641	2.602
(1,6)	41.66	19.63	5.641	2.602
(7,6)	7.136	0.2213	0.9663	0.0293
(6,7)	0.4963	0.1024	0.9663	0.0293
(8,1)	0.1902	0.1980	0.8652	0.9704
(1,8)	0.2616	26.48	0.8652	0.9704
(9,8)	0.0115	0.2830	0.0365	0.0104
(8,9)	NA	0.1559	NA	0.0104
(10,1)	0.1867	0.3090	0.8493	1.516
(1,10)	6.727	15.93	0.8493	1.516
(11,10)	0.3515	0.5270	0.0444	0.0501
(10,11)	0.1805	0.1740	0.0444	0.0501
(12,1)	6.951	3.397	29.08	16.64
(1,12)	4.865	58.95	25.10	11.64
(13,12)	1.284	810.0	3.199	160.0
(12,13)	0.1822	11.58	3.199	160.0
(14,13)	NA	0.0907	NA	1.252
(13,14)	NA	0.0653	NA	1.252
(15,1)	0.0150	0.0122	0.0683	0.0598
(1,15)	0.0311	0.0311	0.0683	0.0598
(16,15)	0.0918	0.0918	0.2013	0.1764
(15,16)	0.4498	0.4498	0.2013	0.1764
(17,1)	0.0047	0.0048	0.0215	0.0235
(1,17)	0.1233	0.2159	0.0215	0.0235
(18,17)	48.75	97.50	8.489	10.60
(17,18)	76.26	78.53	8.489	10.60
(20,12)	0.8740	25.33	3.984	5.003

Shown are model-predicted fractional transfer coefficients [L(I,J)s or the fraction of retinol in compartment J that is transferred to compartment I each day] and transfer rates [R(I,J)s or the nmol of retinol transferred from compartment J to compartment I each day] for rats fed a diet low in vitamin A either with (LA+RA) or without (LA) retinoic acid supplementation. The model is shown in Fig. 3. NA, not applicable.

TABLE 2

Model-predicted compartment masses in rats with low vitamin A status with (LA+RA) or without (LA) retinoic acid supplementation[1]

M(I)	LA+RA	LA	LA+RA/RA
	nmol		
M(1)	4.550	4.900	0.929
M(2)	19.42	1.399	13.9
M(3)	NA	0.394	NA
M(4)	2.023	1.426	1.42
M(5)	4.980	0.668	7.45
M(6)	0.135	0.133	1.01
M(7)	1.947	0.287	6.78
M(8)	3.167	0.037	85.6
M(9)	NA	0.666	NA
M(10)	0.126	0.095	1.33
M(11)	0.246	0.288	0.85
M(12)	5.688	0.197	28.9
M(13)	27.23	13.81	1.97
M(14)	NA	19.17	NA
M(15)	2.193	1.921	1.14
M(16)	0.447	0.392	1.14
M(17)	0.174	0.108	1.61
M(18)	0.111	0.135	0.822

Values are model-predicted masses [M(I); nmol) in compartments shown in Fig. 4 for rats fed a diet low in vitamin A either with (LA+RA) or without (LA) retinoic acid supplementation. NA, not applicable.

TABLE 3

Model-predicted traced masses and residence times in organs of rats with low vitamin A status with (LA+RA) or without (LA) retinoic acid supplementation[1]

	Traced Mass		Residence Time	
	LA+RA	LA	LA+RA	LA
	nmol		d	
Liver	19.42	1.793	4.983	0.358
Kidneys	7.003	2.095	1.454	0.419
Small Intestine	2.086	0.419	0.432	0.084
Lungs	3.168	0.103	1.574	0.021
Eyes	2.641	2.313	0.548	0.462
Testes	0.372	0.383	0.077	0.077
Adrenals	0.285	0.244	0.059	0.049
Carcass	33.18	33.20	9.200	6.630
Total	72.70	45.45	19.28	9.081

Values are model-predicted traced masses (nmol) and residence times (d) or the average of the distribution of times that a molecule of retinol spends in compartment I before irreversibly leaving that compartment after entering the system via compartment J for rats fed a diet low in vitamin A either with (LA+RA) or without (LA) retinoic acid supplementation. The total (system) values include the organs shown plus plasma. Compartments comprising each organ are indicated in the model shown in Fig. 4.

CHAPTER 3:

VA status affects the distribution and catabolism of RA in rats.

INTRODUCTION

Since the 1920's, VA has been characterized as an "anti-infective vitamin" because VA-deficient animals succumbed to infections while VA-sufficient animals survived (182). Subsequent studies have shown that VA-deficiency is associated with decreased resistance to disease (223), prolonged infection (255), and increased mortality (31,220). In humans, VA-deficiency affects approximately 140 – 250 million children in more than 118 countries (256) and is the leading cause of childhood blindness in developing countries (223). Studies have demonstrated that VA supplementation has been effective in treating some infectious diseases, such as measles (23) and diarrhea (257), and leads to a reduction in mortality in children (222). In addition, treatment with RA, the active metabolite of VA, enhances the immune response in both VA-deficient and VA-sufficient animals (6,7,258). Together, these data suggest that supplementation with VA and/or RA may stimulate immune function and reduce the severity of infectious diseases across the entire spectrum of VA status.

Previous work has established that retinol kinetics is directly affected by both VA intake and status (88,235). Specifically, Green and Green (88) showed that retinol utilization was approximately 10x lower in rats with low VA stores as compared to their sufficient counterparts. Similarly, kinetic analysis revealed that the uptake, utilization, and storage of retinol by specific tissues in rats were directly influenced by VA-status (87,238,253). These results suggest that the distribution and utilization of retinol during VA supplementation will be affected by VA status. Furthermore, these studies suggest that VA status will affect the kinetics of other retinoids, such as RA, in a similar manner,

but to date the relationship between VA status and the distribution and metabolism of RA has yet to be fully delineated.

RA is an active metabolite of VA that regulates numerous physiological processes in vivo, including cell proliferation and differentiation, embryonic development, and immune function (96,97). Isomers of RA are used therapeutically for skin disorders (1), to treat acute promyelocytic leukemia (3,4), and to reduce the risk of other cancers (2). Additionally, studies have demonstrated that RA may possess immune adjuvant properties (6,7), suggesting that treatment with RA might reduce the severity of certain infectious diseases. In view of these observations, we were interested in determining how RA distribution and metabolism was affected by VA status and supplementation in rats. Thus, VA-deficient (VAD), VA-marginal (VAM), VA-sufficient (VAS), and supplemented VA-deficient (VAD + RP) rats were examined to determine the effects VA status on RA homeostasis. The present study demonstrates that hepatic VA levels directly affect the distribution and metabolism of RA in a tissue-specific manner.

RESEARCH DESIGN AND METHODS

Animals and Diets

All procedures for animal care and use were approved by The Pennsylvania State University. Female Sprague-Dawley (Charles River Laboratories, Boston, MA) rats with litters of 12 female pups were fed a vitamin A-deficient diet from the time of arrival to reduce the accumulation of vitamin A from milk by their nursing offspring (259). The rats were housed in a room maintained at 22°C with a 12-h dark:light cycle, and food and water were available ad libitum. When the pups were 21 days old, they were weaned in pairs and fed a nutritionally complete AIN-93G diet (260) modified to contain either 0 (VAD; n=15), 0.412 mg (VAM; n=6), or 7.43 mg (VAS; n=9) of retinyl palmitate per kilogram of diet (Research Diets, New Brunswick, NJ). Twenty hours prior to the experiment, 6 VAD rats were given an oral dose of 1.67 μmol of retinyl palmitate prepared in ~ 25 30 µl of vegetable oil (VAD+RP; n=6). Finally, 3 VAS rats were pair fed to 3 VAD rats for 14 days to ensure that VAD-related anorexia did not affect the results.

Experimental Design

When the rats were between 60 and 66 d of age, the rats were lightly anesthetized by isoflurane-oxygen inhalation and 0.15 mL / 100 g BW of the albumin-bound [^{3}H]RA, prepared as described below, was injected into the exposed left common iliac vein. The incision was closed with a sterile surgical staple and the rats were allowed to recover from the anesthesia. Rats were killed 30 minutes post-injection by carbon dioxide asphyxiation. Blood was drawn from the vena cava in heparinized syringes and the liver,

lung, spleen and small intestine were quickly removed, weighed, and aliquots frozen in liquid nitrogen for storage at -80°C before subsequent analysis. Because RA is rapidly metabolized, it is important to collect tissues quickly (140,166); thus, each rat was treated on a defined schedule so that all tissues could collected and frozen within 5 minutes or less after euthanasia. The 10 and 30 minute times chosen for this experiment were based upon previous reports (110) and pilot work (data not shown) that showed that >95% of the [^{3}H]RA dose was cleared from plasma within 10 minutes and that metabolism of [^{3}H]RA to polar metabolites could be readily detected by 30 minutes.

Dose Preparation

All procedures involving retinoids were carried out in either dim or yellow light. The doses of [^{3}H]RA for i.v. injection were prepared fresh immediately prior to injection. To prepare a 1-mL aliquot, 10 μCi of [^{3}H]RA ([11,12(n)-^{3}H]retinoic acid, specific activity, (37.0 Ci / mmol, from PerkinElmer, Boston, MA) was combined with 0.23 μmol of oleic acid and the solvent was evaporated under argon until < 10 μL remained. Next, 5 μL of Tween 20 (Sigma-Aldrich, St. Louis, MO) and 0.9 mL of 0.1% rat serum albumin (Calbiochem, San Diego, CA, diluted in sterile PBS) were added, and the mixture was vortexed. Then, 0.10 mL of whole rat plasma was added to the sample and immediately vortexed. The final concentration of RA in the dose was 0.023 μM, and the dose delivered to each rat was standardized at 0.15 ml (33 pmol RA) / 100 g BW. Following the preparation and administration of each dose, aliquots were analyzed by HPLC (261), which showed them to be > 97% [^{3}H]RA (data not shown).

Determination of Tissue Radioactivity

Aliquots of individual plasma, liver, lung, and small intestine samples were extracted using a modification of the method of Folch et al. (262,263). Half a gram of minced tissue, or 0.5 mL of plasma, was placed in 10 mL of chloroform:methanol (2:1, v/v) overnight, filtered the next day and the filtrate washed four times with 2 mL of the appropriate Folch-wash solution. Following each wash step, the resulting aqueous phases were removed and combined. After the final wash, methanol (100%) was added to the aqueous phase until the total volume was 11 mL per sample. Next, the washes were back extracted with 2 mL of hexane, mixed, and centrifuged at 850 x *g* for 5 minutes. The hexane was removed and added to the Folch organic phase. This backwash was performed in order to remove any non-polar compounds present in the aqueous phase. The organic phase from each sample was dried under argon in an analytical evaporator (Organomation Associates Inc, Northborough, MA) to completeness and the lipophilic compounds were reconstituted in 3 mL of chloroform. Aliquots of the injected dose, along with the organic and aqueous phases from each tissue were analyzed by liquid scintillation spectrometry using a Beckman LS-6500 counter (Beckman Coulter, Fullerton, CA).

Separation of RA from polar organic metabolites (4-oxo- and 4-hydroxy-retinoic acid)

Reverse-phase solid phase extraction (SPE) was employed to separate the parent compound ([^{3}H]RA) from its oxidation products (mainly [^{3}H]-4-*oxo*- and 4-*hydroxy*-RA) (142,143) in the Folch organic phases of liver samples. All-*trans*-retinoic acid and 4-*oxo*-retinoic acid standards (1.34 nmol each) were added to 300 µL (equal to

approximately 50 mg tissue) of Folch-washed organic phase and dried to completeness under argon at ~37°C. The residue was reconstituted in 1.25 mL of HPLC-grade acetonitrile:water (65:35, v/v) containing 10 mM acetic acid. Supelclean LC-18 SPE columns (Supelco, Bellefonte, PA) were conditioned with 8 mL of 100% methanol followed by 2 mL of water. After conditioning the columns, the samples were loaded on to the column. Using the all-*trans* and 4-*oxo*-RA as standards to monitor separation, polar metabolites were eluted first in 3 x 4 mL of acetonitrile:water (65:35, v/v), while all-*trans*-RA was eluted with 3 x 4 mL of acetonitrile:water (80:20, v/v). The elution rate was maintained at ~1 mL / min with negative pressure using an elution vacuum apparatus (Supelco, Bellefonte, PA). After collection, the samples were dried to completeness and analyzed by liquid scintillation spectrometry as described above.

Real Time PCR

Rat liver total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometry. CYP26A1 mRNA levels will quantitatively assessed by RT-PCR at Penn State's Nucleic Acid Facility on a Perkin-Elmer ABI 7700 system using the TaqMan PCR core reagent kit (Perkin-Elmer, Wellesley, MA). The primers used for CYP26A1 were: forward primer, 5'-AGTGATGGGCGCGGATAAT and reverse primer, 5'-TGCACTGACACCAACCGGT. Ribosomal 18S RNA will be measured simultaneously, thus serving as an internal control for each sample.

Statistics

All results are expressed as means ± SEM. One-way ANOVA was used to test for main effects of dietary vitamin A levels on [^{3}H]RA metabolism. Significant differences by ANOVA were then tested by Fisher's Protected LSD to determine which groups differed significantly, $p < 0.05$, from one another.

RESULTS

VA-status affects plasma and liver retinol levels and hepatic CYP26A1 mRNA expression. Throughout the experiment, the rats grew normally and appeared in good health. Body weight did not differ between the VAD, VAS, and VAD + RP rats, but body weight of the VAM was higher than the other groups (**Table 4**). As compared to the VAS and VAM rats, the relative weight of the liver was significantly reduced in the VAD and VAD + RP rats. In contrast, the relative weight of the lung was significantly increased in the VAD rats as compared to the VAS and VAM rats (**Table 4**). There was no difference due to VA-status in the relative weight of the small intestine. Plasma and liver retinol levels (**Table 4**) were measured as an indicator of VA-status. Plasma retinol averaged 1.26 μmol / L in the VAS rats, which was 10x greater than the amount present in the VAD rats. In contrast, plasma retinol levels in the VAM and VAD + RP rats did not differ from the VAS rats. In this experiment, plasma retinol averaged 1.24 μmol / L in the VAM rats, which was about 2x greater than previous studies involving rats with marginal vitamin A deficiency (213). However, studies by Green and Green (14) have shown that plasma retinol levels can be normal even when liver retinol levels are very low. Liver retinol averaged 378.5 nmol / g in the VAS rats, which was approximately 39, 10, and 7 times greater than the amount of retinol in the livers of the VAD, VAM, and VAD + RP rats, respectively (**Table 4**). Previous work in our laboratory (16) has shown that VA-status affects hepatic CYP26A1 mRNA expression. In this experiment, the VAD rats had significantly less hepatic CYP26A1 mRNA levels than the other groups (**Table 4**), while there was no statistical difference between the VAM, VAD + RP, and VAS groups.

Table 4. Body weights, relative organ weights, plasma and liver retinol concentrations, and CYP26A1 mRNA levels in VAD, VAM, VAS, and VAD + RP rats[1]

	VAD	VAM	VAS	VAD + RP
Body Weight (g)	234.7 ± 12.3	280.7 ± 16.0[a,b]	245.67 ± 5.4	243.5 ± 13.6
Relative Liver Weight[2]	3.49 ± 0.10[a]	3.98 ± 0.15[b]	3.99 ± 0.14[b]	3.55 ± 0.07[a]
Relative Lung Weight[2]	0.52 ± 0.02[a]	0.46 ± 0.02[b]	0.47 ± 0.01[b]	0.50 ± 0.02
Relative Small Intestine Weight[2]	2.20 ± 0.04	2.11 ± 0.05	2.21 ± 0.03	2.22 ± 0.04
Plasma Retinol (µmol/L)	0.12 ± 0.01[a]	1.24 ± 0.01[b]	1.26 ± 0.10[b]	1.38 ± 0.09[b]
Liver Retinol (nmol/g)	9.67 ± 3.73[a]	35.7 ± 7.57[a,b]	378.5 ± 38.3[b]	55.9 ± 8.21[a,b]
CYP26A1 mRNA Expression[3]	-2.49 ± 0.51	-0.21 ± 0.17[b]	0.60 ± 0.09[b]	-0.10 ± 0.02[b]

[1] Values are means ± standard error of the mean, n = 9 (VAD and VAS) or n = 6 (VAM and VAD + RP)

[2] Values are expressed as percent body weight.

[3] CYP26A1 mRNA levels were determined via real-time PCR. Data was log transformed prior to statistical analysis

[a] Statistically different from VAS rats ($p < 0.05$)

[b] Statistically different from VAD treated rats ($p < 0.05$)

VAD increases total radioactivity in plasma but decreases the proportion of [^{3}H]-Aqueous phase metabolites. In all groups examined, greater than 96% of the injected dose was eliminated from plasma within 30 minutes (**Figure 5**). However, total [^{3}H]-retinoids were statistically higher in plasma in the VAD than in the other groups examined at 30 minutes post-injection. Using ANOVA, we showed VA-status to be a significant factor affecting both [^{3}H]-aqueous and [^{3}H]-organic phase metabolites ($P <$ 0.005). As compared to the VAS rats, [^{3}H]-Aqueous phase metabolites were significantly lower in the VAD and VAM groups, and, overall, [^{3}H]-aqueous phase metabolites decreased as a function of VA-status (VAS > VAD + RP > VAM > VAD; **Figure 5**). Interestingly, the amount of [^{3}H]-aqueous phase metabolites recovered in the VAD + RP rats was 1.5x greater than the amount recovered in the VAD rats. In contrast to the [^{3}H]-aqueous phase metabolites, the amount of organic phase radioactivity recovered in the plasma of VAD rats was significantly greater that the amount recovered in the other groups (**Figure 5**). Thus, VA-status affected both the total amount of ^{3}H and the amount of aqueous phase radioactivity recovered in plasma.

VA-status affects the total amount and distribution of [^{3}H]-retinoids in liver. The liver of VAS rats contained approximately 14% of the injected dose as total [^{3}H]-retinoids (**Figure 6A**). As compared to the VAS rats, the amount of total [^{3}H]-retinoids recovered in the liver of VAM and VAD were significantly greater and contained approximately 1.5 and 2.5 times more of the total dose, respectively. In contrast, the amount of total [^{3}H]-retinoids recovered in the liver of VAD + RP rats was not different from the VAS rats (**Figure 6A**). Using ANOVA, we showed VA-status to be a significant factor affecting

both [^{3}H]-aqueous and [^{3}H]-organic phase metabolites ($P < 0.0001$) in liver. As compared to the VAS rats, the amount of [^{3}H]-aqueous phase metabolites recovered in liver were significantly lower in the VAD, VAM, and VAD + RP rats (**Figure 6A**). The VAD + RP rats contained significantly more [^{3}H]-aqueous phase metabolites, which was approximately 60% more, than the amount recovered in the VAD rats. In contrast to the [^{3}H]-aqueous phase metabolites, the amount of [^{3}H]-organic phase metabolites was significantly greater in the VAD rats than in the VAM, VAD + RP, and VAS rats (**Figure 6A**).

The proportion of unmetabolized [^{3}H]RA and [^{3}H]-labeled polar retinoids was determined on liver samples from individual rats. As compared to the VAD rats, unmetabolized [^{3}H]RA was much lower in the liver of VAM, VAS, and VAD + RP rats (**Figure 6B**). Specifically, the VAD rats contained significantly more unmetabolized [^{3}H]RA in liver than the VAM rats, which, in turn, contained significantly more unmetabolized [^{3}H]RA than the VAS and VAD + RP rats. In contrast, total [^{3}H]-labeled polar metabolites recovered in liver were highest in the VAS rats and decreased as a function of VA-status (VAS > VAM ≈ VAD + RP > VAD; **Figure 6B**). Taken together, these results suggest that VA-status affects both the distribution and metabolism of RA in the liver.

RA metabolism and distribution in extrahepatic tissues was affected by VA-status. Two extrahepatic tissues, the lungs and small intestine that are intimately involved in VA homeostasis were examined to determine how VA-status affects the distribution and metabolism of RA. Because of the relatively small amount of total radioactivity

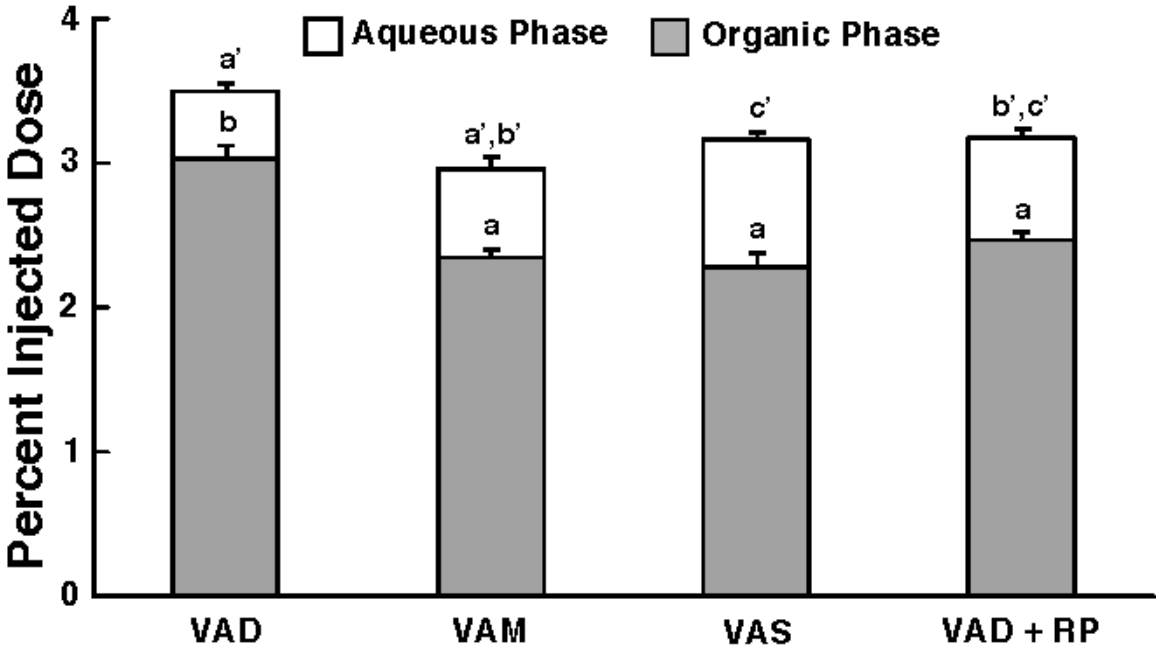

Figure 5. VA-status affects total radioactivity and [^{3}H]-aqueous phase metabolites present in plasma. Female Sprague-Dawley rats (n = 9 for VAD and VAS; n = 6 for VAM and VAD + RP) were fed diets with different amounts of VA as described in the Materials and Methods and injected with [^{3}H]RA (0.023 μM). Plasma was collected 30 minutes later and organic and aqueous phase radioactivity was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

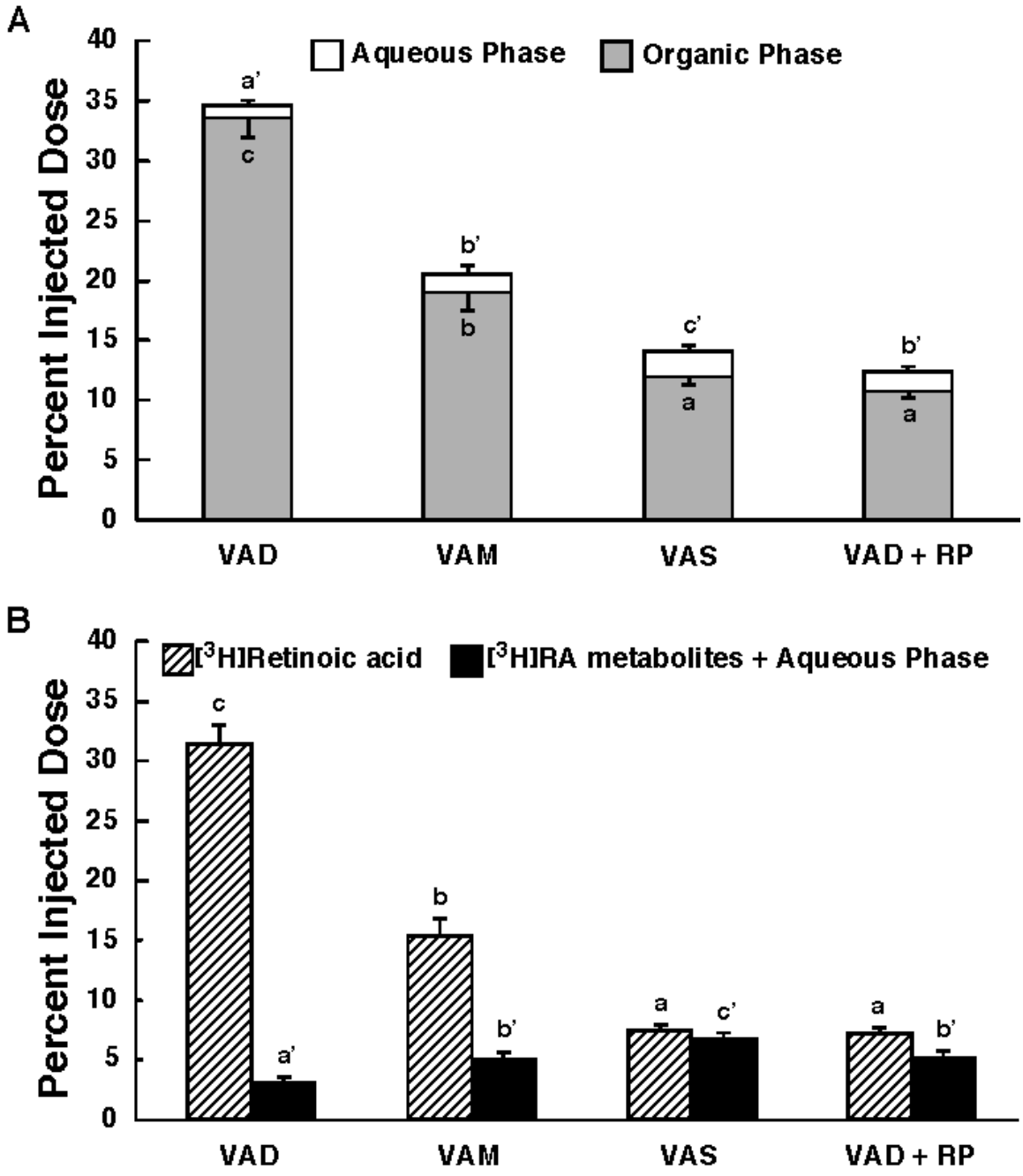

Figure 6. VA-status affects total radioactivity and total [^{3}H]-metabolites present in liver. Female Sprague-Dawley rats (n = 9 for VAD and VAS; n = 6 for VAM and VAD + RP) were fed diets with different amounts of VA as described in the Materials and Methods and injected with [^{3}H]RA (0.023 μM). Tissues were collected 30 minutes later and organic and aqueous phase radioactivity was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

recovered in these two tissues, total ^{3}H-labeled retinoids were only partitioned into and organic and aqueous phase. Less than 0.75% of the injected dose of [^{3}H]RA was recovered in the lungs of VAS rats (**Figure 7**). The total amount of radioactivity recovered in lungs of VAD rats were significantly lower than the VAS rats, while the total amount recovered in the VAM and VAD + RP rats were not different. Using ANOVA, we showed VA-status to be a significant factor affecting [^{3}H]-aqueous phase metabolites in lung. As compared to the VAS rats, the VAD and VAM rats contained significantly less [^{3}H]-aqueous phase metabolites (**Figure 7**). However, the VAD + RP rats contained significantly more [^{3}H]-aqueous phase metabolites than the VAS rats. Thus, unlike the other tissues examined, the highest amount of metabolites recovered in the lung was in the VAD + RP rats than in VAS rats.

Approximately 4.5% of the injected dose of [^{3}H]RA was recovered in the small intestine of VAS rats 30 minutes post-injection (**Figure 8**). As compared to the VAS rats, VAD and VAM rats contained significantly less total ^{3}H in the small intestine, whereas VAD + RP were not statistically different. Using ANOVA, we showed VA-status to be a significant factor affecting both [^{3}H]-aqueous and [^{3}H]-organic phase metabolites ($P < 0.03$) in the small intestine. As compared to the VAS rats, the amount of [^{3}H]-aqueous phase metabolites recovered in small intestine was significantly lower in the VAD rats, but not different in the VAM and VAD + RP rats (**Figure 8**). Similarly, the amount [^{3}H]-organic phase metabolites recovered in the small intestine was lower in the VAD and VAM rats, as compared to the VAS and VAD + RP rats.

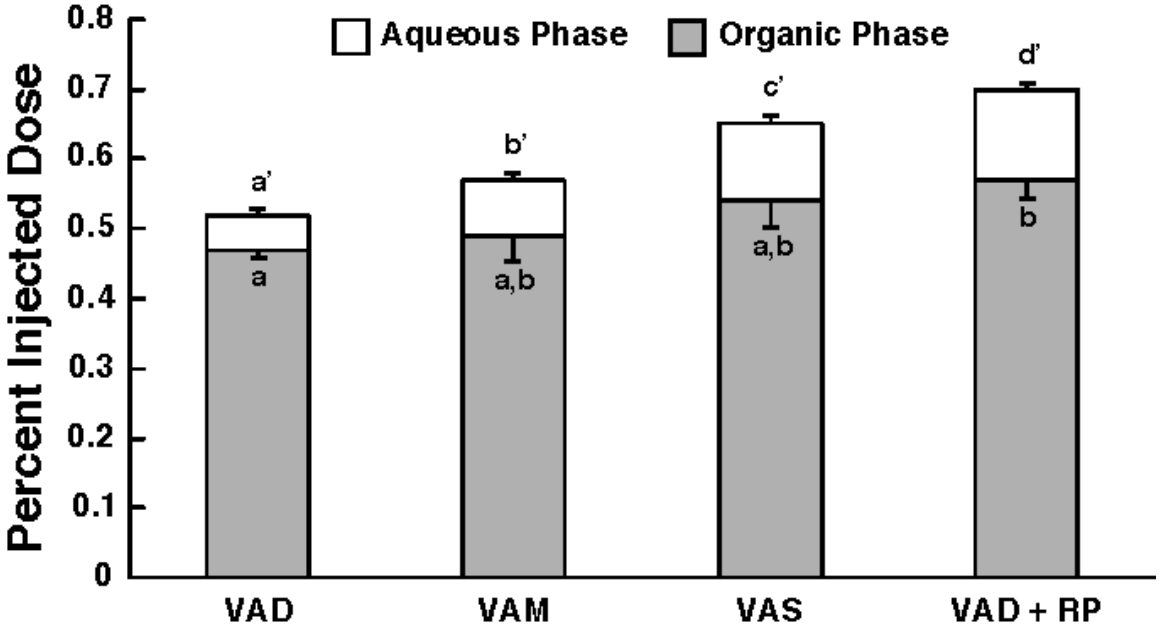

Figure 7. VA-status affects total radioactivity and [^{3}H]-aqueous phase metabolites present in lung. Female Sprague-Dawley rats (n = 9 for VAD and VAS; n = 6 for VAM and VAD + RP) were fed diets with different amounts of VA as described in the Materials and Methods and injected with [^{3}H]RA (0.023 μM). Tissues were collected 30 minutes later and organic and aqueous phase radioactivity was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

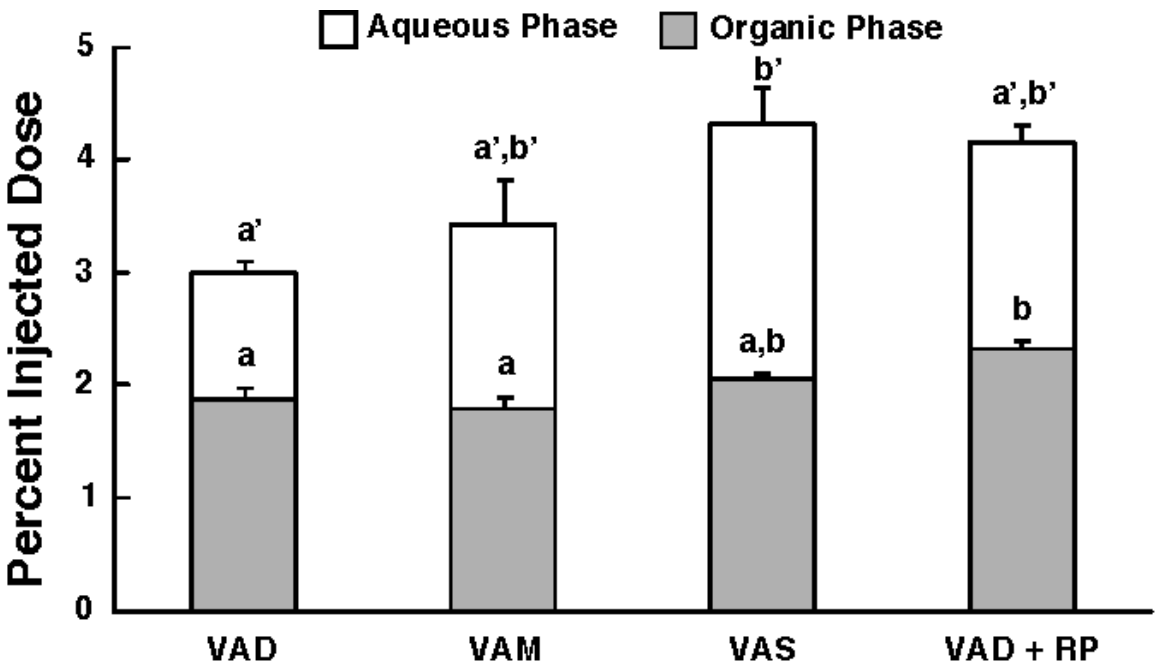

Figure 8. VA-status affects total radioactivity and [^{3}H]-aqueous phase metabolites present in small intestine. Female Sprague-Dawley rats (n = 9 for VAD and VAS; n = 6 for VAM and VAD + RP) were fed diets with different amounts of VA as described in the Materials and Methods and injected with [^{3}H]RA (0.023 μM). Tissues were collected 30 minutes later and organic and aqueous phase was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, $a < b < c$).

DISCUSSION

The current study was conducted to better understand the effect of VA-status on RA homeostasis in rats. To this end, rats were fed diets containing 0 (VAD), 0.412 mg (VAM), or 7.43 mg (VAS) of retinyl palmitate per kilogram of diet in order to establish a range of hepatic VA levels. Moreover, a subset of the VAD group received an oral dose of retinyl palmitate (1.67 μmol, p.o.) to determine how VA repletion influenced RA homeostasis. The amount of VA provided in the diet was similar to that used in previous studies in our lab to deplete hepatic VA stores (13,17,213) and resulted in significant differences in hepatic VA stores (**Table 4**) among the groups. Despite differences in hepatic VA levels, only the VAD rats displayed a significant reduction in plasma retinol levels. This result was surprising since previous studies have shown that a significant reduction (42%) in plasma retinol can occur in VAM rats (213). However, Green and Green (88,89) showed that retinol uptake, recycling, and utilization were different between rats with high and marginal VA stores despite similar plasma retinol levels. Therefore, in the present study, hepatic CYP26A1 mRNA expression was determined to show a functional change with regard to retinoid homeostasis in response to dietary VA manipulation. The results obtained in this study showed that dietary VA intake directly affected CYP26A1 expression, which was similar to a previous study conducted in our lab (16). Taken together, the differences in hepatic VA levels and CYP26A1 expression indicate that both VA status and retinoid homeostasis were different among the groups. By examining the distribution and metabolism of an intravenous dose of [^{3}H]RA in the plasma, liver, lung, and small intestine of VAD, VAM, VAS, and VAD + RP rats, we determined that RA metabolism is affected by VA status.

Plasma [^{3}RA] metabolism is regulated by VA status. Under normal physiological conditions, plasma RA turns over rapidly in both humans and rats (15,264-266) and subsequently appears as various polar metabolites, such as 4-hydroxy-RA, 4-oxo-RA, and retinoyl β-glucuronide (142,166,169). In the present study, greater than 96% of the injected dose of [^{3}RA] was cleared from the plasma in all groups, indicating that plasma RA levels are maintained within a narrow range regardless of VA-status. Despite differences in VA status, plasma [^{3}H]-metabolites were recovered in all groups, confirming the rapid formation of RA metabolites observed previously (169). Plasma [^{3}H]-metabolites were present at a higher level in the VAS rats, while the VAD rats contained the least amount of metabolites. Thus, the injected [^{3}H]RA delivered to the plasma compartment was rapidly cleared and catabolized in all rats, but the amount of [^{3}H]-metabolites recovered was directly affected by the VA status of the rat.

VA-status alters hepatic [^{3}H]RA distribution and metabolism. The liver plays a central role in VA homeostasis by processing dietary VA, storing VA as retinyl esters, synthesizing and secreting plasma retinol-binding protein, and mobilizing retinol for delivery to extrahepatic tissues (267). In view of the liver's importance to VA homeostasis, we were interested in determining the effects of VA-status on the uptake and metabolism of [^{3}H]RA by liver. Previous studies have established that the liver derives a significant amount of RA from the plasma, which is either utilized or recycled (110). On average, approximately 15% of the injected dose was recovered in the liver of VAS rats (**Figure 6A**). In contrast, the livers of VAM and VAD rats contained about 1.3

and 2.3x more of the injected dose, respectively, suggesting the VA-status is inversely related to hepatic RA recycling to plasma. Moreover, the ratio of total [^{3}H]-labeled polar metabolites to [^{3}H]RA was highest in the VAS rats and lowest in the VAD rats. Based on these results, we speculate that two related processes work in concert to cause the observed increase in liver radioactivity and decreased metabolism of RA present in the VAD rats, and to a lesser extent in the VAM rats. First, previous work has shown that the ratio of apo- to holo-cellular retinol binding protein (CRBP) affects retinol metabolism (75,268,269). For instance, as the ratio of apo-CRBP to holo-CRBP increases, retinol storage is inhibited while retinyl ester hydrolysis is increased, whereas if the converse is true retinol storage will increase while retinyl ester mobilization will decrease (268,269). Our results, coupled with the role of CRBP in regulating retinol metabolism, suggest that a parallel mechanism for regulating RA metabolism may also exist in vivo. Specifically, in the VAD rats, we hypothesize that the ratio of apo-cellular retinoic acid binding protein (CRABP) to holo-CRABP is high, resulting in a decrease in hepatic RA metabolism and secretion into plasma, while increasing the residence time of RA in liver. In contrast, the ratio of apo-CRABP to holo-CRABP is lower in the VAS rats, causing an increase in both RA metabolism and the excretion of polar and aqueous-phase metabolites of RA into bile (166,167,169). Second, CYP26A1, a RA-inducible enzyme involved in the degradation of RA (17,150,151) mRNA expression levels are directly related to VA-intake and status (16). In our studies, CYP26A1 mRNA expression was highest in the VAS rats and decreased as a function of VA-status (**Table 1**), confirming our previous results. Additionally, in vitro studies in F9 cells showed that CRABP expression and RA metabolism are correlated (50). Thus, changes in the ratio of

apo- to holo-CRABP and CYP26A1 expression due to VA-status appear to contribute to the regulation of hepatic RA metabolism in order to maintain normal cellular levels of RA.

VA-status affects [^{3}H]RA metabolism in extrahepatic tissues. Previous studies have demonstrated that both lung and small intestine are intimately involved in retinol (67,267) and RA homeostasis (153,267). In terms of RA homeostasis, studies have shown that both the lung and small intestine contain enzymes capable of metabolizing RA (153,270). Therefore, we examined the small intestine and lung for aqueous and organic-phase radioactivity to determine whether VA-status affects RA metabolism in these organs. Similar to liver, the percent injected dose recovered in the lung and small intestine was highest in the VAS rats and decreased as a function of VA-status. Furthermore, the amount of aqueous-phase radioactivity recovered in both organs was lowest in the VAD rats and highest in the VAS rats. Thus, our results suggest that the uptake, recycling, and metabolism of RA by the lung and small intestine is dependent upon VA stores and that the lung and small intestine regulate RA homeostasis in a similar manner to the liver.

VA repletion affects RA distribution and metabolism. In developing countries, nutritional VA-deficiency afflicts millions of children worldwide (256). Deficiency leads to diminished disease resistance (223), prolonged infection (255), and increased mortality (31,220) To combat VA-deficiency, public health programs have been instituted to administer VA supplements to children at risk for VA-deficiency (271). Epidemiological

studies examining the benefits of VA supplements have shown that supplementation is effective in treating measles (272) and diarrhea (257), and leads to a reduction in mortality in children (222). To determine whether VA-supplementation affects RA distribution and metabolism, we compared VAD rats to VAD + RP rats for [^{3}H]RA distribution and metabolism. In the plasma and organs examined we observed two distinct trends with regards to RA homeostasis. First, the total amount of radioactivity recovered in the plasma, liver, lung, and small intestine of the VAD + RP rats was similar to the amount recovered from the VAS rats. Second, the amount of [^{3}H]-metabolites of RA recovered from the VAD + RP was significantly greater than the amount recovered from the VAD rats. Moreover, the amount of [^{3}H]-metabolites recovered from the VAD + RP rats was nearly identical to the amount recovered from the VAM rats, except in the lung, where the VAD + RP rats had a higher amount of aqueous phase radioactivity than the VAM rats. We speculate that the decrease in total radioactivity and increase in RA metabolism observed in the VAD + RP rats was caused by the rapid increase in hepatic VA stores following supplementation. For instance, the sudden increase in hepatic retinol levels could have caused a substantial increase in the hepatic and extrahepatic production of RA from retinol, which would have resulted in a [^{3}H]RA metabolism distribution pattern that was similar to both the VAS and VAM rats.

In conclusion, the results of our study demonstrate that VA status affects both the organ distribution and metabolism of RA in rats. This indicates that the uptake, recycling, and utilization of RA are altered in a tissue-specific manner in response to decreasing stores of vitamin A. Furthermore, the administration of retinyl palmitate to VAD rats, in a 20-h period resulted in increased hepatic stores of retinol, which allowed

them to metabolize the [^{3}H]RA in a manner similar to the VAS rats. Thus, our results suggest that mechanisms regulating RA metabolism are able to adjust rapidly in response to different nutritional states, which helps to maintain homeostatic levels of RA in tissues.

CHAPTER 4:

Exogenous RA can rapidly induce the transcription and translation of CYP26A1 in liver and increase hepatic RA metabolism in rat.

INTRODUCTION

All-*trans*-retinoic acid (RA) is the active metabolite of VA that mediates the majority of VA-dependent functions, such as embryonic development, cellular proliferation and differentiation, testicular function, and immune function (97). RA and its isomers and metabolites have been extensively studied because of their apparent chemorpeventive and therapeutic properties. For instance, RA has been successfully used to treat acute promyelocytic leukemia (3,4), with complete remission occurring in a high proportion of patients (273,274). Additionally, clinical studies have shown that RA and its isomers may reduce the risk of skin, head and neck, lung, and bladder cancers (2,275). However, chronic administration of RA to patients has resulted in a drug-acquired resistance (276). Specifically, pharmacokinetic studies have demonstrated that plasma levels of RA decline rapidly and substantially following long-term drug administration (277). While several mechanisms have been proposed to explain the increase in RA metabolism and clearance in patients receiving long-term RA treatment, the exact molecular mechanism has yet to be delineated.

CYP26A1 is a RA-inducible (150), microsomally located, cytochrome P450 enzyme. In vivo (16) and in vitro (150,151) studies have shown that CYP26A1 is able to metabolize RA to more polar metabolites, such as 4-*hydroxy* and 4-*oxo*-RA. The reactive sites generated by CYP26A1 can be further acted upon to make the substrate more water-soluble, such as by the addition of a molecule of glucuronic acid to form the 4-O-β-glucuronide of 4-hydroxy-RA (155,170,171). CYP26A1 is highly expressed in the liver, but it is also expressed in the duodenum, colon, and placenta (152,179). Thus, CYP26A1 appears to be involved in both hepatic and extrahepatic catabolism of RA through the

generation of both oxidized derivatives and glucuronidated conjugates of RA and their elimination via urine and bile.

Previous work performed in our lab has established that hepatic CYP26A1 mRNA expression is increased following acute RA administration. Yamamoto et al. (16) showed that an acute, intraperitoneal (i.p.) dose of RA significantly increased both hepatic CYP26 mRNA expression and RA metabolism by liver microsomes in control and VA-deficient rats. Subsequently, Wang et al. (17) expanded upon these findings by demonstrating that the induction of CYP26A1 mRNA expression by RA was both time- and dose-dependent *in vivo*. Specifically, the authors showed that a single dose of RA resulted in a significant increase in hepatic CYP26A1 mRNA levels in vitamin A deficient rats by as early as 3 hours post-treatment (17). Additionally, the authors observed that CYP26A1 mRNA increased linearly in response to increasing amounts of exogenous RA (17). In view of these observations, we were interested in determining the rapidity of response to RA in naïve and RA-pretreated rats. The results of these studies demonstrate that a single intravenous dose of RA can increase hepatic CYP26A1 mRNA and protein expression and causes an increase in amount of RA metabolites generated by the liver in both naïve and RA-primed rats. Together, the results suggest that CYP26A1 can respond to exogenous RA rapidly in order to regulate RA concentrations in vivo.

RESEARCH DESIGN AND METHODS

Animals and Diet

All procedures for animal care and use were approved by The Pennsylvania State University. Female Sprague-Dawley (32 days old, Charles River Laboratories, Wilmington, MA) rats were housed in pairs in a room maintained at maintained at 22°C with a 12-h dark:light cycle. Food and water were available ad libitum. The rats were fed a nutritionally complete AIN-93G (260) diet modified to contain 7.43 mg of retinyl palmitate per kilogram of diet (Research Diets, New Brunswick, NJ).

Experimental Design

At 53 d of age, rats were randomly assigned to either the control (naïve) or the all-*trans*-retinoic acid (RA-primed) groups (n = 11 and n = 12, respectively). RA-primed rats received an oral dose of RA (500 μg suspended in 50 μL of canola oil), whereas Naive rats received vehicle alone. Food was removed immediately until the end of the experiment. Sixteen hours post-treatment, the rats were lightly anesthetized by isoflurane-oxygen inhalation and 0.15 mL / 100 g BW of the albumin-bound [^{3}H]RA, prepared as described below, was injected into the exposed left common iliac vein. The incision was closed with a sterile surgical staple and the rats were allowed to recover from the anesthesia. Rats were killed 30, 60, and 90 minutes (n=3/group) post-injection by carbon dioxide asphyxiation. Blood was drawn from the vena cava in heparinized syringes and the liver, lung, spleen and small intestine were quickly removed, weighed, and aliquots frozen in liquid nitrogen for storage at -80°C before subsequent analysis. Because RA is rapidly metabolized, it is important to collect tissues quickly (140,166); thus, each rat was

treated on a defined schedule so that all tissues could collected and frozen within 5 minutes or less after euthanasia.

Previous studies preformed by our lab (17) showed that the maximal induction of CYP26A1 mRNA occurred between 6 and 10 hours following an oral dose of RA. Therefore, 3 additional rats received an oral dose of RA (500 μg in 50 μL of canola oil) and were killed 6 hours later by carbon dioxide asphyxiation. These rats served as our 6-hour positive controls for CYP26A1 expression in the subsequent studies.

Dose Preparation

All procedures involving retinoids were carried out in either dim or yellow light. The doses of RA for i.v. injection were prepared fresh immediately prior to injection. To prepare a 1-mL aliquot, 0.23 μmol of all-*trans*-RA was evaporated under argon until < 10 μL remained. Next, 5 μL of Tween 20 (Sigma-Aldrich, St. Louis, MO) and 0.9 mL of 0.1% rat serum albumin (Calbiochem, San Diego, CA, diluted in sterile PBS) were added, and the mixture was vortexed. Then, 0.10 mL of whole rat plasma was added to the sample and immediately vortexed. The final concentration of RA in the dose was 0.023 mM, and the dose delivered to each rat was standardized at 0.15 ml (33 nmol RA) / 100 g BW. Following the preparation and administration of each dose, aliquots were analyzed by HPLC (261), which showed them to be > 97% all-*trans*-RA (data not shown).

Real Time PCR

Liver CYP26A1 mRNA levels were quantitatively assessed by RT-PCR as described above (see Chapter 3).

Microsomal Preparation and CYP26A1 Western Analysis

Liver microsomes were isolated from 2 g of frozen tissue via differential centrifugation as previously described (54). Protein levels were determined using the Bradford reagent (BioRad Laboratories, Hercules, CA). The CYP26A1 antibody (Alpha Diagnostic International, San Antonio, TX) is a polyclonal antibody from rabbit designed against a 15 amino acid peptide from the N-terminal region of human CYP26A1. The binding of primary antibodies was visualized using peroxidase-conjugated goat anti-rabbit IgG.

Briefly, 30 µg of microsomal protein was dissolved in 2x sodium dodecyl sulfate sample buffer, boiled for 10', and loaded onto a non-denaturing 10% polacrylamide gel for separation (21). Proteins were electroblotted to a nitrocellulose membrane overnight at 20 V. Blots were blocked with 50 mL of 3% nonfat milk in TBS-T (10 mM Tris-HCl, ph 8.0, 150 mM NaCl, and 0.05% Tween 20) for 4 hours at room temperature. Next, the membrane was incubated with the primary antibody (1:1000 dilution in 3% nonfat milk) for 20 h at 4˚ C. Following washing, the blots were incubated with the peroxidase-conjugated goat anti-rabbit IgG and visualized with ECL (Pierce Biotechnology, Rockford IL) solution. Proteins were visualized on Hyperfilm (Amersham Biosciences, Piscataway, NJ) and bands were scanned (OfficeJet G55, Hewlett Packard) and protein expression was semi-quantitatively determined using NIH Image. Microsomal protein

from the 6-hour control rats was analyzed on every blot as a control and naïve and RA-primed CYP26 protein expression was normalized by the mean of the 6-hour control protein expression.

Enzyme Assay

Retinoic acid metabolizing activity was measured as described by Yamamoto et al. (16). Briefly, 200 μg of microsomal protein was incubated with 45 nM of (11,12-[^{3}H(N)]-retinoic acid (specific activity, 1.96 TBq / nmol, from PerkinElmer, Boston, MA) in 0.25 mL of buffer containing 150 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4) and an NADPH regenerating system (2.5 units of glucose 6-phosphate dehydrogenase, 500 nmol of NADP, and 0.5 μmol of glucose-6-phosphate). The reaction was initiated by adding the [^{3}H]RA, incubated at 37° C for 50 minutes, and terminated by the addition of 5 mL of chloroform:methanol (2:1, v/v). The radiolabeled RA was extracted using a modification of the method of Folch et al. (262,263). Subsequently, aliquots of the Folch organic phase were subjected to solid phase extraction to separate the parent compound ([^{3}H]RA) from its oxidation products. The organic and aqueous phases, along with the samples obtained from the solid-phase extraction, were analyzed by liquid scintillation spectrometry using a Beckman LS-6500 counter (Beckman Coulter, Fullerton, CA). Please refer to Chapter 3 for complete experimental details.

Statistics

All results are expressed as means ± SEM. Two-way ANOVA was used to test for main effects and interaction of RA treatment and time on CYP26A1 expression and

activity. Significant differences by ANOVA were then tested by Fisher's Protected LSD to determine which groups differed significantly, $p < 0.05$, from one another.

RESULTS

Status of rats. Rats grew normally and appeared in good health, and groups did not differ in body weight at the time of treatment. Sixteen hours later, after treatment and an overnight fast, there was no difference in final body weight (data not shown). Similarly, there were no differences due to treatment in the relative weights of the liver and lung.

CYP26A1 mRNA levels increase in response to a low dose of intravenous RA in both naïve and RA-primed rats. Using two-way ANOVA, we showed both RA-pretreatment and time to be significant factors, and there was an interaction ($P < 0.001$). As compared to the naïve rats, CYP26A1 mRNA levels were approximately 60x greater in the RA-primed rats at t= 0 (**Figure 9**). Similarly, CYP26A1 mRNA levels were significantly greater in the RA-primed rats than in the naïve rats at 30 minutes, but there was no difference between the groups at 60 and 90 minutes. In response to an i.v. injection of 20 μg of RA, CYP26A1 mRNA levels increased over time in both groups (**Figure 9**). The increase of CYP26A1 mRNA, as determined by the slope of the line, from 30 to 60 minutes and from 60 to 90 minutes in the naïve rats was nearly identical to the rate of increase from 0 to 30 minutes and from 30 to 60 minutes in the RA-primed rats, respectively (**Figure 9**). Therefore, in terms of mRNA levels the initial response to i.v. RA was more rapid in the RA-primed rats than in the naïve rats. However, both the rate and magnitude of the increase in CYP26A1 mRNA levels in the naïve rats was similar to the RA-primed rats following the initial 30-minute delay.

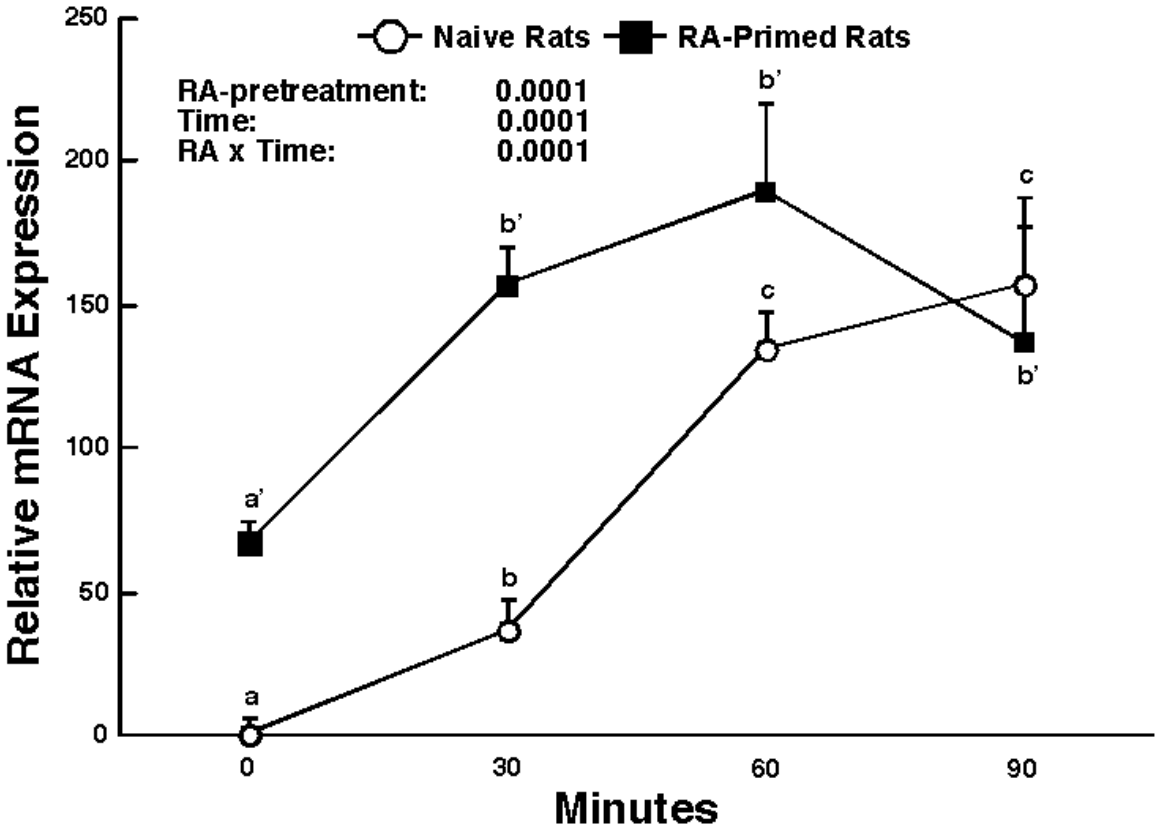

Figure 9. Intravenous RA administration increases CYP26A1 mRNA expression in Naïve and RA-primed rats. Female Sprague-Dawley rats were treated with RA (RA-primed) or oil (Naïve) as described in Materials and Methods, and RA (0.023 mM) bound to rat serum albumin was injected i.v. 16 hours later. Livers were collected 30 (n = 3/group), 60 (n = 3/group), or 90 (n = 3/group) minutes later and CYP26A1 mRNA expression was determined by real-time PCR. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences within treatment groups ($p < 0.05$, $a < b < c$).

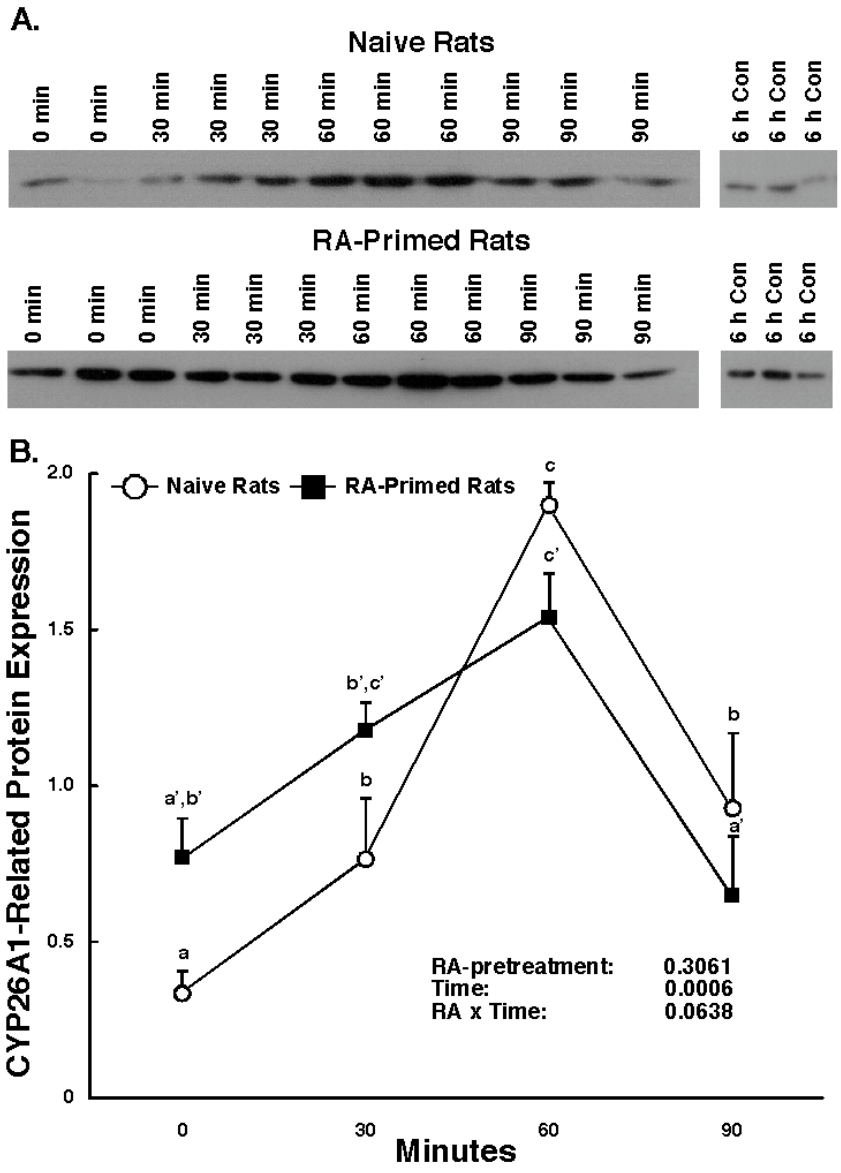

Figure 10. CYP26A1-related protein expression is increased in Naïve and RA-primed rats following intravenous RA administration. Female Sprague-Dawley rats were treated with RA (RA-primed) or oil (Naïve) as described in Materials and Methods, and RA (0.023 mM) bound to rat serum albumin was injected 16 hours later. Livers were collected 30 (n = 3/group), 60 (n = 3/group), or 90 (n = 3/group) minutes later and CYP26A1-related protein expression was determined by Western blot. Naïve and RA-primed protein expression was normalized by the average expression of the 6 hr control rat, which were analyzed on both Western blots. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences within treatment groups ($p < 0.05$, $a < b < c$).

CYP26A1-related protein levels increase in response to a low dose of intravenous RA in both naïve and RA-primed rats. In rat, CYP26A1 is a 497 amino acid polypeptide with a molecular weight of approximately 50 – 55 kD (17,151,152). Since cytochrome P450 enzymes are thought to be associated with and most active in the microsomal fraction (147,278), we attempted to determine CYP26A1 expression in the microsomal fraction of both naïve and RA-primed rat livers using Western analysis. During our analyses, we detected and quantified a single polypeptide band at approximately 40 kD (**Figure 10A**) using a commercially available antibody against human CYP26A1 (Alpha Diagnostic International, San Antonio, TX). The expression of this 40 kD protein increased in response to RA as expected (16,17) in both the naïve and RA-primed rats. However, because the size of our band is about 10 kD smaller the reported molecular weight of CYP26A1, we can not conclusively determine if the polypeptide we detected is in fact CYP26A1. Therefore, throughout the remainder of this paper we will refer to the protein detected on our Western as CYP26A1-related protein.

Using two-way ANOVA, we showed time to be a significant factor affecting CYP26A1-related protein expression, whereas RA-pretreatment was not a factor. As compared to the naïve rats, CYP26A1-related protein levels were significantly greater in the RA-primed rats at t = 0, while there was no statistical difference between the groups at 30, 60, and 90 minutes (**Figure 10B**). In both the naïve and RA-primed rats, CYP26A1-related protein levels increased significantly between 0 and 60 minutes post-injection. However, by 90 minutes CYP26A1-related protein levels had returned to control levels in RA-primed rats, while in the naïve rats the levels had returned to the levels observed at 30 minutes (**Figure 10B**). The increase of CYP26A1-related protein

levels from 0 to 30 minutes was similar in both groups, but between 30 and 60 minutes the increase of CYP26A1-related protein levels in the naïve rats was twice as fast as the rate in RA-primed rats.

Regression analysis indicated that the increase in CYP26A1 mRNA and related-protein levels in response to i.v. RA in naïve ($R^2 = 0.84$) and RA-primed ($R^2 = 0.75$) rats are linearly correlated between 0 and 60 minutes (**Figure 11**). The calculated y-intercept is nearly identical in the Naïve ($y = 0.39$) and RA-primed rats ($y = 0.33$). This indicates that, in the absence of RA, the basal levels of CYP26A1 protein are not different between the groups.

Retinoic acid metabolizing activity increased in response to i.v. RA in both Naïve and RA-primed rats. Using two-way ANOVA, we showed RA-pretreatment to be a significant factor affecting RA metabolizing activity, whereas time was not a factor. RA-metabolizing activity was approximately 3x greater in the RA-primed rats than in the naïve rats at t = 0 (**Figure 12**). While not statistically different, the average formation of [^{3}H]-metabolites of RA was greater in the RA-primed rats than in the naïve rats at 30, 60, and 90 minutes post-injection. As compared to the t = 0 rats, the amount of [^{3}H]-metabolites of RA generated by rat liver microsomes increased over time in both the naïve and RA-primed rats (**Figure 12**). The increase in [^{3}H]-metabolite production was linear in both the naïve ($R^2 = 0.957$) and RA-primed rats ($R^2 = 0.77$). Furthermore, the rate of [^{3}H]-metabolite formation, as determined by the slope of the line, was similar in both groups.

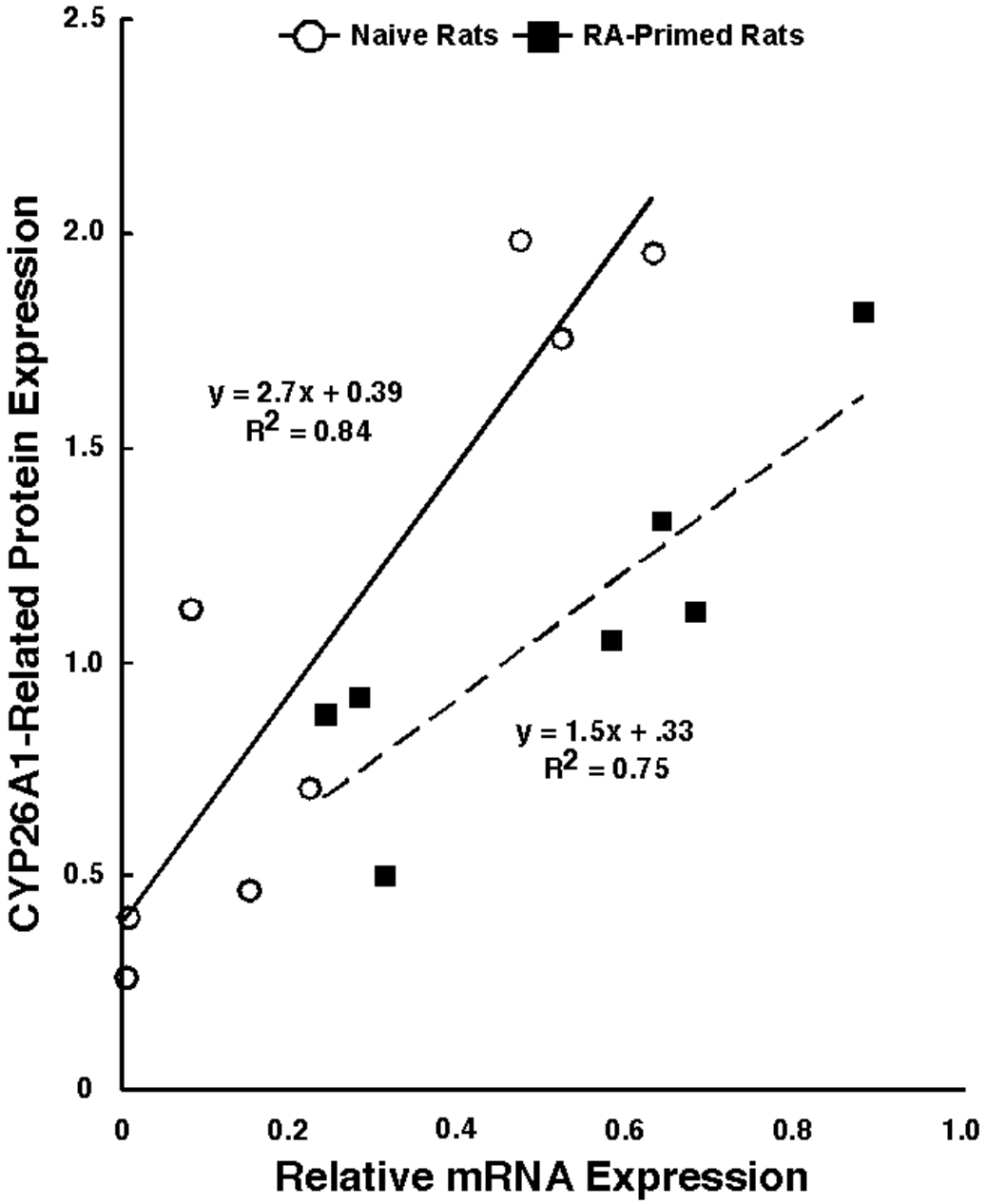

Figure 11. CYP26A1 mRNA and CYP26A1-related protein levels in liver are correlated from 0 to 60 minutes. Female Sprague-Dawley rats were treated with RA (RA-primed) or oil (Naïve) as described in Materials and Methods, and RA (0.023 mM) bound to rat serum albumin was injected 16 hours later. Livers were collected 30 (n = 3/group), 60 (n = 3/group), or 90 (n = 3/group) minutes later and CYP26A1 mRNA and protein expression were determined by real-time PCR and western blot, respectively, and regression analysis was performed using Super ANOVA.

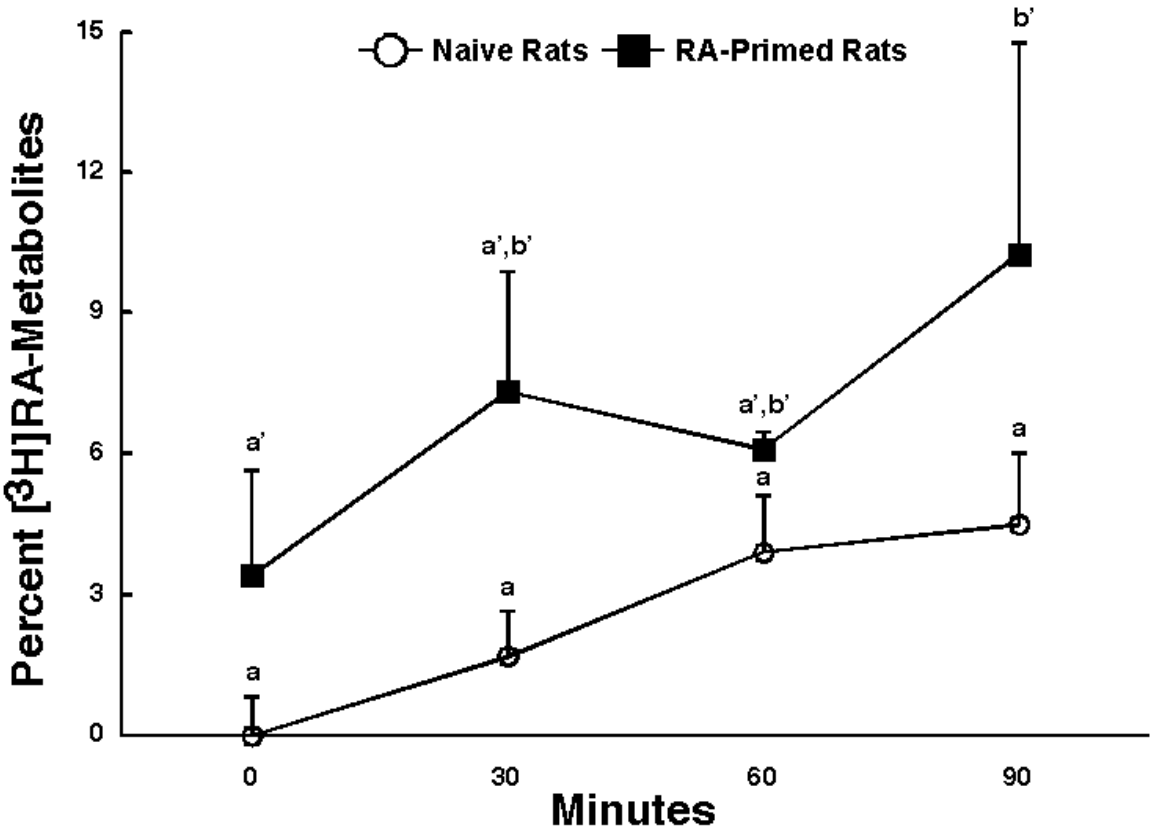

Figure 12. RA metabolism increases in Naïve and RA-primed rats following i.v. RA administration. Female Sprague-Dawley rats were treated with RA (RA-primed) or oil (Naïve) as described in Materials and Methods, and RA (0.023 mM) bound to rat serum albumin was injected 16 hours later. Livers were collected 30 (n = 3/group), 60 (n = 3/group), or 90 (n = 3/group) minutes later and RA-metabolizing activity was determined in microsomes as described in Materials and Methods. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences within treatment group ($p < 0.05$, a < b < c).

DISCUSSION

Retinoids, including RA, have been studied extensively for their ability to treat premalignant lesions and to prevent carcinogenesis. For instance, acute promyelocytic leukemia is distinctively sensitive to oral RA treatment, with complete remission occurring in a high proportion of patients (279,280). Under normal conditions, RA is found at very low concentrations in plasma (4 – 14 nmol/L) of humans (108,109). Following a single dose of RA, peak plasma levels are achieved between 1 – 4 hours post-treatment (281) with a first-order terminal half-life of approximately 45 minutes (15,277). However, several pharmacokinetic studies have shown that chronic RA administration leads to lower peak plasma concentrations and results in clinical resistance to RA in humans (264,265,277). Specifically, plasma RA levels decreased significantly following 7 days of continuous treatment, with a majority of patients displaying RA resistance within 2 days (277,282). Currently, it is believed that RA-metabolizing enzymes, especially the RA-inducible enzyme CYP26A1, are induced during RA treatment and contribute to the clinical resistance observed. Thus, the present study was conducted to expand our understanding of the ability of an intravenous dose of RA to induce hepatic CYP26A1 in naïve and RA-primed rats.

Intravenous RA induces CYP26A1 mRNA and CYP26-related protein levels in both naïve and RA-primed rats. Pharmacokinetic and biochemical studies have shown that both acute and chronic RA administration results in the induction of specific cytochrome P450 enzymes and an increase in RA metabolism (17,142,143,180,181). Moreover, previous studies conducted in our lab have shown that RA can induce CYP26A1 mRNA

expression in VA-deficient rats within 3 hours (17). However, the rapidity of response of CYP26A1 to RA has yet to be fully examined in vivo. In our study, the intravenous dose of 0.23 mM RA significantly increased CYP26A1 mRNA levels within 30 minutes, and remained higher than control levels for the duration of the study (**Figure 10**). Furthermore, this induction in CYP26A1 mRNA expression occurred irrespective of RA-pretreatment. Similar to CYP26A1 mRNA, intravenous RA treatment increased hepatic CYP26A1-related protein expression by 30 minutes in both the naïve and RA-primed rats (**Figure 11**). Additionally, CYP26A1 mRNA and CYP26A1-related protein levels were directly correlated in both the naïve and RA-primed rats between 0 and 60 minutes (**Figure 12**). In contrast to the induction in mRNA expression, CYP26A1-related protein levels returned to (RA-primed) or near (naïve) levels within 90 minutes. Our results confirm and expand upon previous studies by suggesting that: (*a*) exogenous RA alters hepatic RA homeostasis, (*b*) which leads to RA binding to retinoic acid receptors associated with a canonical retinoic acid response element in the promoter region of the CYP26A1 gene (178) (*c*) that, in turn rapidly activates CYP26A1 transcription (*d*) resulting in increased mRNA expression, (*e*) which ultimately leads to increased CYP26A1-related protein translation. Thus, the results presented here clearly show that a single exogenous RA can rapidly increase the transcription of CYP26A1 mRNA and translation of CYP26A1-related protein, regardless of previous RA treatments.

Intravenous RA induces hepatic RA-metabolism in rats. RA is rapidly metabolized in vivo in order to maintain homeostatic concentrations of RA in plasma and tissues. To determine whether the increase in CYP26A1 mRNA and CYP26A1-related protein levels

caused an increase in RA metabolism, we conducted enzyme assays using liver microsomes from naïve and RA-primed rats. Despite substantial variation within the groups examined, our results show that the average formation of [^{3}H]-metabolites of RA was greater in the RA-primed rats than in the naïve rats and that the amount of [^{3}H]-metabolites of RA generated by rat liver microsomes increased over time. Thus, the results presented here demonstrate that the ability of exogenous RA to induce its own metabolism occurs rapidly and that the rapid induction of mRNA and protein had a functional outcome in vivo.

In conclusion, the results of our study demonstrate that CYP26A1 mRNA expression, CYP26A1-related protein expression, and hepatic RA-metabolizing activity all rapidly increased in response to i.v. RA in both naïve and RA-Primed rats. While pretreatment with RA increased basal CYP26A1 mRNA expression, CYP26A1-related protein expression, and hepatic RA-metabolizing activity, the rate of mRNA, protein, and enzymatic activity increase was similar in both groups. Thus, the results presented here demonstrate that the liver maintains RA homeostasis by quickly responding to rapid changes in RA concentrations.

CHAPTER 5:

All-*trans*-retinoic acid distribution and metabolism in vitamin A-marginal rats*

* The results presented in this chapter were published in the American Journal of Physiology
Cifelli CJ and Ross AC. All-trans-retinoic acid distribution and metabolism in vitamin A-marginal rats. *Am J Physiol Gastrointest Liver Physiol*. 2006 Aug; 291(2): G195-202.

INTRODUCTION

Vitamin A and its active metabolites are essential for normal immune competence, and vitamin A deficiency is well known to increase the severity of infectious diseases (220,283). Conversely, retinoids (all-*trans*-retinoic acid (RA), cis isomers, metabolites, and synthetic analogs) have been studied extensively for their ability to treat or prevent numerous disease conditions. Cis and trans isomers of RA are used therapeutically for diseases of the skin, such as cystic acne and hyperkeratotic disorders (1), and all-trans-RA has proved successful in the treatment of acute promyelocytic leukemia (3,4). Similarly, retinoids may reduce the risk of other cancers (2). Moreover, RA may possess immune adjuvant properties, suggesting that it could be administered in conjunction with vaccines to prevent or reduce the severity of various infections (5-7). Surprisingly, however, few studies have addressed the effects of disease states on the metabolism of RA.

Under normal physiological and dietary conditions, RA is present in the circulation at very low levels, in the range of ~4 – 14 nmol/L in humans (108,109) and rats (167), which is approximately 0.2 – 0.7% of plasma retinol levels. Even when RA is administered in pharmacological doses, plasma RA remains quite low (266,284) and turns over rapidly, with a turnover time of approximately 2 minutes in vitamin A-adequate rats (110), and a first-order terminal half-life of approximately 45 minutes in rhesus monkeys given large doses of either all-trans-RA or 9-cis-RA, and in humans (15,265,277). Despite the low concentration and rapid turnover of RA, plasma RA contributes significantly to tissue retinoid pools in a manner that is organ-specific. In rats studied under steady-state conditions, the liver derived more than 75% of its RA pool

from plasma, suggesting that the delivery of RA from plasma to liver is an important process for maintaining RA concentrations in the normal, homeostatically regulated range (110). The liver and biliary system also play central roles in the catabolism and excretion of retinoids (164,169). RA is capable of inducing its own metabolism (16,17,151), and therefore RA turnover is likely to increase during RA therapy.

The acute phase response to inflammation results in marked alterations in hepatic protein synthesis and energy metabolism, which in turn widely affect other organ systems. The levels of albumin, retinol-binding protein (RBP), and other nutrient transport proteins are reduced (212), while other plasma proteins are elevated, during inflammation (211). Previous studies of vitamin A homeostasis during inflammation have established that inflammation induces a state of low plasma retinol (hyporetinolemia), similar to that observed during natural infections, including measles and diarrhea (22-24). In rats with lipopolysaccharide (LPS)-induced inflammation, the level of RBP mRNA in the liver was reduced prior to the reduction in the concentration of the RBP:retinol complex in plasma (214). In studies of similar design, hepatic retinol was increased (226), suggesting that inflammation impairs the transport of retinol from the liver to plasma. Moreover, in rats with marginal vitamin A status the reduction in plasma due to marginal vitamin A status was further compounded by inflammation (213). However, studies have not yet addressed the effects of acute inflammation on the metabolism of plasma RA, the active metabolite of retinol, which mediates the majority of physiological functions of vitamin A.

In the present study, we have used LPS-induced inflammation in rats with marginal vitamin A deficiency, a model of subclinical vitamin A deficiency that affects

many children in the developing world where infection and inflammation are also widespread (223), to study the effects of acute, non-septic inflammation on the distribution and catabolism of plasma RA. We hypothesized that LPS will alter the distribution of RA between plasma and tissues, especially the liver which is centrally involved in retinoid metabolism (267,285). Because RA itself can offset the effects of vitamin A deficiency (8,9), significantly alter the metabolism of retinol (13,286), and induce its own catabolism *in vitro* and *in vivo* (143,166,287), we designed a 2x2 factorial study to examine the effects of RA alone, LPS-induced inflammation alone, and both in combination on RA homeostasis. The results of these studies demonstrate that RA and LPS independently affect the distribution and the metabolism of RA in a tissue specific manner, and that RA and LPS are, for the most part, independent factors in the regulation of RA homeostasis.

RESEARCH DESIGN AND METHODS

Animals and Diet

All procedures for animal care and use were approved by The Pennsylvania State University. Female Sprague-Dawley (Charles River Laboratories, Boston, MA) rats with litters of 12 female pups were fed a vitamin A-deficient diet from the time of arrival to reduce the accumulation of vitamin A from milk by their nursing offspring (259). The rats were housed in a room maintained at 22°C with a 12-h dark:light cycle, and food and water were available ad libitum. When the pups were 21 days old, they were weaned in pairs and fed a nutritionally complete AIN-93G diet (260) modified to contain 0.412 mg of retinyl palmitate per kilogram of diet (Research Diets, New Brunswick, NJ). This amount of vitamin A was shown previously to be adequate for normal growth but result in plasma retinol levels about half that of control-fed rats (213).

Experimental Design

When the rats were between 54 and 60 d of age, they were randomly assigned to one of four treatment groups (n=9 / group): Control, RA alone, LPS alone, or RA+LPS. Rats treated with RA were given an oral dose of 500 μg all-trans-RA (Sigma-Aldrich, St. Louis, MO) prepared in ~30 μl of vegetable oil (17), and all other rats received an equal amount of oil only. Rats treated with LPS received an i.p. injection of 50 μg / 100 g of body weight (BW) of *Pseudomonas aeruginosa*-LPS (List Biologicals, Campbell, CA) (50, 51), while all other rats received an equal volume of sterile phosphate-buffered saline (PBS). Food was removed immediately after treatment until the end of the 12-hour experimental period (214). Twelve hours post-treatment, the rats were lightly

anesthetized by isoflurane-oxygen inhalation and 0.15 mL / 100 g BW of the albumin-bound [^{3}H]RA, prepared as described below, was injected into the exposed left common iliac vein. The incision was closed with a sterile surgical staple and the rats were allowed to recover from the anesthesia. Rats were killed either 10 minutes (n = 5 / group) or 30 minutes (n = 4 / group) post-injection by carbon dioxide asphyxiation. Blood was drawn from the vena cava in heparinized syringes and the liver, lung, spleen and small intestine were quickly removed, weighed, and aliquots frozen in liquid nitrogen for storage at -80°C before subsequent analysis. Because RA is rapidly metabolized, it is important to collect tissues quickly (140,166); thus, each rat was treated on a defined schedule so that all tissues could collected and frozen within 5 minutes or less after euthanasia. The 10 and 30 minute times chosen for this experiment were based upon previous reports (110) and pilot work (data not shown) that showed that >95% of the [^{3}H]RA dose was cleared from plasma within 10 minutes and that metabolism of [^{3}H]RA to polar metabolites could be readily detected by 30 minutes.

Dose Preparation

All procedures involving retinoids were carried out in either dim or yellow light. The doses of [^{3}H]RA for i.v. injection were prepared fresh immediately prior to injection. To prepare a 1-mL aliquot, 10 μCi of [^{3}H]RA ([11,12(n)-^{3}H]retinoic acid, specific activity, 1.96 TBq / nmol, from PerkinElmer, Boston, MA) was combined with 0.23 μmol of all-*trans*-RA and the solvent was evaporated under argon until < 10 μL remained. Next, 5 μL of Tween 20 (Sigma-Aldrich, St. Louis, MO) and 0.9 mL of 0.1% rat serum albumin (Calbiochem, San Diego, CA, diluted in sterile PBS) were added, and

the mixture was vortexed. Then, 0.10 mL of whole rat plasma was added to the sample and immediately vortexed. The final concentration of RA in the dose was 0.023 mM, and the dose delivered to each rat was standardized at 0.15 ml (33 nmol RA) / 100 g BW. Following the preparation and administration of each dose, aliquots were analyzed by HPLC (261), which showed them to be > 97% [^{3}H]RA (data not shown).

Determination of Tissue Radioactivity

Aliquots of individual plasma, liver, lung, and small intestine samples were extracted using a modification of the total lipid extraction method of Folch et al. (262,263) as described in Aim 2. Aliquots of the injected dose, along with the organic and aqueous phases from each tissue were analyzed by liquid scintillation spectrometry using a Beckman LS-3801 counter (Beckman Coulter, Fullerton, CA). Please refer to Chapter 3 for complete experimental design.

Separation of RA from polar organic metabolites (4-oxo- and 4-hydroxy-retinoic acid)

Reverse-phase solid phase extraction (SPE) was employed to separate the parent compound ([^{3}H]RA) from its oxidation products (mainly [^{3}H]-4-*oxo*- and 4-*hydroxy*-RA) (17, 48) in the Folch organic phases of liver and pooled plasma samples. Please see Chapter 3 for complete experimental details.

Statistics

All results are expressed as means ± SEM. Two-way ANOVA was used to test for main effects and interaction of RA treatment and LPS treatment on [^{3}H]RA

metabolism. Significant differences by ANOVA were then tested by Fisher's Protected LSD to determine which groups differed significantly, $p < 0.05$, from one another.

RESULTS

Inflammation- and RA-induced changes in organ weight and plasma retinol. Rats grew normally, appeared in good health, and groups did not differ in BW at the time of treatment (**Table 5**). Twelve hours later, after treatment and an overnight fast, all groups lost ≤ 8.2 g of body weight (**Table 5**). There were no differences due to treatment in the relative weights of the lungs and small intestine. The relative weight of the spleen was higher in LPS-treated rats, indicative of mild inflammation, and the relative weight of the liver of rats treated with RA+LPS was slightly increased (**Table 5**). There was no difference in liver vitamin A, which averaged from 17 to 21 nmol / g (**Table 5**). Plasma retinol (**Table 5**) was measured as an indicator of vitamin A status and to confirm the effect of LPS-induced inflammation on plasma retinol. Plasma retinol averaged 0.54 μmol / L in the control group, which was similar to previous studies of rats with marginal vitamin A deficiency (213), but it was only 42% of that in vitamin A-sufficient rats (214). Plasma retinol was further reduced by 47% in rats with LPS-induced inflammation compared to the marginally vitamin A-deficient control group, whereas RA treatment did not affect plasma retinol in this 12-hour study (**Table 5**). There was no difference due to time (10 minutes versus 30 minutes) in the sum of the total [^{3}H]-retinoids recovered in the plasma, liver, lung, and small intestine (data not shown). However, LPS decreased the sum of the total [^{3}H]-retinoids recovered in the plasma, liver, lung, and small intestine as compared to RA-treated rats (**Table 5**).

Table 5. Body weights, relative organ weights, and plasma retinoid concentrations in RA- and LPS-treated rats[1]

	Control	RA	LPS	RA+LPS
Body weight at dosing (g)	233.8±9.80	224.2±8.67	212.9±5.74	217.9±7.36
Change in body weight (g/12 hr)	-6.8±1.0	-6.4±0.9	-8.0±1.0	-8.2±1.0
Liver weight (%BW[2])	3.64±0.06	3.76±0.12	3.91±0.11	4.03±0.10[a]
Lung weight (%BW)	0.51±0.02	0.53±0.01	0.54±0.02	0.53±0.02
Spleen weight (%BW)	0.22±0.01	0.22±0.01	0.27±0.01[a,b]	0.29±0.01[a,b]
Small Intestine weight (%BW)	2.29±0.09	2.41±0.09	2.43±0.13	2.43±0.14
Plasma retinol (μmol/L)	0.54±0.06	0.44±0.06	0.30±0.04[a,b]	0.29±0.04[a,b]
Liver total retinol (nmol/g)[3]	17±11	21±6	18±5	14±17
Total [^{3}H]-labeled retinoids (% Injected Dose)[4]	30.0±1.23	33.3±1.38	26.5±1.16[b]	28.1±1.15[b]

[1] Values are means ± standard error of the mean, n = 9/group

[2] BW, body weight

[3] Mean ± range of two representative rats per group

[4] Sum of plasma, liver, lung, and small intestine total [^{3}H]-retinoids, expressed as percent injected dose (ID)

[a] Statistically different from control rats ($p < 0.05$)

[b] Statistically different from RA-treated rats ($p < 0.05$)

RA alone and in combination with LPS increases the total radioactivity in plasma and the proportion of [^{3}H]-RA metabolites. In control rats, greater than 95% of the injected dose was eliminated from plasma within 10 minutes (**Figure 13A**). In comparison, total [^{3}H]-retinoids were higher in plasma in the RA, LPS, and RA+LPS groups at this time. By two-way ANOVA, both RA and LPS were significant factors, but there was no interaction. By 30 minutes, total [^{3}H]-retinoids in plasma were similar in the control group and the LPS-treated group, but was higher in the RA group (**Figure 13B**). Furthermore, rats treated with RA+LPS had significantly more (8.5%) of the injected ^{3}H dose in plasma, which was approximately twice the amount in the control group ($P <$ 0.01).

Total [^{3}H]-polar metabolites in plasma, representing lipid-soluble metabolites of RA and its aqueous metabolites combined, were determined on a pooled sample from each treatment group at each time. [^{3}H]-Polar metabolites were increased by RA, as compared to the control group, at both 10 minutes and 30 minutes. At 10 minutes about 60% of the total [^{3}H]-retinoids recovered in plasma already consisted of [^{3}H]-polar metabolites, which increased to 75% at 30 minutes (**Figure 13C and D**). In the plasma of LPS-treated rats, [^{3}H]-polar metabolites were elevated 10 minutes post-injection, but equal to the control level by 30 minutes. In rats treated with the combination of RA+LPS, the proportion of [^{3}H]-polar metabolites was highest (**Figure 13C and D)**, representing approximately 68 and 82% of total [^{3}H]-retinoids at 10 and 30 minutes, respectively. Thus, RA and the combination of RA+LPS resulted in a 2- to 3-fold increase in plasma total ^{3}H, compared to the control group, and the increase was mostly in the form of polar metabolites.

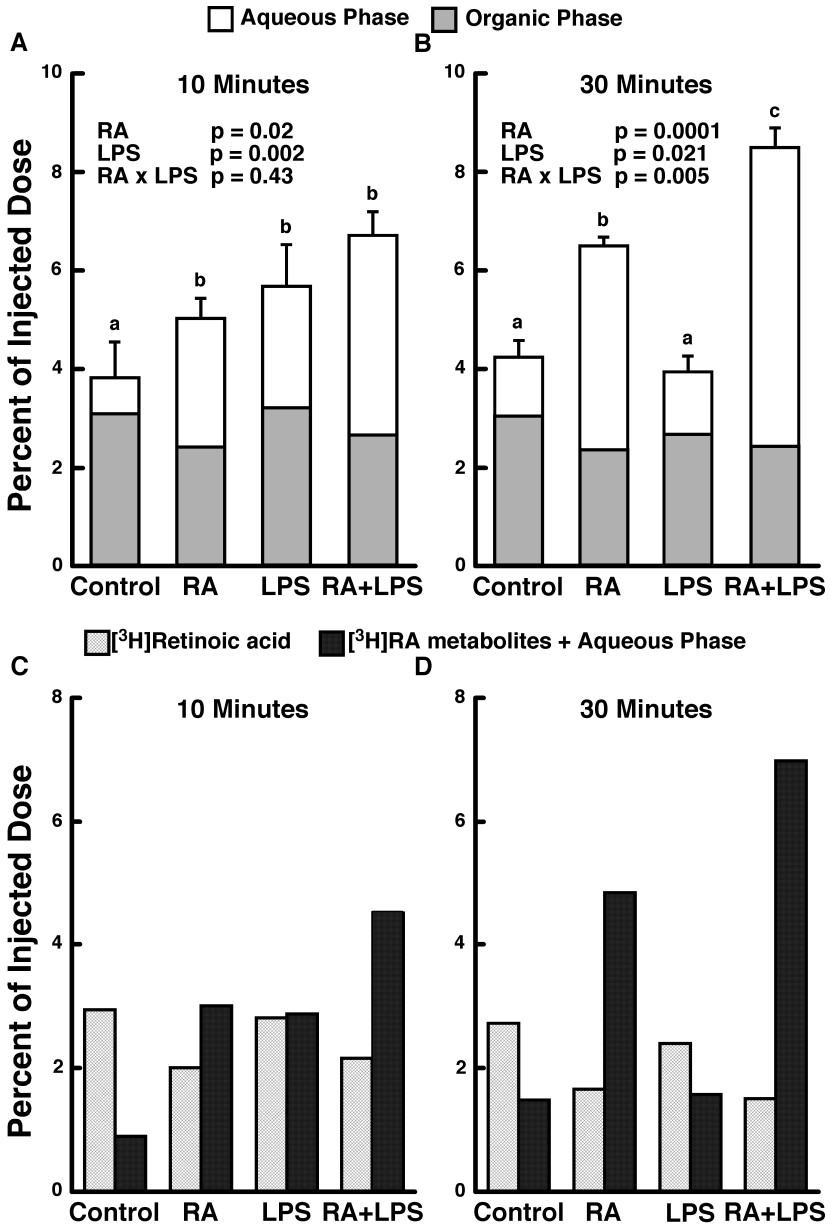

FIGURE 13. RA and LPS increase both the total radioactivity and amount of [^{3}H]-metabolites present in the plasma 10 and 30' post-injection. Female Sprague-Dawley rats were treated with RA, LPS, or the combination of RA+LPS as described in Materials and Methods, and [^{3}H]RA (0.023 mM) bound to rat serum albumin was injected 12 hours later. Tissues were collected 10 (n = 5) or 30 (n = 4) minutes later and total plasma radioactivity (A, 10' and B, 30') and [^{3}H]RA and [^{3}H]-metabolites (C, 10' and D, 30') were determined by liquid scintillation spectrometry. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

LPS and RA independently affect the total amount and distribution of [^{3}H]-retinoids in liver. Ten minutes post-injection, the liver of control rats contained approximately 24% of the injected dose as total [^{3}H]-retinoids (**Figure 14A**). The liver of LPS- and RA+LPS-treated rats contained less of the total dose, suggesting that LPS-induced inflammation may delay the uptake of [^{3}H]RA by the liver. By 30 minutes, total [^{3}H]-retinoids fell by 4.3% in the control group (**Figure 14B**), and a similar reduction was observed in each treatment group (**Figure 14A and B**). By 2-way ANOVA, LPS reduced the total [^{3}H]-retinoids at both 10 and 30 minutes (**Table 5, below; Figure 14A and B**).

The proportion of unmetabolized [^{3}H]RA and [^{3}H]-polar retinoids was determined on liver samples from individual rats. LPS reduced the amount of unmetabolized [^{3}H]RA in liver at both 10 and 30 minutes (**Figure 14C; Table 2**). Although there was no effect of RA on unmetabolized [^{3}H]RA in liver at 10 minutes, unmetabolized [^{3}H]RA was much lower in the liver of all RA-treated rats (RA alone and RA+LPS), compared to the control group, at 30 minutes (**Figure 14D; Table 5**). The percent of total [^{3}H]-polar metabolites did not differ among treatments at 10 minutes (**Table 5**), although the ratio of total [^{3}H]-polar metabolites to [^{3}H]RA was greater in all RA- and LPS-treated rats compared to the control group. By 30 minutes, total [^{3}H]-polar metabolites increased in the liver of all RA-treated rats, regardless of inflammation (**Figure 14D; Table 5**). At this time, total [^{3}H]-metabolites represented approximately 82% of the total radioactivity present in the liver in both of these groups. Thus, although LPS significantly affected the total amount of ^{3}H present in the liver at both times, only RA affected the amount of [^{3}H]-polar metabolites in the liver (**Table 5**).

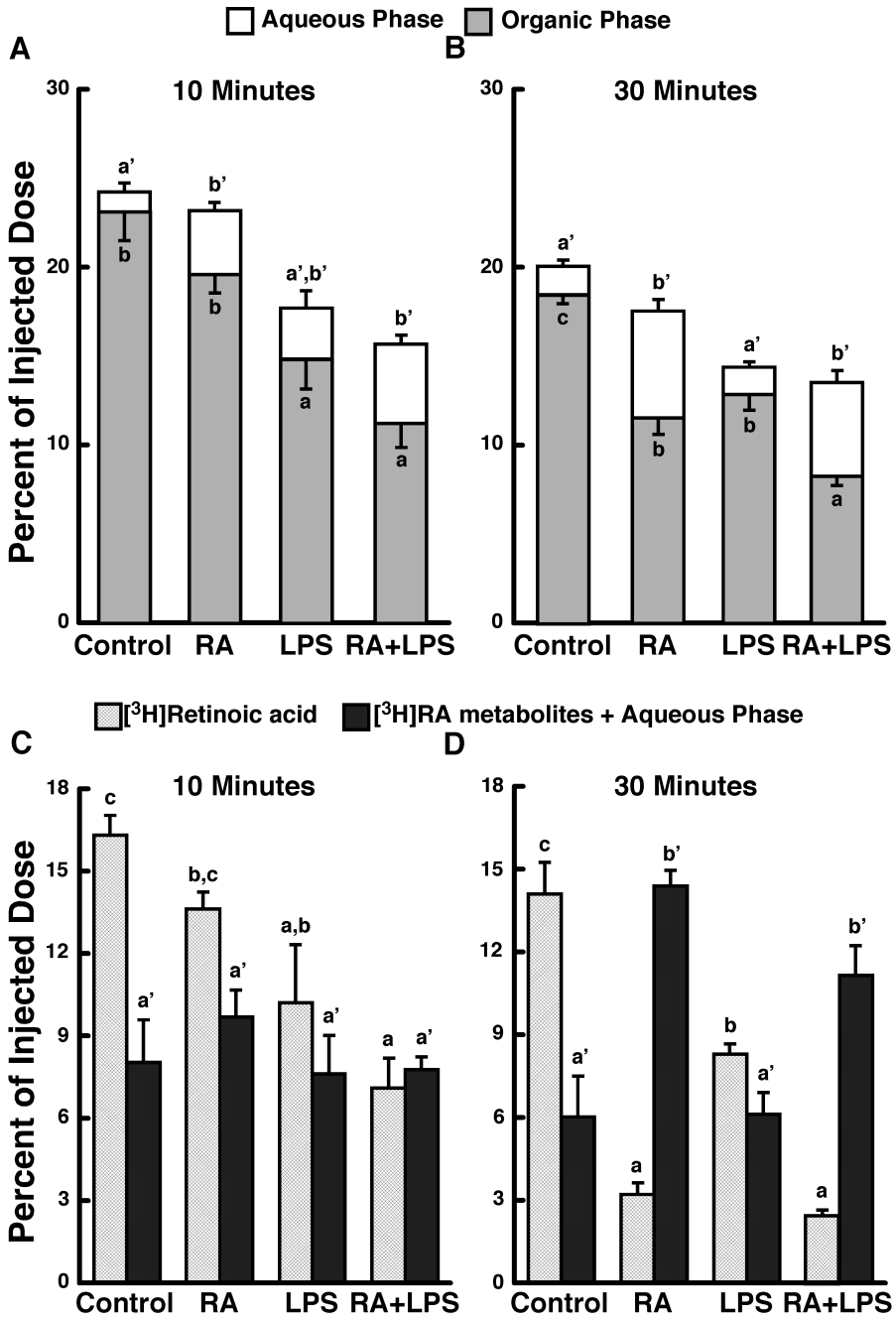

FIGURE 14. RA and LPS independently affect both the total radioactivity and amount of [^{3}H]-metabolites present in the liver 10 and 30' post-injection. Liver was collected 10 (n = 5) or 30 (n = 4) minutes after injection of [^{3}H]RA and organic phase and aqueous phase radioactivity (A, 10' and B, 30'), along with [^{3}H]RA and [^{3}H]-metabolites (C, 10' and D, 30') was determined by liquid scintillation spectrometry. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

Table 6. Probability values for two-way ANOVA of liver, lung, and small intestine radioactivity 10 and 30 minutes post-injection[1]

Liver	10 Minutes			30 Minutes		
	RA	LPS	RA x LPS	RA	LPS	RA x LPS
Total ^{3}H	0.23	0.002	0.87	0.93	0.0002	0.37
Organic Phase ^{3}H	0.03	0.0001	0.99	0.0001	0.0001	0.18
Aqueous Phase ^{3}H	0.004	0.05	0.42	0.0001	0.51	0.64
[^{3}H]RA[2]	0.04	0.0002	0.89	0.0001	0.0005	0.004
[^{3}H]-Polar metabolites[3]	0.46	0.32	0.54	0.0001	0.16	0.15
Lung	10 Minutes			30 Minutes		
	RA	LPS	RA x LPS	RA	LPS	RA x LPS
Total ^{3}H	0.63	0.01	0.28	0.02	0.04	0.85
Organic Phase ^{3}H	0.03	0.23	0.64	0.0001	0.17	0.24
Aqueous Phase ^{3}H	0.009	0.06	0.43	0.0001	0.04	0.06
Small Intestine	10 Minutes			30 Minutes		
	RA	LPS	RA x LPS	RA	LPS	RA x LPS
Total ^{3}H	0.16	0.0004	0.11	0.01	0.57	0.33
Organic Phase ^{3}H	0.21	0.03	0.34	0.01	0.42	0.07
Aqueous Phase ^{3}H	0.10	0.21	0.96	0.005	0.48	0.43

[1]: *P*-value obtained via two-way ANOVA
[2]: Amount of ^{3}H that eluted with an all-*trans*-RA standard during SPE
[3]: Amount of ^{3}H that eluted with a 4-*oxo*-RA standard during SPE

RA metabolism in extrahepatic tissues. Next, we examined two extrahepatic tissues, the lungs and small intestine, both of which are intimately involved in vitamin A homeostasis (267,285), to determine their response to RA and LPS-induced inflammation (**Figure 15 and 16**). Due to the relatively small amount of total ^{3}H recovered in the lungs and small intestine (see below), total [^{3}H]-retinoids were only partitioned into an organic phase and an aqueous phase; thus the organic phase may contain unmetabolized [^{3}H]RA and [^{3}H]-polar metabolites, whereas all ^{3}H in the aqueous phase has undergone metabolism. Less than 1% of the injected dose of [^{3}H]RA was recovered in the lungs of control rats at either time (**Figure 15**). However, this still represented an enrichment of 1.34 ± 0.12 and 1.25 ± 0.05 at 10 and 30 minutes, respectively, based on the relative weight of the lung. RA treatment did not affect total [^{3}H]-retinoids present in lung at 10 minutes, but it significantly decreased the amount of total [^{3}H]-retinoids at 30 minutes (**Figure 15A and B; Table 5**). In contrast, lung total [^{3}H]-retinoids were higher in LPS rats at both 10 and 30 minutes (**Figure 15A and B; Table 5**). Partitioning of the lipid extract showed that organic phase radioactivity was lower and [^{3}H]-aqueous phase metabolites were higher in the lungs of RA rats at both times (**Figure 15A and B; Table 5**). LPS treatment did not affect the amount of [^{3}H]-aqueous phase metabolites in the lungs at either time (**Figure 15**). Thus, while LPS affected total ^{3}H in the lung at both times, only RA increased the amount of [^{3}H]-aqueous phase metabolites.

Approximately 3.5% of the injected dose of [^{3}H]RA was recovered in the small intestine of control rats at 10 and 30 minutes (**Figure 16A and B**), representing an enrichment of 1.15 ± 0.07 and 1.92 ± 0.15, respectively. Treatment with RA increased the amount of ^{3}H recovered in the small intestine at 30 minutes (**Figure 16A and B;**

Table 5), whereas LPS increased the total amount of ^{3}H present in the small intestine only at 10 minutes (**Figure 16A and B; Table 5**). The amount of [^{3}H]-aqueous phase metabolites in the small intestine was significantly increased by RA, but not by LPS, at 30 minutes (**Figure 16B; Table 5**). In fact, [^{3}H]-aqueous phase metabolites recovered in the small intestine of RA-treated rats exceeded the total amount of radioactivity recovered from each of the other groups examined at either time (**Figure 16A and B**). Therefore, although LPS affected the total ^{3}H in the small intestine at 10 minutes, only RA affected both total ^{3}H and [^{3}H]-aqueous phase metabolites at 30 minutes.

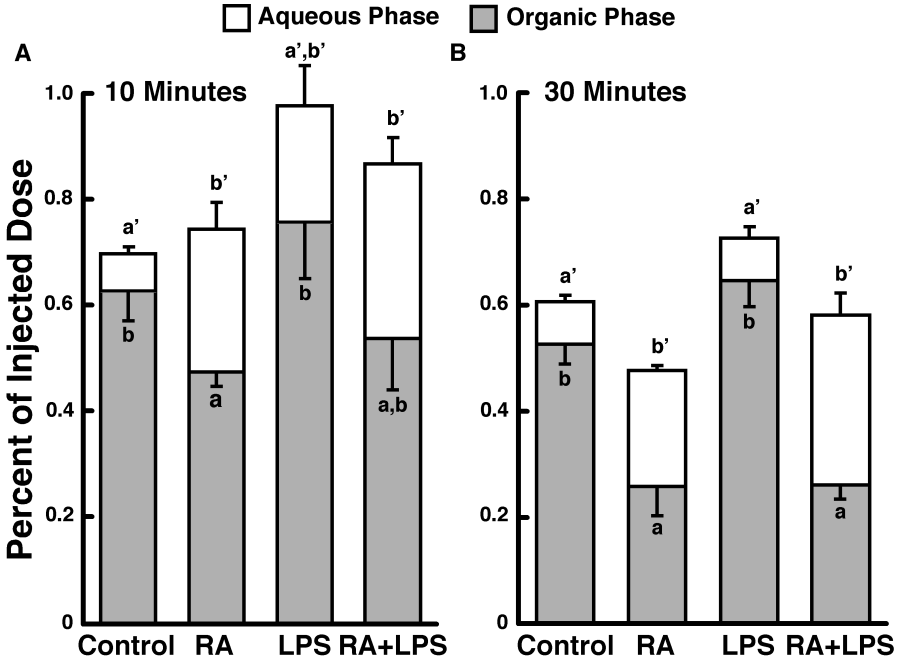

FIGURE 15. RA and LPS independently affect total radioactivity and [^{3}H]-aqueous phase metabolites present in the lungs. Lung was collected 10 (n = 5; panel A) or 30 (n = 4; panel B) minutes after injection of [^{3}H]RA and organic phase and aqueous phase (A, 10' and B, 30') radioactivity was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

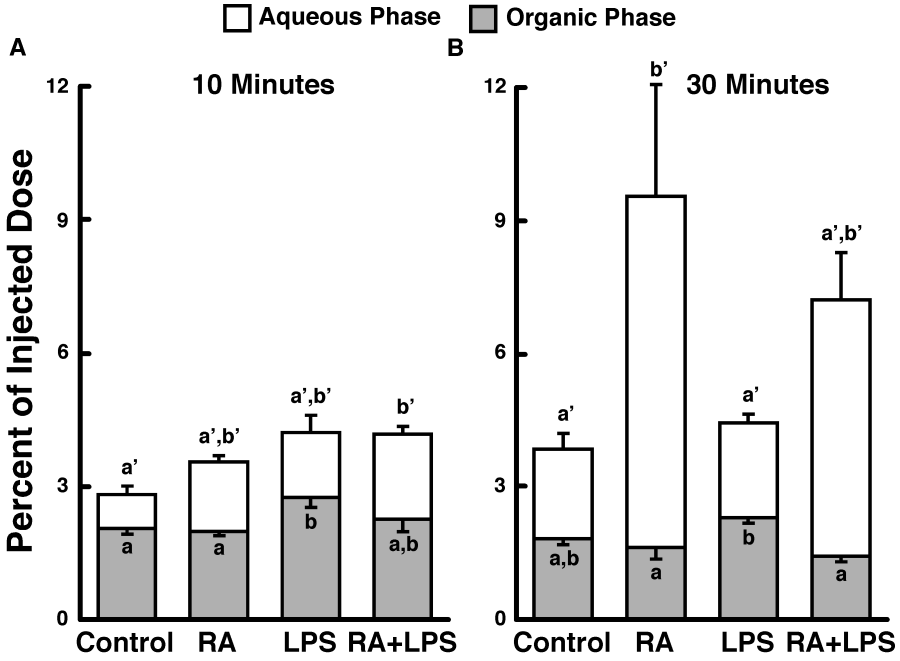

FIGURE 16. RA and LPS independently affect total radioactivity and [^{3}H]-aqueous phase metabolites present in the small intestine. Small intestine was collected 10 (n = 5; panel A) or 30 (n = 4; panel B) minutes after injection of [^{3}H]RA and organic phase and aqueous phase (A, 10' and B, 30') radioactivity was determined. Bars represent means ± SEM. Different letters above bars within panels indicate significant differences ($p < 0.05$, a < b < c).

DISCUSSION

The present study was conducted to better understand the regulation of RA homeostasis during inflammation. To this end, we designed our study to investigate the effects of RA and LPS each alone, as well as combination, on the distribution and metabolism of a dose of [^{3}H]RA in vitamin A-marginal rats, a model of subclinical vitamin A deficiency relevant to human populations in which inflammation is also prominent. The amount of LPS administered (50 μg/100 g body weight) was similar to that used in previous experiments to elicit a moderate inflammatory response in rats, which included a significant reduction of ~ 7.5% in body weight over 24 h, a moderate increase in body temperature (0.6°C), and significant hyporetinolemia (213,214). However, the LPS treatment protocol we have used does not induce endotoxemia or septic shock, as are induced by 10 - 20-fold higher doses of LPS (288,289). Inflammation-induced hyporetinemia was confirmed in our experiment by a 47% reduction in plasma retinol. Inflammation was also evident by a small but significant increase in relative spleen weight (**Table 1**), which is consistent with immune cell activation by LPS as shown in other studies (190,290). The dose of RA used in this study was shown previously to increase the expression of several retinoid-responsive genes in both vitamin A deficient and sufficient rats (17) and, therefore, would be expected to have similar effects in vitamin A marginal rats. By examining the distribution of intravenously administered [^{3}H]RA in the plasma, liver, and extrahepatic organs of vitamin A-marginal rats, we determined that [^{3}H]RA is cleared from plasma and metabolized very rapidly, and that each organ differs in its pattern of RA metabolism.

RA and LPS each significantly modified the metabolism of [^{3}H]RA. However, for the most part, RA and LPS were independent determinants of RA metabolism.

Plasma retinoid metabolites are predominantly regulated by RA

Reports prior to this study had established that under normal physiological conditions, the plasma pools of RA turns over rapidly in both rats and humans (15,110,265,277). Several investigators have shown that following the clearance of all-trans-RA from plasma, this retinoid subsequently appears as various polar metabolites, including 4-hydroxy-RA, 4-oxo-RA, and retinoyl ß-glucuronic acid (142,166,169). In present study, greater than 95% of the injected dose of [^{3}H]RA was cleared from plasma within 10 minutes, confirming the rapid clearance of RA from plasma observed in rats and other species. Although treatment with RA resulted in a higher percentage of ^{3}H in plasma, especially at 30 minutes, analysis of retinoids after reverse-phase solid phase extraction showed that the increase is accounted for by the combination of [^{3}H]-organic and aqueous phase metabolites (**Figure 13**). It is evident that plasma [^{3}H]RA was maintained within a narrow range regardless of pretreatment, while plasma [^{3}H]-metabolites were rapidly formed and were present at a higher level in RA-treated rats. In contrast, LPS had a modest (at 10 minutes), or no (at 30 minutes) effect on total ^{3}H and [^{3}H]-organic metabolites in plasma. Thus, [^{3}H]RA delivered into the plasma compartment was rapidly metabolized by all rats, but [^{3}H]RA metabolism was accelerated in RA-treated rats, regardless of concurrent inflammation.

Inflammation-induced changes in hepatic [^{3}H]RA metabolism

The liver is directly involved in the maintenance of retinol homeostasis by regulating its storage, mobilization, and utilization (291). Previous work has established that hepatic retinol mobilization is impaired during acute inflammation (214,226). Since RA mediates most of the physiologic functions of vitamin A, we were interested in understanding the effects of LPS-induced inflammation on the uptake and metabolism of [^{3}H]RA by liver. Significant amounts of RA are taken up by liver from plasma and presumably utilized or recycled (110). On average, 24% of the injected dose was recovered in the liver of control rats at 10 minutes (**Figure 14A**). This represents an enrichment of 6.48 ± 0.50 based on the relative weight of the liver. The amount of total [^{3}H]-retinoids recovered in liver was lower at 30 minutes than at 10 minutes in all four groups (**Figure 14**). This reduction could be due in part to the loss from liver back into plasma of the parent compound, [^{3}H]RA, or more polar metabolites, such as retinoyl _-glucuronide, (292). Alternatively or additionally, the decline in liver radioactivity could be caused by the formation and excretion of polar and aqueous-phase metabolites of RA, which have been shown to appear in the bile of normal rats 2 hours after RA administration (166,167,169). In our study, LPS significantly reduced the total amount of ^{3}H present in liver at both 10 and 30 minutes (**Figure 14A and B**). We speculate that the observed reduction in liver radioactivity could be a consequence of three primary causes. First, LPS-induced inflammation has been shown to increase vascular permeability, which could alter organ blood flow and thus influencing the uptake, utilization, and recycling of RA by the liver (230,231). Second, acute inflammation is known to diminish the expression of fatty acid transport protein (FATP) and fatty acid translocase

(FAT), both of which are involved in the uptake of long-chain fatty acids from plasma (232,234). Similarly, LPS administration has been shown to reduce the expression of cellular fatty acid binding protein, L-FABP (233). While it is currently believed that RA readily diffuses across plasma membranes in a nonfacilitated manner, the effects of inflammation on different fatty acid transporters suggest that these or similar changes could affect RA homeostasis as well. Third, specific transporters involved in the uptake and secretion of chemicals by the liver are altered following LPS administration (229). Thus, vascular alteration coupled with changes in membrane-bound transporters involved in fat and xenobiotic homeostasis, known to be affected by LPS, may have contributed to the LPS-induced decrease in liver [^{3}H]-retinoid in our LPS-treated rats.

RA-induced changes in hepatic [^{3}H]RA metabolism

Pharmacokinetic studies have determined that long-term administration of RA results in a decrease in plasma RA and subsequent increase in RA metabolism (15,266,284). Specific cytochrome P450 enzymes are induced in response to high cellular concentrations of RA, which may work in concert to regulate the degradation of RA, and to return the concentration of RA to normal cellular levels (17,142,143,151,180,181). In our studies, pretreatment of marginally vitamin A-deficient rats with oral RA significantly increased the amount of [^{3}H]-metabolites present in the liver. Furthermore, this induction in RA metabolism occurred irrespective of LPS administration. The inability of LPS to affect the amount of [^{3}H]-metabolites in the liver was surprising since it has been shown that the detoxification capacity of the liver for drugs and xenobiotics is diminished during inflammation (18,19), evidenced by an LPS-

induced reduction in both the expression and enzymatic activity of various members of the cytochrome P450 family (20). Thus, the results presented here suggest that the ability of RA to induce its own catabolism is maintained during periods of physiological stress, which would serve to prevent the elevation of RA concentrations when acute or chronic RA treatment is used therapeutically as an adjuvant or to treat different disease conditions.

Inflammation- and RA-induced changes in extrahepatic [^{3}H]RA metabolism

Similar to liver, both lung and small intestine have been implicated in retinol (67,267,285) and RA homeostasis (153,267). To determine if LPS-induced inflammation affects these organs in a manner similar to its effects in liver, we examined the small intestine and lung for organic and aqueous phase radioactivity. Similar to liver, both lung and small intestine exhibited enrichment of the injected [^{3}H]RA dose soon after administration, indicating these organs have the capacity to take up and concentrate RA. LPS initially increased the total amount of ^{3}H present in the lung and small intestine, but by 30 minutes the amount returned to control levels in both organs (**Figure 15 and 16**). In contrast, only RA administration increased the amount of aqueous phase radioactivity at 10 and 30 minutes in both organs, irrespective of LPS treatment. The increased aqueous phase radioactivity could be caused by enhanced RA oxidation because both lung and small intestine contain members of the cytochrome P450 family capable of oxidizing RA (153,270). Interestingly, in the small intestine, the amount of aqueous phase radioactivity increased in each treatment group over time, suggesting the presence of biliary metabolites of RA in the small intestine by 30 minutes (166,167,169).

In conclusion, the results of our study demonstrate that LPS-induced inflammation affects the organ distribution of RA which may reflect differences in either the rate of uptake or the recycling of RA in a tissue-specific manner. The greatest effect was observed in the liver, where LPS reduced the amount of radioactivity recovered by nearly 30%. However, despite differences in the initial distribution of RA, LPS did not affect the total amount of [^{3}H]-metabolites present in the liver, lung, and small intestine by 30 minutes. The interval from 10 to 30 minutes may have been sufficient for all tissues to metabolize most of the RA to metabolites. In contrast, while RA administration did not influence tissue radioactivity levels, it did cause a significant increase in the recovery of [^{3}H]-metabolites, irrespective of LPS. Taken together, our results suggest that acute inflammation can alter systemic RA homeostasis by disrupting the normal physiologic distribution of RA. Nevertheless, the moderate degree of inflammation induced in our rat model did not greatly compromise the metabolism of [^{3}H]RA, while the conversion of [^{3}H]RA to [^{3}H]-metabolites metabolism was significantly increased after treatment with RA.

CHAPTER 6

DISCUSSION and CONCLUSIONS

Introduction

Vitamin A, which is comprised of a family of compounds all structurally related to retinol, regulates numerous physiological processes in vivo including vision, embryonic development, cellular proliferation and differentiation, and immune function. With the exception of the visual cycle (30), the majority of the biological effects of VA are mediated by all-*trans*-retinoic acid (RA), an oxidation product in the physiological metabolism of retinol. Thus, alterations in retinol and/or RA homeostasis can have drastic effects on overall health. For instance, clinical VA-deficiency is characterized by night blindness, Bitot's spots, epithelial keratinization, dedifferentiation, and xerophthalmia (293). Moreover, subclinical VA-deficiency, which is characterized by low serum and liver retinol levels in the absence of overt clinical symptoms, has been shown to compromise immune function (294,295). In contrast, VA-toxicity is characterized by headaches, vomiting, alopecia, bone and joint pain, and, at high doses, liver damage, hemorrhage, and coma (296). Additionally, therapeutic doses of RA increase the risk of birth defects in humans during the first trimester of pregnancy and limb and organ development are extremely sensitive to intracellular RA concentrations (297). In view of these observations, it is apparent that proper regulation of VA metabolism is essential to maintaining normal physiological functioning. Our long-term goal is to delineate the underlying mechanisms that regulate retinol and RA metabolism under diverse conditions in vivo and to better describe the dynamic relationship between retinol and RA homeostasis. Therefore, in the studies presented here, our main hypothesis is that RA levels regulate VA (retinol and RA) homeostasis in rats. Specifically, we hypothesize that (*1*) exogenous RA alters retinol kinetics in rats with low

VA stores, (*2*) plasma and tissue RA levels are maintained regardless of dietary status, (*3*) hepatic CYP26A1 mRNA, protein, and enzymatic activity are rapidly increased in response to an intravenous dose of RA, and (*4*) RA distribution and metabolism are independently affected by exogenous RA and LPS-induced inflammation in rats. Our results confirm and expand upon previous studies, suggesting that tissue and plasma RA levels are homeostatically defended and that RA is the main determinant with regards to VA metabolism.

Hepatic metabolism of VA is essential to maintaining whole-body VA homeostasis by processing newly absorbed dietary VA in chylomicron remnants, storing VA as retinyl esters, synthesizing and secreting RBP, and mobilizing retinol for delivery to peripheral tissues (298). Moreover, it has been hypothesized (11,267,285) that the liver can "sense" whole-body VA status and rapidly adjust retinol oxidation, storage, and mobilization in response to different physiological and nutritional states. While the exact molecular mechanisms underlying the liver's ability to "sense" VA status have yet to be fully delineated, previous studies in our lab have shown that two enzymes, LRAT and CYP26, play a critical role in regulating the availability of retinol (LRAT) and for RA degradation (CYP26).

LRAT and CYP26 regulate retinoid homeostasis

LRAT and CYP26 are situated at opposite ends of the VA spectrum (**Figure 17A; see below**). Specifically, LRAT is located at the beginning of the pathway and is the principle enzyme involved in the synthesis of retinyl esters (57,59,62). Additionally, it is believed that LRAT, in conjunction with CRBP, diverts retinol away from oxidation

pathway that generates RA especially in the presence of excess VA or RA (75,268,269). In contrast, CYP26 is located at the end of the VA pathway and is involved in the phase I oxidation of RA (16,150). The CYP26-mediated oxidation of RA, coupled with subsequent glucuronidation (166,169), facilitates the elimination of RA to maintain normal physiological levels of VA. While the molecular processes that maintain VA homeostasis may involve the regulation of retinoid receptors, retinoid-binding proteins, and specific enzymes, our work has focused on LRAT and CYP26 because of their sensitivity to VA status and endogenous and exogenous RA. Our results suggest that LRAT and CYP26 function in concert to regulate retinol and RA levels, thus maintaining VA homeostasis. The work presented here focused on the terminal end of the VA pathway. Specifically, we examined (*1*) the effect of RA on retinol homeostasis and (*2*) RA metabolism under different conditions. The results presented here demonstrate that RA levels are homeostatically defended and that RA is the main determinant in the regulation of whole-body VA metabolism.

Dietary retinoic acid alters vitamin A kinetics in rats with low vitamin A status

Before the discovery of the molecular mechanisms of RA action, it was shown that dietary RA could partially substitute for retinol, influence liver VA levels, and spare whole-body VA stores (8,11). Using model-based compartmental analysis, we showed that chronic RA supplementation resulted in a slight positive VA-balance. This indicates that the LA+RA rats more efficiently utilized dietary retinol and the combination of retinol and RA provided in the diet was sufficient to meet the VA requirements of the rats, confirming the previously published results (8,9,11). Additionally, we showed that

the whole-body changes in VA kinetics observed in the LA+RA rats occurred due to tissue-specific changes in VA kinetics. Specifically, by as early as 2 hours post-injection, the tracer response curves for the liver, kidneys, small intestine, and lung of the LA+RA rats were visually different from the LA rats. These differences were reflected in the model-derived kinetic parameters, which showed that the tissue residence times and total traced mass of VA associated with the liver, kidneys, small intestine, and lung were significantly greater in the LA+RA rats than in the LA rats (Figure 2). We hypothesize that the difference in tissue and whole-body VA kinetics was caused by two mechanisms. First, our model predicts that chronic RA administration reduced the oxidation of retinol to RA, thus sparing the retinol and allowing it to accumulate. For instance, the model-predicted VA disposal rate was 20% lower in the LA+RA rats as compared to the LA rats. Furthermore, both the model-predicted system residence time and total traced mass for VA were significantly higher in the LA+RA rats. Second, our results suggest that the continuous availability of dietary RA enhanced the expression and activity of LRAT. Previous work in our lab has demonstrated that acute RA administration significantly increased LRAT mRNA and activity in VAD rats (13,286). Our results tie in well with this work, by suggesting that chronic RA administration can maintain LRAT in an active state, which results in increased retinol storage. Together, the present analysis suggests that long-term RA supplementation affects retinol homeostasis by reducing retinol oxidation and increasing retinol storage in rats with low VA stores.

Vitamin A status affects retinoic acid distribution and metabolism

CYP26A1 is a RA-inducible (150) cytochrome P450 enzyme that is expressed in both embryonic and adult tissues (152). Previous studies have established that CYP26A1 mRNA expression and activity responds to changes in both retinol and RA homeostasis. Yamamoto et al. (16) showed that the expression of CYP26A1 was directly correlated to dietary VA intake and increased VA storage. That is, CYP26A1 mRNA expression was not detected in VAD rats whereas it was strongly expressed in VAS rats (16). Similarly, our results (Table 4) show that CYP26A1 expression increased progressively as a function of VA stores (VAD < VAM ≤ VAD + RP < VAS). In addition, we used [^{3}H]RA as a tracer to determine how VA status affects the distribution and metabolism of RA. Our results show that, similar to CYP26 expression, both the distribution and metabolism of RA are related to VA status. Specifically, the total amount of [^{3}H]-metabolites recovered increased as a function of VA status (VAD < VAM ≤ VAD + RP < VAS), whereas the total amount of radioactivity recovered in liver was inversely related to VA status (VAS ≤ VAD + RP < VAM < VAD). We hypothesize that the observed difference in liver radioactivity is related to the larger pools of RA present in the VAS rats. Under normal conditions, the liver derives more than 75% of its RA from plasma, suggesting that the production and secretion of RA by extrahepatic tissues, coupled with the subsequent delivery of RA to the liver is important for maintaining normal RA concentrations (110). Based on the radioactive data obtained from the liver, our results suggest that the amount of RA recycling to liver from extrahepatic tissues was significantly lower in the VAD rats than all other groups. Therefore, a greater percentage of the injected dose was cleared and stored by the liver in the VAD rats. In contrast, the

feedback of RA to the liver from peripheral tissues was normal in the VAS rats, resulting in increased metabolism and clearance of the radioactive dose. Moreover, previous studies have shown that VAD rats repleted with retinol display a significant rise in plasma RBP and retinol levels within minutes (299-301), resulting in increased delivery of retinol to tissues. Thus, we propose that the rate of RA formation in extrahepatic tissues increased in response to retinol administration, causing more RA to recycle back to the liver, which led to the observed decrease in hepatic radioactivity and increase in metabolism. To our knowledge, this is the first study to show that both the distribution and metabolism of RA are directly affected by VA status and retinol repletion. Our results tie in well with the work of Yamamoto et al. (16) and together suggest that the molecular mechanisms controlling whole-body RA homeostasis respond both to chronic and acute changes in VA intake to maintain normal physiological concentrations of RA.

Exogenous retinoic acid rapidly induces CYP26A1 mRNA and protein expression

One of the hallmarks of CYP26A1 is that all-*trans*-RA strongly induces its expression in vivo. Wang et al. (17) showed that exogenous RA substantially increased CYP26A1 mRNA levels in VAD rats by as early as 3 hours post-treatment. Expanding upon these results, we show that an intravenous dose (10 μg / 100 g BW) of RA significantly increased CYP26A1 mRNA expression within 30 minutes irrespective of prior RA administration and remained higher than control levels throughout the study (Figure 9). The rapid induction of CYP26A1 mRNA in response to RA mirrors the induction pattern of other proteins, such as the MAFF transcription factor (302), heat shock protein 70 (303), cyclooxygenase-2 (304). Moreover, other members of the

cytochrome P450 superfamily display a similar rate of induction in response to exogenous substrate (305). For example, the induction pattern of CYP24, which catalyzes the hydroxylation of 1,25-dihydroxyvitamin D_3, parallels the pattern observed for CYP26. Specifically, Tashiro et al., (306) showed that exogenous vitamin D increased CYP24 mRNA levels within 60 minutes in vitro. Thus, similar to other members of the cytochrome P450 superfamily, CYP26A1 responds rapidly to changes in substrate concentration enabling cells to maintain homeostatic levels of RA.

Previous studies have delineated some of the underlying mechanisms regulating CYP26A1 transcription (17,178). However, the ability of exogenous RA to affect CYP26A1 translation and subsequent protein levels has yet to be fully explored. We detected a single polypeptide band of approximately 40 kD that displayed an expected expression pattern (17). That is, the expression of this protein in liver was higher in the RA-primed rats at baseline and increased over time in both groups. Moreover, the induction in CYP26A1 mRNA correlated to the increase in this 40 kD protein. But, in rat, CYP26 is a 497 amino acid polypeptide with a molecular weight of approximately 50 – 55 kD (17,151,152). In addition, recently performed in vitro transcription/translation studies detected CYP26 at 50 kD (Zolfaghari R, unpublished results). Thus, the band we detected was about 10 kD smaller than both the reported molecular weight of CYP26A1 and the expressed CYP26A1 during the in vitro studies. The antibody used in this experiment was obtained commercially and is directed against a N-terminal region of CYP26. Analysis of the amino acid sequence in CYP26A1 against which the antibody was developed showed that the region did not match any other proteins in the protein database, including other members of the CYP26 family. We speculate that the observed

difference in molecular weight is due to processing of the protein, such as cleavage of a part of the C-terminal region of CYP26. In fact, there are several amino acid sequences within the C-terminal region of CYP26 that are sites for endopeptidase cleavage (Appendix, **Figure A1**). Despite the contradiction in molecular weight, to our knowledge this is the first study to examine the affect of RA on CYP26A1 protein expression. Our results show that hepatic CYP26A1 protein levels rapidly increase in response to exogenous RA enabling the liver to degrade RA thus maintaining normal physiological levels of RA.

Pharmacokinetic analysis of RA has shown that chronic administration of RA results in increased RA metabolism (15,266,284). Similarly, acute RA administration induces specific members of the cytochrome P450 superfamily, including CYP26A1, that degrade RA (17,142,143,151,180,181). Therefore, in an attempt to correlate CYP26A1 mRNA and protein expression to RA metabolism, we conducted enzyme assays using liver microsomes from naïve and RA-primed rats. Our results show that the average formation of RA-metabolites was statistically greater in the RA-primed rats than in the naïve rats and that the amount of metabolites tended to increase over time (Figure 12). Moreover, neither CYP26A1 mRNA nor protein levels were correlated with the amount of metabolites recovered (data not shown). One possibility for this finding is that various members of the cytochrome P450 superfamily can metabolize RA in vitro (180). Thus, differences in their expression would lead to differences in the amount of metabolites recovered following the assay, especially in the RA-pretreated rats. Within our treatment groups, there was a high amount of variance associated with our means, potentially contributing to weaker statistical power. In retrospect, our design would have been

strengthened by increasing the number of rats per group, which would have increased the statistical power. Despite the variability associated with the enzyme assay, the results suggest that hepatic RA-metabolizing activity rapidly increases in response to i.v. RA in both naïve and RA-Primed rats. While pretreatment with RA increased basal hepatic RA-metabolizing activity, the rate enzymatic activity increases similarly in both groups allowing the liver to respond to rapidly to changes in RA concentrations.

Inflammation-induced changes in retinoic acid metabolism

During an inflammatory-response, plasma retinol levels are significantly reduced (213,214) while hepatic retinol levels are increased (226). These results indicate that retinol homeostasis is altered during inflammation, which is caused by an inability to mobilize hepatic retinol stores (214,226). In addition, in vitro work performed in our lab has shown that CYP26A1 mRNA levels are reduced following LPS administration (21). In view of these observations, we were interested in determining the effects of acute inflammation on the regulation of RA metabolism. Therefore, we examined the effects of RA and LPS, both alone and in combination, on the distribution and metabolism of RA in VA-marginal rats. While LPS-induced inflammation significantly altered the organ distribution of RA, it did not affect the production of RA-metabolites. As discussed above, we hypothesize that LPS-induced vascular changes in conjunction with changes in cellular transporters involved in fat and xenobiotic homeostasis caused the LPS-induced decrease in liver radioactivity. In contrast, while RA administration did not affect the organ distribution of RA, it significantly increased the amount of metabolites recovered, regardless of LPS treatment. Previous studies have demonstrated that the detoxification

capacity of the liver is diminished during inflammation (18,19) due to a reduction in both the expression and activity of various members of the cytochrome P450 family (20). Moreover, in a similar study conducted in VAS rats, we showed that LPS was able to partially attenuate hepatic CYP26A1 mRNA levels in the presence RA co-treatment (Appendix, **Figure A2**). Our results suggest that the ability of the liver to metabolize a pharmacological dose of RA is maintained during an inflammatory response. We speculate that observed maintenance in RA metabolism could be a consequence of two independent mechanisms. First, the mass of RA administered orally (500 μg) perturbed plasma and tissue RA levels to the point where it was able to overcome the CYP26 transcriptional repression observed following LPS administration (21). For instance, previous studies have shown that AP-1, a transcription factor that directly regulates genes important to cell growth, activity is disrupted by overexpression of RA and/or RAR (307,308). In vivo, LPS enhances the expression of the transcription factor NF-κβ, which mediates the majority of the biological response to LPS. Thus, the relationship between AP-1 and RA suggests that high levels of RA could disrupt NF-κβ function leading to RAR-mediated gene transcription and CYP26 up-regulation. Second, other cytochrome P450 enzymes have been identified that are capable of converting RA into 4-hydroxy- and 4-oxo-RA (180,181). Thus, the cumulative action of each of the various members of the cytochrome P450 family involved in RA metabolism could enable the body to respond adequately to elevated RA levels. Overall, our results suggest LPS-induced inflammation and exogenous RA independently affect whole-body RA metabolism. Furthermore, we determined that homeostatic concentrations of RA are

maintained during periods of physiological stress, thus preventing plasma or tissue RA levels to elevate following acute RA administration.

Comparison of RA doses

In our studies, four distinct RA doses were examined for their affect on retinol or RA distribution and metabolism. Therefore, to compare the RA doses used in this experiment to each other and to the amount of RA used clinically in humans, the RA doses administered in our studies were normalized per kilogram body weight and are presented in **Table 7**. All-*trans*-retinoic acid is used therapeutically to treat various cancers, including acute promyelocytic leukemia (274,279,280) and Kaposi's sarcoma (282). In humans, the oral administration of 45 mg RA/m^2/ day successfully treats acute promyelocytic leukemia in a high proportion of patients (266,274,279,280). Pharmacokinetic analysis has shown that following a single dose of RA (45 mg RA / m^2), the peak plasma concentration of RA is reached within 1 – 4 hours (265) and that the terminal half-life of RA in plasma is approximately 45 minutes (15,277). However, chronic administration of RA to humans and animals results in a drug-acquired resistance to RA, presumably via an induction in RA metabolism and elimination in vivo (276). The results presented here show that low doses of RA are capable of eliciting both molecular and physiological changes in rats (Table 7). For instance, the chronic administration of 0.6 mg RA / kg BW to animals with low liver VA stores altered whole-body and tissue-specific VA kinetics. Additionally, the acute administration (intravenously) of 0.1 mg RA / kg BW significantly increased both CYP26 expression and RA metabolism in rats. Thus, our results demonstrate that the administration of low

Table 7. Comparison of exogenous RA doses in rats from experiments 1, 2, 3, and 4

Rats	Length of Administration	Dose Amount
Experiment 1		
Oral[1]	Chronic (25 – 26 d)	0.6 mg RA / kg BW
Experiment 2		
Intravenous Injection[2]	Acute	100 ng RA / kg BW
Experiment 3		
Oral[3]	Acute	2.5 mg RA / kg BW
Intravenous Injection[2]	Acute	100 μg RA / kg BW
Experiment 4		
Oral[3]	Acute	2.5 mg RA / kg BW
Intravenous Injection[2]	Acute	100 μg RA / kg BW
Average Male[4]		
Oral	Chronic (4 – 10 wks)	1.15 mg RA / kg BW
	Acute	1.15 mg RA / kg BW

[1] RA was provided in diet and food was available ad libitum

[2] RA was administered intravenous bound to rat serum albumin and dose volume was 0.15 mL / 100 g BW

[3] RA was suspended in 50 μL of canola oil and administered orally

[4] Average male is 5' 10", 170 lbs; for review, please see Regazzi MB et al. (266)

doses of RA (Table 7) can significantly affect retinol and RA distribution and metabolism in rats.

Experimental limitations and future directions

The present study was conducted to further elucidate the underlying mechanisms regulating retinol and RA metabolism in vivo. Although our study demonstrated that VA-status and exogenous RA affect retinol and RA metabolism, many questions remain to be answered. First, the present study showed that exogenous RA altered retinol kinetics in rats with low VA stores, increased hepatic CYP26 expression, and increased the amount of RA-metabolites generated in liver and extrahepatic tissues. Additionally, we showed that dietary VA intake alters RA metabolism and distribution in rats. However, the effect of VA status and exogenous RA administration on plasma and tissue RA levels was not directly determined. Previous studies have shown that under normal dietary and physiological conditions, plasma RA levels are approximately 4 – 14 nmol/L (108,109), whereas liver RA levels are in the range of 13 – 20 pmol/g (226). Therefore, it will be interesting to determine if dietary VA intake and/or exogenous RA administration affect tissue and plasma RA levels. To address this question, RA levels in tissue and plasma samples from each of the studies will be determined using solid phase extraction (226,309) followed by high performance liquid chromatography (226). This study will further our understanding of the regulation of homeostatic levels of RA under various conditions.

In the present study, [^{3}H]RA was used to determine the distribution and metabolism of RA at one or two time-points. However, this approach did not allow for

an estimation of RA uptake, recycling, residence time, or fractional catabolic rate. Specifically, the limited number of time points sampled coupled with the use of a non-ideal tracer prevented us from examining the whole-body and tissue-specific kinetics of RA in rats. Therefore, in a future study we will use model-based compartmental analysis to analyze RA kinetic data in rats. Previous studies have utilized an accelerator mass spectrometer to kinetically analyze β-carotene (310), folate (311), and lutein (312) in humans. The accelerator mass spectrometer is able to detect extremely low levels of ^{14}C, enabling the investigator to use a non-perturbing dose of tracer and to sample frequently. Thus, using [^{14}C]-labeled RA as a tracer, we will be able to use mathematical modeling to kinetically analyze RA kinetics in humans. Moreover, we will compare control to RA-supplemented subjects to determine how a pharmacological dose of RA affects the recycling, uptake, and utilization of RA in humans. The results of this study will enable us to better understand RA kinetics and dynamics in humans, which will advance the therapeutic use of retinoids.

Conclusions

Prior to the discovery of the molecular mechanisms of RA action, Keilson et al. (11) postulated that VA homeostasis is regulated by a positive feedback mechanism tied to RA. The subsequent discovery of the retinoic acid receptors provided the necessary mechanism by which RA could physiologically regulate VA homeostasis. Specifically, RA is capable of directly enhancing the expression of two enzymes that directly affect VA homeostasis, LRAT and CYP26. Thus, modulation of LRAT and CYP26 expression and activity enables the body to rapidly adjust VA metabolism in response to different

dietary and/or physiological conditions. The present study was conducted to better understand the regulation of RA in vivo and expand the current model that describes VA regulation. Our results show that chronic RA administration altered VA kinetics, acute RA rapidly increased CYP26 expression, and RA metabolism is maintained during physiological stress. Moreover, we show that the distribution and metabolism of RA are adjusted in response to changes in dietary VA intake and retinol supplementation. Together, the results presented here suggest that VA homeostasis is maintained through an autoregulatory process, with RA acting as the principle messenger that enables the liver to "sense" VA status (**Figure 17A, - C**). Furthermore, we propose that tissue and plasma RA levels are homeostatically defended within a narrow range, allowing the liver to respond to minor changes in VA status. This hypothesis is based on our observation that increased RA levels, whether administered through diet or intravenous injection, directly affect retinol and RA homeostasis. In addition, RA homeostasis was apparently maintained despite nutritional and physiological stress, thus enabling the body to preserve VA homeostasis. In sum, our results demonstrate that RA is the signal that enables the body to "sense" VA status and that molecular mechanisms regulating RA levels adjust rapidly in response to different physiological, nutritional, and metabolic states to preserve RA homeostasis.

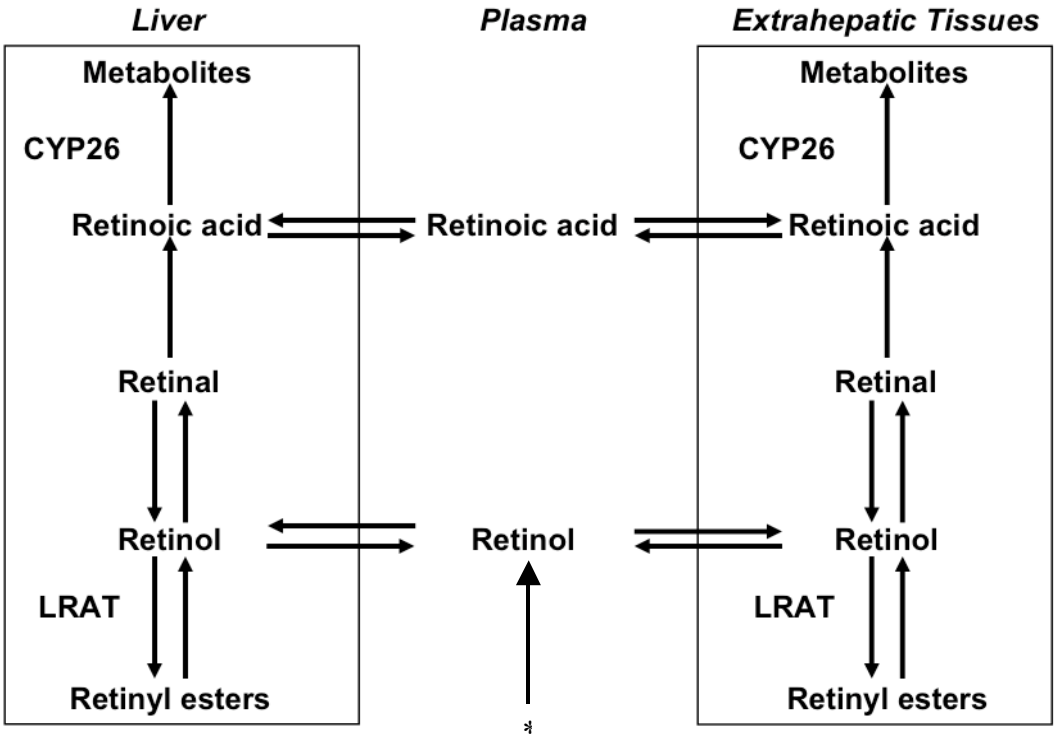

Figure 17A. Retinol and retinoic acid transport, storage, and degradation in a vitamin A-sufficient rat. Homeostatic levels of retinol and retinoic acid are maintained by regulating the expression of LRAT and CYP26, which rapidly respond to changes in tissue and plasma retinoic acid levels. Asterisk denotes input of retinol from diet.

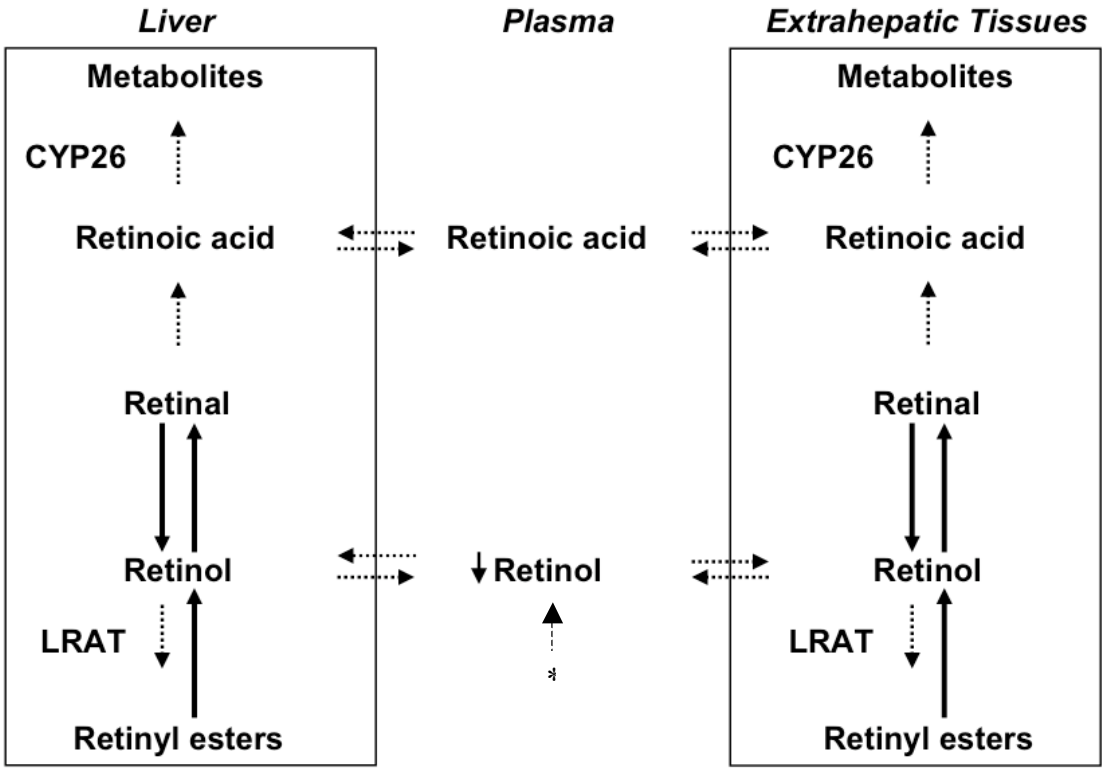

Figure 17B. Retinol and retinoic acid transport, storage, and degradation in a vitamin A-deficient rat. In response to reduced or negligible vitamin A intake, LRAT expression is suppressed, tissue retinol stores are mobilized, and, as hepatic stores are depleted, plasma retinol levels decrease. Additionally, CYP26 expression and activity is reduced resulting in less retinoic acid catabolism and, presumably, more retinoic acid recycling. Thus, changes in LRAT and CYP26 expression allow the body to maintain retinoic acid levels, even as retinol levels are depleted. Asterisk denotes input of retinol from diet.

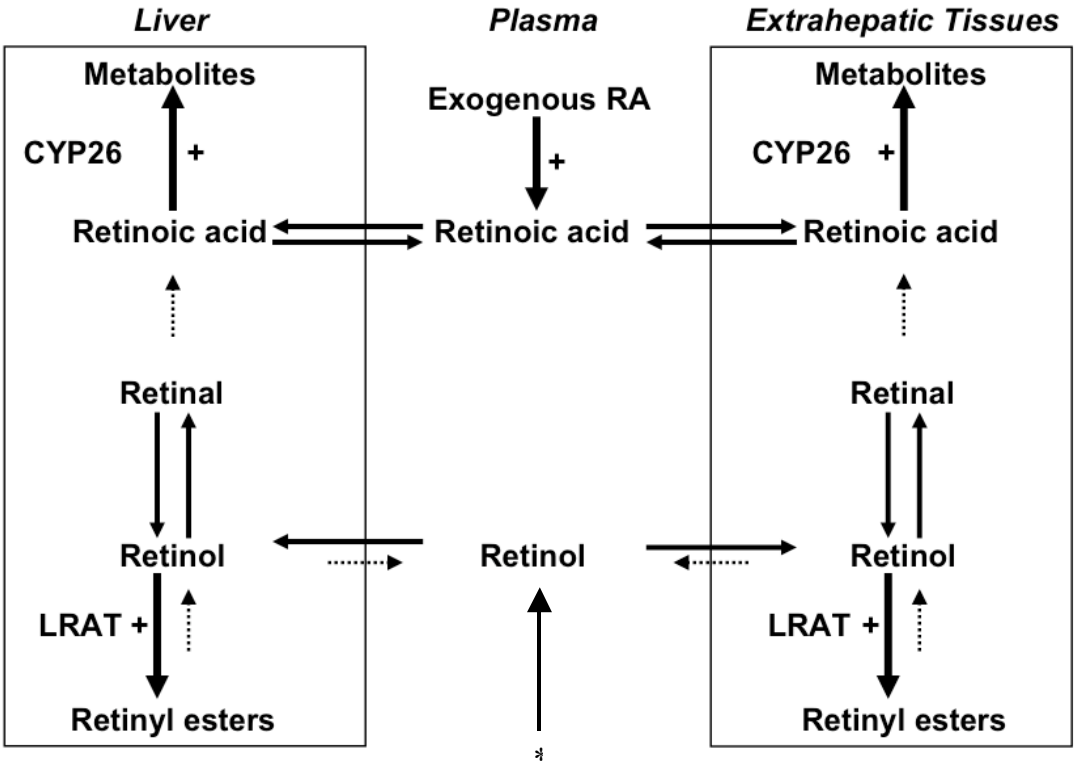

Figure 17C. Retinol and retinoic acid transport, storage, and degradation in a vitamin A-deficient, vitamin A-marginal, and or vitamin A-sufficient rat treated with exogenous retinoic acid. Chronic or acute exogenous retinoic acid will increase plasma retinoic acid levels. In response to increased retinoic acid levels, the expression of both LRAT and CYP26 are increased. Thus, retinol storage is increased in a tissue-specific manner, leading to retinol "sparing" in rats with low vitamin A status, whereas retinoic acid catabolism is increased in order to maintain homeostatic levels of retinoic acid in plasma and tissues. Asterisk denotes input of retinol from diet.

WORKS CITED

1. Peck, G. L. & DiGiovanna, J. J. (1994) Synthetic retinoids in dermatology. In: The Retinoids: Biology, Chemistry, and Medicine, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 631 - 658. Raven Press, New York.

2. Okuno, M., Kojima, S., Matsushima-Nishiwaki, R., Tsurumi, H., Muto, Y., Friedman, S. L. & Moriwaki, H. (2004) Retinoids in cancer chemoprevention. Curr Cancer Drug Targets 4: 285-298.

3. Jimenez-Lara, A. M., Clarke, N., Altucci, L. & Gronemeyer, H. (2004) Retinoic-acid-induced apoptosis in leukemia cells. Trends Mol Med 10: 508-515.

4. Ortega, J. J., Madero, L., Martin, G., Verdeguer, A., Garcia, P., Parody, R., Fuster, J., Molines, A., Novo, A. et al. (2005) Treatment with all-trans retinoic acid and anthracycline monochemotherapy for children with acute promyelocytic leukemia: a multicenter study by the PETHEMA Group. J Clin Oncol 23: 7632-7640.

5. Benn, C. S., Balde, A., George, E., Kidd, M., Whittle, H., Lisse, I. M. & Aaby, P. (2002) Effect of vitamin A supplementation on measles-specific antibody levels in Guinea-Bissau. Lancet 359: 1313-1314.

6. Ma, Y., Chen, Q. & Ross, A. C. (2005) Retinoic acid and polyriboinosinic:polyribocytidylic acid stimulate robust anti-tetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations. J Immunol 174: 7961-7969.

7. Ma, Y. & Ross, A. C. (2005) The anti-tetanus immune response of neonatal mice is augmented by retinoic acid combined with polyriboinosinic:polyribocytidylic acid. Proc Natl Acad Sci U S A 102: 13556-13561.

8. Lamb, A. J., Apiwatanaporn, P. & Olson, J. A. (1974) Induction of rapid, synchronous vitamin A deficiency in the rat. J Nutr 104: 1140-1148.

9. Dowling, J. E. & Wald, G. (1960) The role of vitamin A acid. Vitam Horm 18: 515-541.

10. Anonymous (1960) The Biological Function of Vitamin a Acid. Proc Natl Acad Sci U S A 46: 587-608.

11. Keilson, B., Underwood, B. A. & Loerch, J. D. (1979) Effects of retinoic acid on the mobilization of vitamin A from the liver in rats. J Nutr 109: 787-795.

12. Matsuura, T. & Ross, A. C. (1993) Regulation of hepatic lecithin: retinol acyltransferase activity by retinoic acid. Arch Biochem Biophys 301: 221-227.

13. Zolfaghari, R. & Ross, A. C. (2000) Lecithin:retinol acyltransferase from mouse and rat liver. CDNA cloning and liver-specific regulation by dietary vitamin a and retinoic acid. J Lipid Res 41: 2024-2034.

14. Green, M. H., Green, J. B. & Lewis, K. C. (1992) Model-based compartmental analysis of retinol kinetics in organs of rats at different levels of vitamin A status. In: Retinoids: Progress in Research and Clinical Applications (Livrea, M. A. & Packer, L., eds.), pp. 185 - 204. Marcel Dekker, New York.

15. Smith, M. A., Adamson, P. C., Balis, F. M., Feusner, J., Aronson, L., Murphy, R. F., Horowitz, M. E., Reaman, G., Hammond, G. D. et al. (1992) Phase I and pharmacokinetic evaluation of all-trans-retinoic acid in pediatric patients with cancer. J Clin Oncol 10: 1666-1673.

16. Yamamoto, Y., Zolfaghari, R. & Ross, A. C. (2000) Regulation of CYP26 (cytochrome P450RAI) mRNA expression and retinoic acid metabolism by retinoids and dietary vitamin A in liver of mice and rats. Faseb J 14: 2119-2127.

17. Wang, Y., Zolfaghari, R. & Ross, A. C. (2002) Cloning of rat cytochrome P450RAI (CYP26) cDNA and regulation of its gene expression by all-trans-retinoic acid in vivo. Arch Biochem Biophys 401: 235-243.

18. Gorodischer, F., Krasner, J., McDevitt, J. J., Nolan, J. P. & Yaffe, S. J. (1976) Hepatic microsomal drug metabolism after administration of endotoxin in rats. Biochem Pharmacol 25: 351-353.

19. Ghezzi, P., Saccardo, B., Villa, P., Rossi, V., Bianchi, M. & Dinarello, C. A. (1986) Role of interleukin-1 in the depression of liver drug metabolism by endotoxin. Infect Immun 54: 837-840.

20. Sewer, M. B., Koop, D. R. & Morgan, E. T. (1996) Endotoxemia in rats is associated with induction of the P4504A subfamily and suppression of several other forms of cytochrome P450. Drug Metab Dispos 24: 401-407.

21. Chen, Q., Ma, Y. & Ross, A. C. (2002) Opposing cytokine-specific effects of all trans-retinoic acid on the activation and expression of signal transducer and activator of transcription (STAT)-1 in THP-1 cells. Immunology 107: 199-208.

22. Neuzil, K. M., Gruber, W. C., Chytil, F., Stahlman, M. T., Engelhardt, B. & Graham, B. S. (1994) Serum vitamin A levels in respiratory syncytial virus infection. J Pediatr 124: 433-436.

23. Hussey, G. D. & Klein, M. (1990) A randomized, controlled trial of vitamin A in children with severe measles. N Engl J Med 323: 160-164.

24. Salazar-Lindo, E., Salazar, M. & Alvarez, J. O. (1993) Association of diarrhea and low serum retinol in Peruvian children. Am J Clin Nutr 58: 110-113.

25. Kelley, S. K. & Green, M. H. (1998) Plasma retinol is a major determinant of vitamin A utilization in rats. J Nutr 128: 1767-1773.

26. Wolbach, S. B. & Howe, P. R. (1925) Tissue changes following deprivation of fat-soluble A vitamin. Journal of Experimental Medicine 42: 753 - 777.

27. Bloch, C. E. (1924) Blindness and other diseases in children arising from deficient nutrition (lack of fat-soluble A factor). Am J Dis Child 27: 140 - 148.

28. Wald, G. (1934 - 1935) Vitamin A in eye tissues. J Gen Physiol 18: 905.

29. Wald, G. & Hubbard, R. (1950) The Synthesis of Rhodopsin from Vitamin A(1). Proc Natl Acad Sci U S A 36: 92-102.

30. Wald, G. (1960) The visual function of the vitamins A. Vitam Horm 18: 417-430.

31. Sommer, A. (1983) Mortality associated with mild, untreated xerophthalmia. Trans Am Ophthalmol Soc 81: 825-853.

32. Goodman, D. S. & Huang, H. S. (1965) Biosynthesis of Vitamin a with Rat Intestinal Enzymes. Science 149: 879-880.

33. Goodman, D. S., Blomstrand, R., Werner, B., Huang, H. S. & Shiratori, T. (1966) The intestinal absorption and metabolism of vitamin A and beta-carotene in man. J Clin Invest 45: 1615-1623.

34. Rigtrup, K. M. & Ong, D. E. (1992) A retinyl ester hydrolase activity intrinsic to the brush border membrane of rat small intestine. Biochemistry 31: 2920-2926.

35. van Bennekum, A. M., Kako, Y., Weinstock, P. H., Harrison, E. H., Deckelbaum, R. J., Goldberg, I. J. & Blaner, W. S. (1999) Lipoprotein lipase expression level influences tissue clearance of chylomicron retinyl ester. J Lipid Res 40: 565-574.

36. Goodman, D. W., Huang, H. S. & Shiratori, T. (1965) Tissue Distribution and Metabolism of Newly Absorbed Vitamin a in the Rat. J Lipid Res 6: 390-396.

37. Blomhoff, R., Helgerud, P., Rasmussen, M., Berg, T. & Norum, K. R. (1982) In vivo uptake of chylomicron [3H]retinyl ester by rat liver: evidence for retinol transfer from parenchymal to nonparenchymal cells. Proc Natl Acad Sci U S A 79: 7326-7330.

38. Napoli, J. L. (1997) Retinoid binding-proteins redirect retinoid metabolism: biosynthesis and metabolism of retinoic acid. Semin Cell Dev Biol 8: 403-415.

39. Bashor, M. M., Toft, D. O. & Chytil, F. (1973) In vitro binding of retinol to rat-tissue components. Proc Natl Acad Sci U S A 70: 3483-3487.

40. MacDonald, P. N. & Ong, D. E. (1987) Binding specificities of cellular retinol-binding protein and cellular retinol-binding protein, type II. J Biol Chem 262: 10550-10556.

41. Ong, D. E. & Chytil, F. (1978) Cellular retinol-binding protein from rat liver. Purification and characterization. J Biol Chem 253: 828-832.

42. Levin, M. S., Li, E., Ong, D. E. & Gordon, J. I. (1987) Comparison of the tissue-specific expression and developmental regulation of two closely linked rodent genes encoding cytosolic retinol-binding proteins. J Biol Chem 262: 7118-7124.

43. Ong, D. E. & Page, D. L. (1987) Cellular retinol-binding protein (type two) is abundant in human small intestine. J Lipid Res 28: 739-745.

44. Rajan, N., Kidd, G. L., Talmage, D. A., Blaner, W. S., Suhara, A. & Goodman, D. S. (1991) Cellular retinoic acid-binding protein messenger RNA: levels in rat tissues and localization in rat testis. J Lipid Res 32: 1195-1204.

45. Ong, D. E. & Chytil, F. (1975) Retinoic acid-binding protein in rat tissue. Partial purification and comparison to rat tissue retinol-binding protein. J Biol Chem 250: 6113-6117.

46. Sani, B. P. & Hill, D. L. (1974) Retinoic acid: a binding protein in chick embryo metatarsal skin. Biochem Biophys Res Commun 61: 1276-1282.

47. Ross, A. C. & Goodman, D. S. (1979) Intracellular binding proteins for retinol and retinoic acid: comparison with each other and with serum retinol-binding protein. Fed Proc 38: 2515-2518.

48. Chytil, F. & Ong, D. E. (1979) Cellular retinol- and retinoic acid-binding proteins in vitamin A action. Fed Proc 38: 2510-2514.

49. Ong, D. E., Newcomer, M. E. & Chytil, F. (1994) Cellular Retinoid-Binding Proteins. In: The Retinoids: Biology, Chemistry, and Medicine, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 283 - 318. Raven Press, New York.

50. Boylan, J. F. & Gudas, L. J. (1992) The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. J Biol Chem 267: 21486-21491.

51. Dong, D., Ruuska, S. E., Levinthal, D. J. & Noy, N. (1999) Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. J Biol Chem 274: 23695-23698.

52. Ross, A. C. (1982) Retinol esterification by mammary gland microsomes from the lactating rat. J Lipid Res 23: 133-144.

53. Helgerud, P., Petersen, L. B. & Norum, K. R. (1982) Acyl CoA:retinol acyltransferase in rat small intestine: its activity and some properties of the enzymic reaction. J Lipid Res 23: 609-618.

54. Ross, A. C. (1982) Retinol esterification by rat liver microsomes. Evidence for a fatty acyl coenzyme A: retinol acyltransferase. J Biol Chem 257: 2453-2459.

55. Rasmussen, M., Helgerud, P., Petersen, L. B. & Norum, K. R. (1984) Microsomal esterification of retinol in human liver. Acta Med Scand 216: 403-407.

56. Ong, D. E., Kakkad, B. & MacDonald, P. N. (1987) Acyl-CoA-independent esterification of retinol bound to cellular retinol-binding protein (type II) by microsomes from rat small intestine. J Biol Chem 262: 2729-2736.

57. MacDonald, P. N. & Ong, D. E. (1988) Evidence for a lecithin-retinol acyltransferase activity in the rat small intestine. J Biol Chem 263: 12478-12482.

58. Yost, R. W., Harrison, E. H. & Ross, A. C. (1988) Esterification by rat liver microsomes of retinol bound to cellular retinol-binding protein. J Biol Chem 263: 18693-18701.

59. Randolph, R. K. & Ross, A. C. (1991) Vitamin A status regulates hepatic lecithin: retinol acyltransferase activity in rats. J Biol Chem 266: 16453-16457.

60. Dawson, H. D., Yamamoto, Y., Zolfaghari, R., Rosales, F. J., Dietz, J., Shimada, T., Li, N. & Ross, A. C. (2000) Regulation of hepatic vitamin A storage in a rat model of controlled vitamin A status during aging. J Nutr 130: 1280-1286.

61. Zolfaghari, R., Wang, Y., Chen, Q., Sancher, A. & Ross, A. C. (2002) Cloning and molecular expression analysis of large and small lecithin:retinol acyltransferase mRNAs in the liver and other tissues of adult rats. Biochem J 368: 621-631.

62. Zolfaghari, R. & Ross, A. C. (2002) Lecithin:retinol acyltransferase expression is regulated by dietary vitamin A and exogenous retinoic acid in the lung of adult rats. J Nutr 132: 1160-1164.

63. Shimada, T., Ross, A. C., Muccio, D. D., Brouillette, W. J. & Shealy, Y. F. (1997) Regulation of hepatic lecithin:retinol acyltransferase activity by retinoic acid receptor-selective retinoids. Arch Biochem Biophys 344: 220-227.

64. Green, M. H., Green, J. B., Berg, T., Norum, K. R. & Blomhoff, R. (1988) Changes in hepatic parenchymal and nonparenchymal cell vitamin A content during vitamin A depletion in the rat. J Nutr 118: 1331-1335.

65. Blomhoff, R. & Wake, K. (1991) Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. Faseb J 5: 271-277.

66. Moriwaki, H., Blaner, W. S., Piantedosi, R. & Goodman, D. S. (1988) Effects of dietary retinoid and triglyceride on the lipid composition of rat liver stellate cells and stellate cell lipid droplets. J Lipid Res 29: 1523-1534.

67. Nagy, N. E., Holven, K. B., Roos, N., Senoo, H., Kojima, N., Norum, K. R. & Blomhoff, R. (1997) Storage of vitamin A in extrahepatic stellate cells in normal rats. J Lipid Res 38: 645-658.

68. Harrison, E. H., Smith, J. E. & Goodman, D. S. (1979) Unusual properties of retinyl palmitate hydrolase activity in rat liver. J Lipid Res 20: 753-759.

69. Harrison, E. H. & Gad, M. Z. (1989) Hydrolysis of retinyl palmitate by enzymes of rat pancreas and liver. Differentiation of bile salt-dependent and bile salt-independent, neutral retinyl ester hydrolases in rat liver. J Biol Chem 264: 17142-17147.

70. Gad, M. Z. & Harrison, E. H. (1991) Neutral and acid retinyl ester hydrolases associated with rat liver microsomes: relationships to microsomal cholesteryl ester hydrolases. J Lipid Res 32: 685-693.

71. Cooper, D. A. & Olson, J. A. (1986) Properties of liver retinyl ester hydrolase in young pigs. Biochim Biophys Acta 884: 251-258.

72. Prystowsky, J. H., Smith, J. E. & Goodman, D. S. (1981) Retinyl palmitate hydrolase activity in normal rat liver. J Biol Chem 256: 4498-4503.

73. Blaner, W. S., Smith, J. E., Dell, R. B. & Goodman, D. S. (1985) Spatial distribution of retinol-binding protein and retinyl palmitate hydrolase activity in normal and vitamin A-deficient rat liver. J Nutr 115: 856-864.

74. Blomhoff, R., Rasmussen, M., Nilsson, A., Norum, K. R., Berg, T., Blaner, W. S., Kato, M., Mertz, J. R., Goodman, D. S. et al. (1985) Hepatic retinol metabolism. Distribution of retinoids, enzymes, and binding proteins in isolated rat liver cells. J Biol Chem 260: 13560-13565.

75. Boerman, M. H. & Napoli, J. L. (1991) Cholate-independent retinyl ester hydrolysis. Stimulation by Apo-cellular retinol-binding protein. J Biol Chem 266: 22273-22278.

76. Kanai, M., Raz, A. & Goodman, D. S. (1968) Retinol-binding protein: the transport protein for vitamin A in human plasma. J Clin Invest 47: 2025-2044.

77. Peterson, P. A. & Rask, L. (1971) Studies on the fluorescence of the human vitamin A-transporting plasma protein complex and its individual components. J Biol Chem 246: 7544-7550.

78. Rask, L., Anundi, H. & Peterson, P. A. (1979) The primary structure of the human retinol-binding protein. FEBS Lett 104: 55-58.

79. Rask, L., Anundi, H., Bohme, J., Eriksson, U., Ronne, H., Sege, K. & Peterson, P. A. (1981) Structural and functional studies of vitamin A-binding proteins. Ann N Y Acad Sci 359: 79-90.

80. Smith, F. R., Raz, A. & Goodman, D. S. (1970) Radioimmunoassay of human plasma retinol-binding protein. J Clin Invest 49: 1754-1761.

81. Smith, F. R. & Goodman, D. S. (1971) The effects of diseases of the liver, thyroid, and kidneys on the transport of vitamin A in human plasma. J Clin Invest 50: 2426-2436.

82. Goodman, D. S. & Leslie, R. B. (1972) Fluorescence studies of human plasma retinol-binding protein and of the retinol-binding protein-prealbumin complex. Biochim Biophys Acta 260: 670-678.

83. Goodman, D. S. & Raz, A. (1972) Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein. J Lipid Res 13: 338-347.

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

85. Soprano, D. R. & Blaner, W. S. (1994) Plasma retinol-binding protein. In: The Retinoids: Biology, Chemistry, and Medicine, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 257 - 281. Raven Press, New York.

86. Lewis, K. C., Green, M. H. & Underwood, B. A. (1981) Vitamin A turnover in rats as influenced by vitamin A status. J Nutr 111: 1135-1144.

87. Green, M. H., Uhl, L. & Green, J. B. (1985) A multicompartmental model of vitamin A kinetics in rats with marginal liver vitamin A stores. J Lipid Res 26: 806-818.

88. Green, M. H., Green, J. B. & Lewis, K. C. (1987) Variation in retinol utilization rate with vitamin A status in the rat. J Nutr 117: 694-703.

89. Green, M. H. & Green, J. B. (1990) Experimental and kinetic methods for studying vitamin A dynamics in vivo. Methods Enzymol 190: 304-317.

90. Blomhoff, R., Green, M. H., Green, J. B., Berg, T. & Norum, K. R. (1991) Vitamin A metabolism: new perspectives on absorption, transport, and storage. Physiol Rev 71: 951-990.

91. Jang, G. F., McBee, J. K., Alekseev, A. M., Haeseleer, F. & Palczewski, K. (2000) Stereoisomeric specificity of the retinoid cycle in the vertebrate retina. J Biol Chem 275: 28128-28138.

92. Underwood, B. A., Loerch, J. D. & Lewis, K. C. (1979) Effects of dietary vitamin A deficiency, retinoic acid and protein quantity and quality on serially obtained plasma and liver levels of vitamin A in rats. J Nutr 109: 796-806.

93. Torrisi, R. & Decensi, A. (2000) Fenretinide and cancer prevention. Curr Oncol Rep 2: 263-270.

94. Formelli, F., Carsana, R., Costa, A., Buranelli, F., Campa, T., Dossena, G., Magni, A. & Pizzichetta, M. (1989) Plasma retinol level reduction by the synthetic retinoid fenretinide: a one year follow-up study of breast cancer patients. Cancer Res 49: 6149-6152.

95. Adams, W. R., Smith, J. E. & Green, M. H. (1995) Effects of N-(4-hydroxyphenyl)retinamide on vitamin A metabolism in rats. Proc Soc Exp Biol Med 208: 178-185.

96. Goodman, D. S. (1984) Vitamin A and retinoids in health and disease. N Engl J Med 310: 1023-1031.

97. Gudas, L. J., Sporn, M. B. & Roberts, A. B. (1994) Cellular biology and biochemistry of the retinoids. In: The Retinoids, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 443 - 520. Raven Press, New York.

98. Smith, J. E., Milch, P. O., Muto, Y. & Goodman, D. S. (1973) The plasma transport and metabolism of retinoic acid in the rat. Biochem J 132: 821-827.

99. Zachman, R. D. & Olson, J. A. (1961) A comparison of retinene reductase and alcohol dehydrogenase of rat liver. J Biol Chem 236: 2309-2313.

100. Napoli, J. L. (1986) Quantification of physiological levels of retinoic acid. Methods Enzymol 123: 112-124.

101. Posch, K. C., Burns, R. D. & Napoli, J. L. (1992) Biosynthesis of all-trans-retinoic acid from retinal. Recognition of retinal bound to cellular retinol binding protein (type I) as substrate by a purified cytosolic dehydrogenase. J Biol Chem 267: 19676-19682.

102. Zhai, Y., Higgins, D. & Napoli, J. L. (1997) Coexpression of the mRNAs encoding retinol dehydrogenase isozymes and cellular retinol-binding protein. J Cell Physiol 173: 36-43.

103. Zhai, Y., Sperkova, Z. & Napoli, J. L. (2001) Cellular expression of retinal dehydrogenase types 1 and 2: effects of vitamin A status on testis mRNA. J Cell Physiol 186: 220-232.

104. Ziegler, T. L. & Vasiliou, V. (1999) Aldehyde dehydrogenase gene superfamily. The 1998 update. Adv Exp Med Biol 463: 255-263.

105. Penzes, P., Wang, X., Sperkova, Z. & Napoli, J. L. (1997) Cloning of a rat cDNA encoding retinal dehydrogenase isozyme type I and its expression in E. coli. Gene 191: 167-172.

106. Penzes, P., Wang, X. & Napoli, J. L. (1997) Enzymatic characteristics of retinal dehydrogenase type I expressed in Escherichia coli. Biochim Biophys Acta 1342: 175-181.

107. Blaner, W. S. & Olson, J. A. (1994) Retinol and retinoic acid metabolism. In: The Retinoids: Biology, Chemistry, and Medicine, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 229 - 255. Raven Press, New York.

108. De Leenheer, A. P., Lambert, W. E. & Claeys, I. (1982) All-trans-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. J Lipid Res 23: 1362-1367.

109. Eckhoff, C. & Nau, H. (1990) Identification and quantitation of all-trans- and 13-cis-retinoic acid and 13-cis-4-oxoretinoic acid in human plasma. J Lipid Res 31: 1445-1454.

110. Kurlandsky, S. B., Gamble, M. V., Ramakrishnan, R. & Blaner, W. S. (1995) Plasma delivery of retinoic acid to tissues in the rat. J Biol Chem 270: 17850-17857.

111. Giguere, V., Ong, E. S., Segui, P. & Evans, R. M. (1987) Identification of a receptor for the morphogen retinoic acid. Nature 330: 624-629.

112. Petkovich, M., Brand, N. J., Krust, A. & Chambon, P. (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 330: 444-450.

113. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. Science 240: 889-895.

114. Brand, N., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P. & Dejean, A. (1988) Identification of a second human retinoic acid receptor. Nature 332: 850-853.

115. Ishikawa, T., Umesono, K., Mangelsdorf, D. J., Aburatani, H., Stanger, B. Z., Shibasaki, Y., Imawari, M., Evans, R. M. & Takaku, F. (1990) A functional retinoic acid receptor encoded by the gene on human chromosome 12. Mol Endocrinol 4: 837-844.

116. Mangelsdorf, D. J., Umesono, K. & Evans, R. M. (1994) The retinoid receptors. In: The Retinoids: Biology, Chemistry. and Medicine, 2nd ed. (Sporn, M. B., Roberts, A. B. & Goodman, D. S., eds.), pp. 319 - 349. Raven Press, New York.

117. Mangelsdorf, D. J., Ong, E. S., Dyck, J. A. & Evans, R. M. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345: 224-229.

118. Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A. & Evans, R. M. (1992) Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev 6: 329-344.

119. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M. & Thaller, C. (1992) 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 68: 397-406.

120. Levin, A. A., Sturzenbecker, L. J., Kazmer, S., Bosakowski, T., Huselton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M. et al. (1992) 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. Nature 355: 359-361.

121. Kastner, P., Mark, M., Ghyselinck, N., Krezel, W., Dupe, V., Grondona, J. M. & Chambon, P. (1997) Genetic evidence that the retinoid signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development 124: 313-326.

122. Murray, M. B. & Towle, H. C. (1989) Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol Endocrinol 3: 1434-1442.

123. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J. M., Glass, C. K. & Rosenfeld, M. G. (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell 67: 1251-1266.

124. Kliewer, S. A., Umesono, K., Mangelsdorf, D. J. & Evans, R. M. (1992) Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. Nature 355: 446-449.

125. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A. & Mangelsdorf, D. J. (1995) LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev 9: 1033-1045.

126. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. & Evans, R. M. (1992) Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358: 771-774.

127. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J. Y., Staub, A., Garnier, J. M. et al. (1992) Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68: 377-395.

128. Glass, C. K. & Rosenfeld, M. G. (2000) The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev 14: 121-141.

129. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M. et al. (1995) Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377: 397-404.

130. Chen, J. D. & Evans, R. M. (1995) A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377: 454-457.

131. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J. M. & Chambon, P. (1997) Stimulation of RAR alpha activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. Cell 90: 97-107.

132. Taneja, R., Rochette-Egly, C., Plassat, J. L., Penna, L., Gaub, M. P. & Chambon, P. (1997) Phosphorylation of activation functions AF-1 and AF-2 of RAR alpha and RAR gamma is indispensable for differentiation of F9 cells upon retinoic acid and cAMP treatment. Embo J 16: 6452-6465.

133. Rochette-Egly, C., Plassat, J. L., Taneja, R. & Chambon, P. (2000) The AF-1 and AF-2 activating domains of retinoic acid receptor-alpha (RARalpha) and their phosphorylation are differentially involved in parietal endodermal differentiation of F9 cells and retinoid-induced expression of target genes. Mol Endocrinol 14: 1398-1410.

134. Adam-Stitah, S., Penna, L., Chambon, P. & Rochette-Egly, C. (1999) Hyperphosphorylation of the retinoid X receptor alpha by activated c-Jun NH2-terminal kinases. J Biol Chem 274: 18932-18941.

135. Keriel, A., Stary, A., Sarasin, A., Rochette-Egly, C. & Egly, J. M. (2002) XPD mutations prevent TFIIH-dependent transactivation by nuclear receptors and phosphorylation of RARalpha. Cell 109: 125-135.

136. Zhu, J., Gianni, M., Kopf, E., Honore, N., Chelbi-Alix, M., Koken, M., Quignon, F., Rochette-Egly, C. & de The, H. (1999) Retinoic acid induces proteasome-dependent degradation of retinoic acid receptor alpha (RARalpha) and oncogenic RARalpha fusion proteins. Proc Natl Acad Sci U S A 96: 14807-14812.

137. Boudjelal, M., Wang, Z., Voorhees, J. J. & Fisher, G. J. (2000) Ubiquitin/proteasome pathway regulates levels of retinoic acid receptor gamma and retinoid X receptor alpha in human keratinocytes. Cancer Res 60: 2247-2252.

138. Dunagin, P. E., Jr., Zachman, R. D. & Olson, J. A. (1964) Identification of Free and Conjugated Retinoic Acid as a Product of Retinal (Vitamin a Aldehyde) Metabolism in the Rat in Vivo. Biochim Biophys Acta 90: 432-434.

139. Anonymous (1966) The identification of metabolites of retinal and retinoic acid in rat bile. Biochim Biophys Acta 124: 71-85.

140. Roberts, A. B. & DeLuca, H. F. (1967) Pathways of retinol and retinoic acid metabolism in the rat. Biochem J 102: 600-605.

141. Zachman, R. D., Singer, M. B. & Olson, J. A. (1966) Biliary secretion of metabolites of retinol and of retinoic acid in the guinea pig and chick. J Nutr 88: 137-142.

142. Frolik, C. A., Roberts, A. B., Tavela, T. E., Roller, P. P., Newton, D. L. & Sporn, M. B. (1979) Isolation and identification of 4-hydroxy- and 4-oxoretinoic acid. In vitro metabolites of all-trans-retinoic acid in hamster trachea and liver. Biochemistry 18: 2092-2097.

143. Roberts, A. B., Frolik, C. A., Nichols, M. D. & Sporn, M. B. (1979) Retinoid-dependent induction of the in vivo and in vitro metabolism of retinoic acid in tissues of the vitamin A-deficient hamster. J Biol Chem 254: 6303-6309.

144. Williams, J. B. & Napoli, J. L. (1987) Inhibition of retinoic acid metabolism by imidazole antimycotics in F9 embryonal carcinoma cells. Biochem Pharmacol 36: 1386-1388.

145. Van Wauwe, J. P., Coene, M. C., Goossens, J., Van Nijen, G., Cools, W. & Lauwers, W. (1988) Ketoconazole inhibits the in vitro and in vivo metabolism of all-trans-retinoic acid. J Pharmacol Exp Ther 245: 718-722.

146. Van Wauwe, J. P., Coene, M. C., Goossens, J., Cools, W. & Monbaliu, J. (1990) Effects of cytochrome P-450 inhibitors on the in vivo metabolism of all-trans-retinoic acid in rats. J Pharmacol Exp Ther 252: 365-369.

147. Guengerich, F. P. (1992) Human cytochrome P-450 enzymes. Life Sci 50: 1471-1478.

148. Anonymous (2001) Uncommon P450-catalyzed reactions. Curr Drug Metab 2: 93-115.

149. Anonymous (1993) The 1992 Bernard B. Brodie Award Lecture. Bioactivation and detoxication of toxic and carcinogenic chemicals. Drug Metab Dispos 21: 1-6.

150. White, J. A., Guo, Y. D., Baetz, K., Beckett-Jones, B., Bonasoro, J., Hsu, K. E., Dilworth, F. J., Jones, G. & Petkovich, M. (1996) Identification of the retinoic acid-inducible all-trans-retinoic acid 4-hydroxylase. J Biol Chem 271: 29922-29927.

151. White, J. A., Beckett-Jones, B., Guo, Y. D., Dilworth, F. J., Bonasoro, J., Jones, G. & Petkovich, M. (1997) cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. J Biol Chem 272: 18538-18541.

152. Ray, W. J., Bain, G., Yao, M. & Gottlieb, D. I. (1997) CYP26, a novel mammalian cytochrome P450, is induced by retinoic acid and defines a new family. J Biol Chem 272: 18702-18708.

153. Lampen, A., Meyer, S., Arnhold, T. & Nau, H. (2000) Metabolism of vitamin A and its active metabolite all-trans-retinoic acid in small intestinal enterocytes. J Pharmacol Exp Ther 295: 979-985.

154. Marikar, Y., Wang, Z., Duell, E. A., Petkovich, M., Voorhees, J. J. & Fisher, G. J. (1998) Retinoic acid receptors regulate expression of retinoic acid 4-hydroxylase that specifically inactivates all-trans retinoic acid in human keratinocyte HaCaT cells. J Invest Dermatol 111: 434-439.

155. Samokyszyn, V. M., Gall, W. E., Zawada, G., Freyaldenhoven, M. A., Chen, G., Mackenzie, P. I., Tephly, T. R. & Radominska-Pandya, A. (2000) 4-hydroxyretinoic acid, a novel substrate for human liver microsomal UDP-glucuronosyltransferase(s) and recombinant UGT2B7. J Biol Chem 275: 6908-6914.

156. White, J. A., Ramshaw, H., Taimi, M., Stangle, W., Zhang, A., Everingham, S., Creighton, S., Tam, S. P., Jones, G. & Petkovich, M. (2000) Identification of the human cytochrome P450, P450RAI-2, which is predominantly expressed in the adult cerebellum and is responsible for all-trans-retinoic acid metabolism. Proc Natl Acad Sci U S A 97: 6403-6408.

157. Nelson, D. R. (1999) A second CYP26 P450 in humans and zebrafish: CYP26B1. Arch Biochem Biophys 371: 345-347.

158. Gu, X., Xu, F., Wang, X., Gao, X. & Zhao, Q. (2005) Molecular cloning and expression of a novel CYP26 gene (cyp26d1) during zebrafish early development. Gene Expr Patterns 5: 733-739.

159. Gu, X., Xu, F., Song, W., Wang, X., Hu, P., Yang, Y., Gao, X. & Zhao, Q. (2006) A Novel Cytochrome P450, Zebrafish Cyp26D1, Is Involved in Metabolism of All-trans Retinoic Acid. Mol Endocrinol.

160. Tahayato, A., Dolle, P. & Petkovich, M. (2003) Cyp26C1 encodes a novel retinoic acid-metabolizing enzyme expressed in the hindbrain, inner ear, first branchial arch and tooth buds during murine development. Gene Expr Patterns 3: 449-454.

161. Taimi, M., Helvig, C., Wisniewski, J., Ramshaw, H., White, J., Amad, M., Korczak, B. & Petkovich, M. (2004) A novel human cytochrome P450, CYP26C1, involved in metabolism of 9-cis and all-trans isomers of retinoic acid. J Biol Chem 279: 77-85.

162. Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M. J. et al. (2006) Retinoid signaling determines germ cell fate in mice. Science 312: 596-600.

163. Lippel, K. & Olson, J. A. (1968) Biosynthesis of beta-glucuronides of retinol and of retinoic acid in vivo and in vitro. J Lipid Res 9: 168-175.

164. Zile, M. H., Inhorn, R. C. & DeLuca, H. F. (1982) Metabolites of all-trans-retinoic acid in bile: identification of all-trans- and 13-cis-retinoyl glucuronides. J Biol Chem 257: 3537-3543.

165. Barua, A. B., Gunning, D. B. & Olson, J. A. (1991) Metabolism in vivo of all-trans-[11-3H]retinoic acid after an oral dose in rats. Characterization of retinoyl beta-glucuronide in the blood and other tissues. Biochem J 277 (Pt 2): 527-531.

166. Dunagin, P. E., Jr., Meadows, E. H., Jr. & Olson, J. A. (1965) Retinoyl Beta-Glucuronic Acid: A Major Metabolite of Vitamin a in Rat Bile. Science 148: 86-87.

167. Swanson, B. N., Frolik, C. A., Zaharevitz, D. W., Roller, P. P. & Sporn, M. B. (1981) Dose-dependent kinetics of all-trans-retinoic acid in rats. Plasma levels and excretion into bile, urine, and faeces. Biochem Pharmacol 30: 107-113.

168. Skare, K. L., Schnoes, H. K. & DeLuca, H. F. (1982) Biliary metabolites of all-trans-retinoic acid in the rat: isolation and identification of a novel polar metabolite. Biochemistry 21: 3308-3317.

169. Skare, K. L. & DeLuca, H. F. (1983) Biliary metabolites of all-trans-retinoic acid in the rat. Arch Biochem Biophys 224: 13-18.

170. Olson, J. A., Moon, R. C., Anders, M. W., Fenselau, C. & Shane, B. (1992) Enhancement of biological activity by conjugation reactions. J Nutr 122: 615-624.

171. Genchi, G., Wang, W., Barua, A., Bidlack, W. R. & Olson, J. A. (1996) Formation of beta-glucuronides and of beta-galacturonides of various retinoids catalyzed by induced and noninduced microsomal UDP-glucuronosyltransferases of rat liver. Biochim Biophys Acta 1289: 284-290.

172. MacLean, G., Abu-Abed, S., Dolle, P., Tahayato, A., Chambon, P. & Petkovich, M. (2001) Cloning of a novel retinoic-acid metabolizing cytochrome P450, Cyp26B1, and comparative expression analysis with Cyp26A1 during early murine development. Mech Dev 107: 195-201.

173. Abu-Abed, S., Dolle, P., Metzger, D., Beckett, B., Chambon, P. & Petkovich, M. (2001) The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes Dev 15: 226-240.

174. de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J. & van der Saag, P. T. (1999) Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and Xenopus laevis embryogenesis. Mech Dev 82: 205-211.

175. Abu-Abed, S., MacLean, G., Fraulob, V., Chambon, P., Petkovich, M. & Dolle, P. (2002) Differential expression of the retinoic acid-metabolizing enzymes CYP26A1 and CYP26B1 during murine organogenesis. Mech Dev 110: 173-177.

176. Sonneveld, E., van den Brink, C. E., van der Leede, B. M., Schulkes, R. K., Petkovich, M., van der Burg, B. & van der Saag, P. T. (1998) Human retinoic acid (RA) 4-hydroxylase (CYP26) is highly specific for all-trans-RA and can be induced through RA receptors in human breast and colon carcinoma cells. Cell Growth Differ 9: 629-637.

177. Popa, C., Dicker, A. J., Dahler, A. L. & Saunders, N. A. (1999) Cytochrome P450, CYP26AI, is expressed at low levels in human epidermal keratinocytes and is not retinoic acid-inducible. Br J Dermatol 141: 460-468.

178. Loudig, O., Babichuk, C., White, J., Abu-Abed, S., Mueller, C. & Petkovich, M. (2000) Cytochrome P450RAI(CYP26) promoter: a distinct composite retinoic acid response element underlies the complex regulation of retinoic acid metabolism. Mol Endocrinol 14: 1483-1497.

179. Lampen, A., Meyer, S. & Nau, H. (2001) Effects of receptor-selective retinoids on CYP26 gene expression and metabolism of all-trans-retinoic acid in intestinal cells. Drug Metab Dispos 29: 742-747.

180. Leo, M. A., Lasker, J. M., Raucy, J. L., Kim, C. I., Black, M. & Lieber, C. S. (1989) Metabolism of retinol and retinoic acid by human liver cytochrome P450IIC8. Arch Biochem Biophys 269: 305-312.

181. Roberts, E. S., Vaz, A. D. & Coon, M. J. (1992) Role of isozymes of rabbit microsomal cytochrome P-450 in the metabolism of retinoic acid, retinol, and retinal. Mol Pharmacol 41: 427-433.

182. Green, H. N. & Mellanby, E. (1928) Vitamin A as an anti-infective agent. Br Med J 306: 366 - 370.

183. Chaplin, D. D. (2006) 1. Overview of the human immune response. J Allergy Clin Immunol 117: S430-435.

184. Anonymous (2003) 1. Overview of the immune response. J Allergy Clin Immunol 111: S442-459.

185. Dauphinee, S. M. & Karsan, A. (2006) Lipopolysaccharide signaling in endothelial cells. Lab Invest 86: 9-22.

186. Tobias, P. S., Gegner, J., Han, J., Kirkland, T., Kravchenko, V., Leturcq, D., Lee, J. D., Moriarty, A., Mathison, J. C. et al. (1994) LPS binding protein and CD14 in the LPS dependent activation of cells. Prog Clin Biol Res 388: 31-39.

187. Jack, R. S., Fan, X., Bernheiden, M., Rune, G., Ehlers, M., Weber, A., Kirsch, G., Mentel, R., Furll, B. et al. (1997) Lipopolysaccharide-binding protein is required to combat a murine gram-negative bacterial infection. Nature 389: 742-745.

188. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249: 1431-1433.

189. Kirkland, T. N., Finley, F., Leturcq, D., Moriarty, A., Lee, J. D., Ulevitch, R. J. & Tobias, P. S. (1993) Analysis of lipopolysaccharide binding by CD14. J Biol Chem 268: 24818-24823.

190. Miyake, K., Ogata, H., Nagai, Y., Akashi, S. & Kimoto, M. (2000) Innate recognition of lipopolysaccharide by Toll-like receptor 4/MD-2 and RP105/MD-1. J Endotoxin Res 6: 389-391.

191. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. & Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 189: 1777-1782.

192. Viriyakosol, S., Kirkland, T., Soldau, K. & Tobias, P. (2000) MD-2 binds to bacterial lipopolysaccharide. J Endotoxin Res 6: 489-491.

193. Akashi, S., Shimazu, R., Ogata, H., Nagai, Y., Takeda, K., Kimoto, M. & Miyake, K. (2000) Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol 164: 3471-3475.

194. Akashi, S., Ogata, H., Kirikae, F., Kirikae, T., Kawasaki, K., Nishijima, M., Shimazu, R., Nagai, Y., Fukudome, K. et al. (2000) Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. Biochem Biophys Res Commun 268: 172-177.

195. Hashimoto, C., Hudson, K. L. & Anderson, K. V. (1988) The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52: 269-279.

196. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. & Hoffmann, J. A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86: 973-983.

197. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C. A., Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388: 394-397.

198. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A. & Bazan, J. F. (1998) A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A 95: 588-593.

199. Akira, S., Hoshino, K. & Kaisho, T. (2000) The role of Toll-like receptors and MyD88 in innate immune responses. J Endotoxin Res 6: 383-387.

200. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413: 732-738.

201. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M. et al. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 282: 2085-2088.

202. Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. & Malo, D. (1999) Endotoxin-tolerant mice have mutations in Toll-like receptor 4 (Tlr4). J Exp Med 189: 615-625.

203. Muzio, M., Bosisio, D., Polentarutti, N., D'Amico, G., Stoppacciaro, A., Mancinelli, R., van't Veer, C., Penton-Rol, G., Ruco, L. P. et al. (2000) Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells. J Immunol 164: 5998-6004.

204. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S. (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 162: 3749-3752.

205. Haziot, A., Ferrero, E., Lin, X. Y., Stewart, C. L. & Goyert, S. M. (1995) CD14-deficient mice are exquisitely insensitive to the effects of LPS. Prog Clin Biol Res 392: 349-351.

206. Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C. L. & Goyert, S. M. (1996) Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. Immunity 4: 407-414.

207. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G. et al. (1999) TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev 13: 1015-1024.

208. Klasing, K. C. (1984) Effect of inflammatory agents and interleukin 1 on iron and zinc metabolism. Am J Physiol 247: R901-904.

209. Argiles, J. M. & Lopez-Soriano, F. J. (1989) Effects of tumour necrosis factor on hepatic amino acid uptake. Biochem Soc Trans 17: 1045-1046.

210. Schreiber, G., Tsykin, A., Aldred, A. R., Thomas, T., Fung, W. P., Dickson, P. W., Cole, T., Birch, H., De Jong, F. A. & Milland, J. (1989) The acute phase response in the rodent. Ann N Y Acad Sci 557: 61-85; discussion 85-66.

211. Pepys, M. B. & Baltz, M. L. (1983) Acute phase proteins with special reference to C-reactive protein and related proteins (pentaxins) and serum amyloid A protein. Adv Immunol 34: 141-212.

212. Birch, H. E. & Schreiber, G. (1986) Transcriptional regulation of plasma protein synthesis during inflammation. J Biol Chem 261: 8077-8080.

213. Rosales, F. J. & Ross, A. C. (1998) Acute inflammation induces hyporetinemia and modifies the plasma and tissue response to vitamin A supplementation in marginally vitamin A-deficient rats. J Nutr 128: 960-966.

214. Rosales, F. J., Ritter, S. J., Zolfaghari, R., Smith, J. E. & Ross, A. C. (1996) Effects of acute inflammation on plasma retinol, retinol-binding protein, and its mRNA in the liver and kidneys of vitamin A-sufficient rats. J Lipid Res 37: 962-971.

215. Siewert, E., Bort, R., Kluge, R., Heinrich, P. C., Castell, J. & Jover, R. (2000) Hepatic cytochrome P450 down-regulation during aseptic inflammation in the mouse is interleukin 6 dependent. Hepatology 32: 49-55.

216. Cheng, P. Y., Wang, M. & Morgan, E. T. (2003) Rapid transcriptional suppression of rat cytochrome P450 genes by endotoxin treatment and its inhibition by curcumin. J Pharmacol Exp Ther 307: 1205-1212.

217. Whiting, J. F., Green, R. M., Rosenbluth, A. B. & Gollan, J. L. (1995) Tumor necrosis factor-alpha decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. Hepatology 22: 1273-1278.

218. Hartmann, G., Kim, H. & Piquette-Miller, M. (2001) Regulation of the hepatic multidrug resistance gene expression by endotoxin and inflammatory cytokines in mice. Int Immunopharmacol 1: 189-199.

219. Hartmann, G., Cheung, A. K. & Piquette-Miller, M. (2002) Inflammatory cytokines, but not bile acids, regulate expression of murine hepatic anion transporters in endotoxemia. J Pharmacol Exp Ther 303: 273-281.

220. Sommer, A., Tarwotjo, I., Hussaini, G. & Susanto, D. (1983) Increased mortality in children with mild vitamin A deficiency. Lancet 2: 585-588.

221. Sommer, A. (1984) Vitamin A deficiency and mortality risk. Lancet 1: 347-348.

222. Sommer, A., Tarwotjo, I., Djunaedi, E., West, K. P., Jr., Loeden, A. A., Tilden, R. & Mele, L. (1986) Impact of vitamin A supplementation on childhood mortality. A randomised controlled community trial. Lancet 1: 1169-1173.

223. Stephensen, C. B. (2001) Vitamin A, infection, and immune function. Annu Rev Nutr 21: 167-192.

224. Bankson, D. D. & Russell, R. M. (1988) Protein energy malnutrition and taurine supplementation: effects on vitamin A nutritional status and electroretinogram of young rats. J Nutr 118: 23-32.

225. Basu, T. K. & Basualdo, C. (1997) Vitamin A homeostasis and diabetes mellitus. Nutrition 13: 804 - 806.

226. Gieng, S. H., Raila, J. & Rosales, F. J. (2005) Accumulation of retinol in the liver after prolonged hyporetinolemia in the vitamin A-sufficient rat. J Lipid Res 46: 641-649.

227. Morgan, E. T., Li-Masters, T. & Cheng, P. Y. (2002) Mechanisms of cytochrome P450 regulation by inflammatory mediators. Toxicology 181-182: 207-210.

228. Morgan, E. T. (1997) Regulation of cytochromes P450 during inflammation and infection. Drug Metab Rev 29: 1129-1188.

229. Cherrington, N. J., Slitt, A. L., Li, N. & Klaassen, C. D. (2004) Lipopolysaccharide-mediated regulation of hepatic transporter mRNA levels in rats. Drug Metab Dispos 32: 734-741.

230. Cousins, S. W., Guss, R. B., Howes, E. L., Jr. & Rosenbaum, J. T. (1984) Endotoxin-induced uveitis in the rat: observations on altered vascular permeability, clinical findings, and histology. Exp Eye Res 39: 665-676.

231. Edamitsu, S., Matsukawa, A., Ohkawara, S., Takagi, K., Nariuchi, H. & Yoshinaga, M. (1995) Role of TNF alpha, IL-1, and IL-1ra in the mediation of leukocyte infiltration

and increased vascular permeability in rabbits with LPS-induced pleurisy. Clin Immunol Immunopathol 75: 68-74.

232. Memon, R. A., Feingold, K. R., Moser, A. H., Fuller, J. & Grunfeld, C. (1998) Regulation of fatty acid transport protein and fatty acid translocase mRNA levels by endotoxin and cytokines. Am J Physiol 274: E210-217.

233. Memon, R. A., Bass, N. M., Moser, A. H., Fuller, J., Appel, R., Grunfeld, C. & Feingold, K. R. (1999) Down-regulation of liver and heart specific fatty acid binding proteins by endotoxin and cytokines in vivo. Biochim Biophys Acta 1440: 118-126.

234. Feingold, K., Kim, M. S., Shigenaga, J., Moser, A. & Grunfeld, C. (2004) Altered expression of nuclear hormone receptors and coactivators in mouse heart during the acute-phase response. Am J Physiol Endocrinol Metab 286: E201-207.

235. Varma, R. N. & Beaton, G. H. (1972) Quantitative aspects of the urinary and fecal excretion of radioactive metabolites of vitamin A in the rat. Can J Physiol Pharmacol 50: 1026-1037.

236. Hicks, V. A., Gunning, D. B. & Olson, J. A. (1984) Metabolism, plasma transport and biliary excretion of radioactive vitamin A and its metabolites as a function of liver reserves of vitamin A in the rat. J Nutr 114: 1327-1333.

237. Lewis, K. C. (1987) Multicompartmental Tracer Kinetic Analysis of Retinol Metabolism in Rats with Low Vitamin A Status. The Pennsylvania State University, University Park.

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

239. Wastney, M. E., Patterson, B. H., Linares, O. A., Greif, P. C. & Boston, R. C. (1999) Investigating Biological Systems Using Modeling. Academic Press, San Diego.

240. Novotny, J. A., Green, M. H. & Boston, R. C., eds. (2003) Mathematical Modeling in Nutrition and Health Sciences. Kluwer Academic/Plenum Publishers, New York.

241. Foster, D. M. & Boston, R. C. (1983) The use of computers in compartmental analysis: the SAAM and CONSAM programs. In: Compartmental Distribution of Radiotracers (Robertson, J. S., ed.), pp. 73 - 142. CRC Press, Boca Raton.

242. Birge, S. J., Peck, W. A., Berman, M. & Whedon, G. D. (1969) Study of calcium absorption in man: a kinetic analysis and physiologic model. J Clin Invest 48: 1705-1713.

243. Wastney, M. E., Angelus, P., Barnes, R. M. & Subramanian, K. N. (1996) Zinc kinetics in preterm infants: a compartmental model based on stable isotope data. Am J Physiol 271: R1452-1459.

244. Pinna, K., Woodhouse, L. R., Sutherland, B., Shames, D. M. & King, J. C. (2001) Exchangeable zinc pool masses and turnover are maintained in healthy men with low zinc intakes. J Nutr 131: 2288-2294.

245. Adiels, M., Packard, C., Caslake, M. J., Stewart, P., Soro, A., Westerbacka, J., Wennberg, B., Olofsson, S. O., Taskinen, M. R. & Boren, J. (2005) A new combined multicompartmental model for apolipoprotein B-100 and triglyceride metabolism in VLDL subfractions. J Lipid Res 46: 58-67.

246. Malmendier, C. L., Delcroix, C. & Berman, M. (1974) Interrelations in the oxidative metabolism of free fatty acids, glucose, and glycerol in normal and hyperlipemic patients. A compartmental model. J Clin Invest 54: 461-476.

247. DiStefano, J. J., 3rd & Landaw, E. M. (1984) Multiexponential, multicompartmental, and noncompartmental modeling. I. Methodological limitations and physiological interpretations. Am J Physiol 246: R651-664.

248. Carson, E. R., Cobelli, C. & Finkelstein, L. (1983) The Mathematical Modeling of Metabolic and Endocrine Systems: Model Formulation, Identification, and Validation. John Wiley and Sons, New York.

249. Landaw, E. M. & DiStefano, J. J., 3rd (1984) Multiexponential, multicompartmental, and noncompartmental modeling. II. Data analysis and statistical considerations. Am J Physiol 246: R665-677.

250. Thompson, J. N., Erdody, P., Brien, R. & Murray, T. K. (1971) Fluorometric determination of vitamin A in human blood and liver. Biochem Med 5: 67-89.

251. Hara, A. & Radin, N. S. (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90: 420-426.

252. Duncan, T. E., Green, J. B. & Green, M. H. (1993) Liver vitamin A levels in rats are predicted by a modified isotope dilution technique. J Nutr 123: 933-939.

253. Green, M. H. & Green, J. B. (2005) The Application of Model-based Compartmental Analysis to the Study of Kinetic Behavior of Vitamin A in Vitamin A-sufficient Rats: Validation of a Theoretical Model. In: Mathematical Modeling in Nutrition and Toxicology (Hargrove, J. L. & Berdanier, C. D., eds.), pp. 175 - 187. Mathematical Biology Press, Athens.

254. Lewis, K. C., Green, M. H., Green, J. B. & Zech, L. A. (1988) Effects of retinoic acid supplementation on retinol kinetics in rats with low vitamin A status. Faseb J 2: A844.

255. Scrimshaw, N. S., Taylor, C. E. & Gordon, J. E. (1968) Interactions of nutrition and infection. Monogr Ser World Health Organ 57: 3-329.

256. Goodman, T., Dalmiya, N., de Benoist, B. & Schultink, W. (2000) Polio as a platform: using national immunization days to deliver vitamin A supplements. Bull World Health Organ 78: 305-314.

257. Villamor, E. & Fawzi, W. W. (2000) Vitamin A supplementation: implications for morbidity and mortality in children. J Infect Dis 182 Suppl 1: S122-133.

258. DeCicco, K. L., Zolfaghari, R., Li, N. & Ross, A. C. (2000) Retinoic acid and polyriboinosinic acid act synergistically to enhance the antibody response to tetanus toxoid during vitamin A deficiency: possible involvement of interleukin-2 receptor-beta, signal transducer and activator of transcription-1, and interferon regulatory factor-1. J Infect Dis 182 Suppl 1: S29-36.

259. Gardner, E. M. & Ross, A. C. (1993) Dietary vitamin A restriction produces marginal vitamin A status in young rats. J Nutr 123: 1435-1443.

260. Reeves, P. G., Rossow, K. L. & Lindlauf, J. (1993) Development and testing of the AIN-93 purified diets for rodents: results on growth, kidney calcification and bone mineralization in rats and mice. J Nutr 123: 1923-1931.

261. Disdier, B., Bun, H., Catalin, J. & Durand, A. (1996) Simultaneous determination of all-trans-, 13-cis-, 9-cis-retinoic acid and their 4-oxo-metabolites in plasma by high-performance liquid chromatography. J Chromatogr B Biomed Appl 683: 143-154.

262. Folch, J., Lees, M. & Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226: 497-509.

263. Folch, J., Ascoli, I., Lees, M., Meath, J. A. & Le, B. N. (1951) Preparation of lipide extracts from brain tissue. J Biol Chem 191: 833-841.

264. Adamson, P. C. (1994) Clinical and pharmacokinetic studies of all-trans-retinoic acid in pediatric patients with cancer. Leukemia 8: 1813-1816.

265. Anonymous (1994) Pharmacokinetics of all-trans-retinoic acid: clinical implications in acute promyelocytic leukemia. Semin Hematol 31: 14-17.

266. Regazzi, M. B., Iacona, I., Gervasutti, C., Lazzarino, M. & Toma, S. (1997) Clinical pharmacokinetics of tretinoin. Clin Pharmacokinet 32: 382-402.

267. Ross, A. C. (2003) Retinoid production and catabolism: role of diet in regulating retinol esterification and retinoic Acid oxidation. J Nutr 133: 291S-296S.

268. Posch, K. C., Boerman, M. H., Burns, R. D. & Napoli, J. L. (1991) Holocellular retinol binding protein as a substrate for microsomal retinal synthesis. Biochemistry 30: 6224-6230.

269. Herr, F. M. & Ong, D. E. (1992) Differential interaction of lecithin-retinol acyltransferase with cellular retinol binding proteins. Biochemistry 31: 6748-6755.

270. Liu, C., Russell, R. M. & Wang, X. D. (2003) Exposing ferrets to cigarette smoke and a pharmacological dose of beta-carotene supplementation enhance in vitro retinoic acid catabolism in lungs via induction of cytochrome P450 enzymes. J Nutr 133: 173-179.

271. Underwood, B. A. & Arthur, P. (1996) The contribution of vitamin A to public health. Faseb J 10: 1040-1048.

272. Hussey, G. D. & Klein, M. (1992) Measles-induced vitamin A deficiency. Ann N Y Acad Sci 669: 188-194; discussion 194-186.

273. Degos, L., Chomienne, C., Daniel, M. T., Berger, R., Dombret, H., Fenaux, P. & Castaigne, S. (1990) Treatment of first relapse in acute promyelocytic leukaemia with all-trans retinoic acid. Lancet 336: 1440-1441.

274. Warrell, R. P., Jr., Frankel, S. R., Miller, W. H., Jr., Scheinberg, D. A., Itri, L. M., Hittelman, W. N., Vyas, R., Andreeff, M., Tafuri, A. et al. (1991) Differentiation therapy of acute promyelocytic leukemia with tretinoin (all-trans-retinoic acid). N Engl J Med 324: 1385-1393.

275. Hill, D. L. & Grubbs, C. J. (1992) Retinoids and cancer prevention. Annu Rev Nutr 12: 161-181.

276. Warrell, R. P., Jr. (1993) Retinoid resistance in acute promyelocytic leukemia: new mechanisms, strategies, and implications. Blood 82: 1949-1953.

277. Muindi, J., Frankel, S. R., Miller, W. H., Jr., Jakubowski, A., Scheinberg, D. A., Young, C. W., Dmitrovsky, E. & Warrell, R. P., Jr. (1992) Continuous treatment with all-trans retinoic acid causes a progressive reduction in plasma drug concentrations: implications for relapse and retinoid "resistance" in patients with acute promyelocytic leukemia. Blood 79: 299-303.

278. Guengerich, F. P. (1992) Characterization of human cytochrome P450 enzymes. Faseb J 6: 745-748.

279. Huang, M. E., Ye, Y. C., Chen, S. R., Chai, J. R., Lu, J. X., Zhoa, L., Gu, L. J. & Wang, Z. Y. (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. Blood 72: 567-572.

280. Castaigne, S., Chomienne, C., Daniel, M. T., Ballerini, P., Berger, R., Fenaux, P. & Degos, L. (1990) All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. Blood 76: 1704-1709.

281. Lazzarino, M., Regazzi, M. B. & Corso, A. (1996) Clinical relevance of all-trans retinoic acid pharmacokinetics and its modulation in acute promyelocytic leukemia. Leuk Lymphoma 23: 539-543.

282. Adamson, P. C., Bailey, J., Pluda, J., Poplack, D. G., Bauza, S., Murphy, R. F., Yarchoan, R. & Balis, F. M. (1995) Pharmacokinetics of all-trans-retinoic acid administered on an intermittent schedule. J Clin Oncol 13: 1238-1241.

283. Sommer, A., Katz, J. & Tarwotjo, I. (1984) Increased risk of respiratory disease and diarrhea in children with preexisting mild vitamin A deficiency. Am J Clin Nutr 40: 1090-1095.

284. Ozpolat, B., Lopez-Berestein, G., Adamson, P., Fu, C. J. & Williams, A. H. (2003) Pharmacokinetics of intravenously administered liposomal all-trans-retinoic acid (ATRA) and orally administered ATRA in healthy volunteers. J Pharm Pharm Sci 6: 292-301.

285. Ross, A. C. & Zolfaghari, R. (2004) Regulation of hepatic retinol metabolism: perspectives from studies on vitamin A status. J Nutr 134: 269S-275S.

286. Matsuura, T., Gad, M. Z., Harrison, E. H. & Ross, A. C. (1997) Lecithin:retinol acyltransferase and retinyl ester hydrolase activities are differentially regulated by retinoids and have distinct distributions between hepatocyte and nonparenchymal cell fractions of rat liver. J Nutr 127: 218-224.

287. Takatsuka, J., Takahashi, N. & de Luca, L. M. (1996) Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. Cancer Res 56: 675-678.

288. Kalitsky-Szirtes, J., Shayeganpour, A., Brocks, D. R. & Piquette-Miller, M. (2004) Suppression of drug-metabolizing enzymes and efflux transporters in the intestine of endotoxin-treated rats. Drug Metab Dispos 32: 20-27.

289. Lin, S. L., Lee, Y. M., Chang, H. Y., Cheng, Y. W. & Yen, M. H. (2005) Effects of naltrexone on lipopolysaccharide-induced sepsis in rats. J Biomed Sci 12: 431-440.

290. Tokuyama, H. & Tokuyama, Y. (1995) Endogenous cytokine expression profiles in retinoic acid-induced IgA production by LPS-stimulated murine splenocytes. Cell Immunol 166: 247-253.

291. Ross, A. C., Zolfaghari, R. & Weisz, J. (2001) Vitamin A: recent advances in the biotransformation, transport, and metabolism of retinoids. Curr Opin Gastroenterol 17: 184-192.

292. Barua, A. B. & Olson, J. A. (1986) Retinoyl beta-glucuronide: an endogenous compound of human blood. Am J Clin Nutr 43: 481-485.

293. Sommer, A. & West, K. P., Jr. (1996). In: Vitamin A deficiency: health, survival, and vision. Oxford University Press, Inc., New York.

294. Dawson, H. D. & Ross, A. C. (1999) Chronic marginal vitamin A status affects the distribution and function of T cells and natural T cells in aging Lewis rats. J Nutr 129: 1782-1790.

295. Semba, R. D. (1994) Vitamin A, immunity, and infection. Clin Infect Dis 19: 489-499.

296. Biesalski, H. K. (1989) Comparative assessment of the toxicology of vitamin A and retinoids in man. Toxicology 57: 117-161.

297. Soprano, D. R. & Soprano, K. J. (1995) Retinoids as teratogens. Annu Rev Nutr 15: 111-132.

298. Ross, A. C. (2003) Hepatic Metabolism of Vitamin A. In: Hepatology: A Textbook of Liver Disease (Zakim, D. & Boyer, T., eds.), pp. 149 - 168. Saunders, Philadelphia.

299. Smith, J. E., DeMoor, L. M., Handler, C. E., Green, E. L. & Ritter, S. J. (1998) The complex between retinol and retinol-binding protein is formed in the rough microsomes of liver following repletion of vitamin A-depleted rats. Biochim Biophys Acta 1380: 10-20.

300. Ronne, H., Ocklind, C., Wiman, K., Rask, L., Obrink, B. & Peterson, P. A. (1983) Ligand-dependent regulation of intracellular protein transport: effect of vitamin a on the secretion of the retinol-binding protein. J Cell Biol 96: 907-910.

301. Peterson, P. A., Rask, L., Ostberg, L., Andersson, L., Kamwendo, F. & Pertoft, H. (1973) Studies on the transport and cellular distribution of vitamin A in normal and vitamin A-deficient rats with special reference to the vitamin A-binding plasma protein. J Biol Chem 248: 4009-4022.

302. Massrieh, W., Derjuga, A., Doualla-Bell, F., Ku, C. Y., Sanborn, B. M. & Blank, V. (2006) Regulation of the MAFF transcription factor by proinflammatory cytokines in myometrial cells. Biol Reprod 74: 699-705.

303. Ammon-Treiber, S., Grecksch, G., Stumm, R., Riechert, U., Tischmeyer, H., Reichenauer, A. & Hollt, V. (2004) Rapid, transient, and dose-dependent expression of hsp70 messenger RNA in the rat brain after morphine treatment. Cell Stress Chaperones 9: 182-197.

304. Duque, J., Diaz-Munoz, M. D., Fresno, M. & Iniguez, M. A. (2006) Up-regulation of cyclooxygenase-2 by interleukin-1beta in colon carcinoma cells. Cell Signal 18: 1262-1269.

305. Krusekopf, S., Roots, I., Hildebrandt, A. G. & Kleeberg, U. (2003) Time-dependent transcriptional induction of CYP1A1, CYP1A2 and CYP1B1 mRNAs by H+/K+ -ATPase inhibitors and other xenobiotics. Xenobiotica 33: 107-118.

306. Tashiro, K., Abe, T., Oue, N., Yasui, W. & Ryoji, M. (2004) Characterization of vitamin D-mediated induction of the CYP 24 transcription. Mol Cell Endocrinol 226: 27-32.

307. Soprano, D. R., Chen, L. X., Wu, S., Donigan, A. M., Borghaei, R. C. & Soprano, K. J. (1996) Overexpression of both RAR and RXR restores AP-1 repression in ovarian adenocarcinoma cells resistant to retinoic acid-dependent growth inhibition. Oncogene 12: 577-584.

308. Zhou, X. F., Shen, X. Q. & Shemshedini, L. (1999) Ligand-activated retinoic acid receptor inhibits AP-1 transactivation by disrupting c-Jun/c-Fos dimerization. Mol Endocrinol 13: 276-285.

309. Tang, G. W. & Russell, R. M. (1990) 13-cis-retinoic acid is an endogenous compound in human serum. J Lipid Res 31: 175-182.

310. Lemke, S. L., Dueker, S. R., Follett, J. R., Lin, Y., Carkeet, C., Buchholz, B. A., Vogel, J. S. & Clifford, A. J. (2003) Absorption and retinol equivalence of beta-carotene in humans is influenced by dietary vitamin A intake. J Lipid Res 44: 1591-1600.

311. Lin, Y., Dueker, S. R., Follett, J. R., Fadel, J. G., Arjomand, A., Schneider, P. D., Miller, J. W., Green, R., Buchholz, B. A. et al. (2004) Quantitation of in vivo human folate metabolism. Am J Clin Nutr 80: 680-691.

312. de Moura, F. F., Ho, C. C., Getachew, G., Hickenbottom, S. & Clifford, A. J. (2005) Kinetics of 14C distribution after tracer dose of 14C-lutein in an adult woman. Lipids 40: 1069-1073.

Appendix A. Supplemental Figures

1 MGLPA**LL**ASA LCTFVLP**LLL** FLAALKLWDL YCVSSRDRSC ALPLPPGTMG

51 FPFFGETLQM VLQ**RR**KFLQM K**RR**KYGFIYK THLFGRPTVR VMGADNV**RR**I

101 **LL**GEHRLVSV HWPASVRTIL *GAGCLSNLHD SSHKQRKK*VI MQAFNREALQ

151 CYVPVIAEEV SGCLEQWLSC GERG**LL**VYPE VKRLMFRIAM RI**LL**GCEPGP

201 AGGGEDEQQL VEAFEEMTRN LFSLPIDVPF SGLYRGVKPR NLIHARIEEN

251 IRAKI**RR**LQA AERNAGCKDA LQ**LL**IEHSWE RGERLDMQAL KQSSTE**LL**FG

301 GHETTASAAT SLITYLGLYP HVLQKVREEI KSKG**LL**CKSH HEDKLDMETL

351 EQLKYIGCVI KETLRLNPPV PGGFRVALKT FELNGYQIPK GWNVIYSICD

401 THDVADSFTN KEEFNPDRFT SLHPEDTSRF SFIPFGGGLR SCRSKEFAKI

451 **LL**KIFTVELA **RR**CDWQ**LL**NG PPTMKTSPTV YPVDNLPARF THFQGDI **497**

Figure A1. The amino acid sequence of rat CYP26A1. The antigenic peptide is underlined and in italics, (Alpha Diagnostic, Inc., San Antonio, TX) while proposed sites for microsomal endoproteases are underlined and in bold (Kawabata and Davie, 1992).

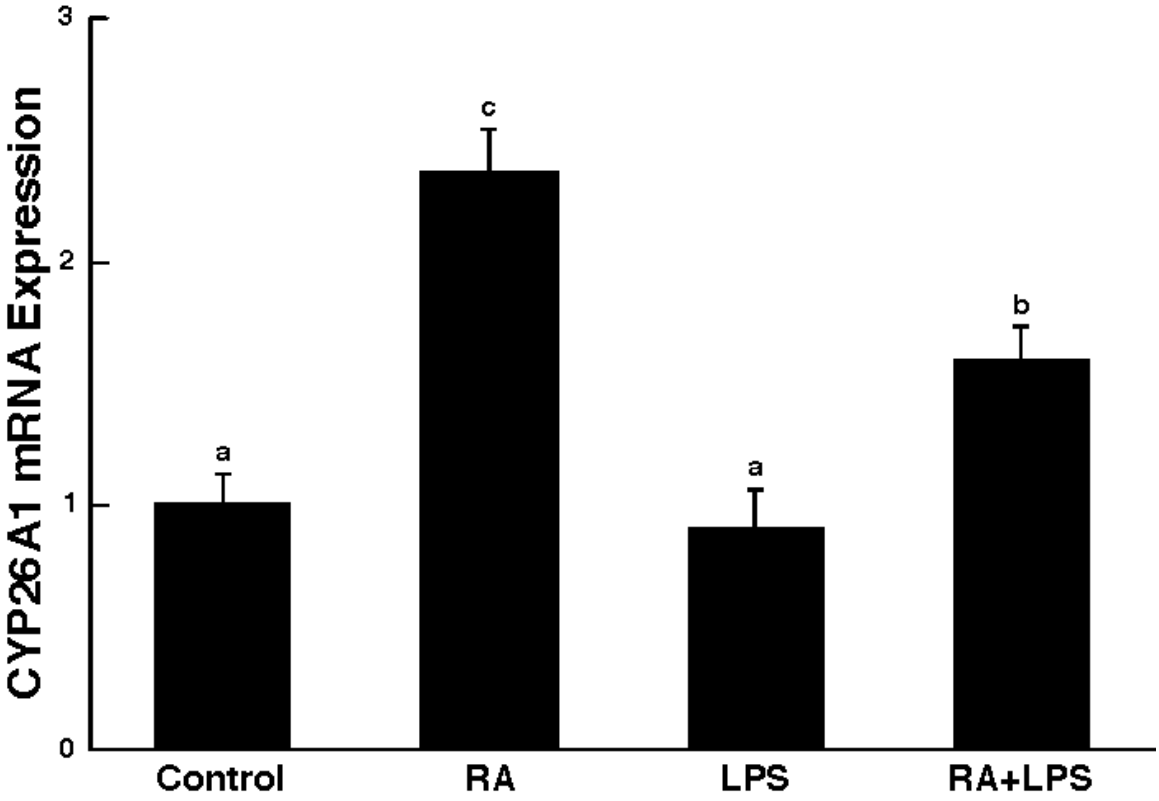

Figure A2. LPS attenuates the RA-induced increase in hepatic CYP26A1 mRNA expression in VA-sufficient rats. Female Spague-Dawley rats were treated with RA, LPS, or the combination of RA + LPS and [^{3}H]RA (0.23 nM) bound to rat serum albumin was injected 6 hours later. Liver was collected, RNA was extracted using TRIzol, and CYP26A1 mRNA expression was determined via RT-PCR (n = 6 / group). Bars are means ± SEM. Different letters above bars indicate significant differences ($P < 0.05$, a < b < c).

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Cifelli CJ, Green JB, Green MH. Dietary retinoic acid alters vitamin A kinetics in both the whole body and in specific organs of rats with low vitamin A status. *J Nutr*. 2005 Apr; 135(4):746-52.

Zolfaghari R, Cifelli CJ, Banta MD, Ross AC. Fatty acid Δ^5-desaturase mRNA is regulated by dietary vitamin A and exogenous retinoic acid in liver of adult rats. *Arch Biochem Biophys*. 2001; 391:8-15.

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