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Diet-dependent retinoid effects on liver gene expression include stellate and inflammation markers and parallel effects of the nuclear repressor *Shp*☆

Meghan Maguire[a,b], Justin R. Bushkofsky[a], Michele Campaigne Larsen[b], Yee Hoon Foong[a], Sherry A. Tanumihardjo[c], Colin R. Jefcoate[a,b,*]

[a]*Endocrinology and Reproductive Physiology Program, University of Wisconsin-Madison, Madison, WI 53705*
[b]*Department of Cell and Regenerative Biology, University of Wisconsin-Madison, Madison, WI 53705*
[c]*Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, WI 53705*

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Abstract

For mice, a maternal vitamin A (VA)-deficient diet initiated from midgestation (GVAD) produces serum retinol deficiency in mature offspring. We hypothesize that the effects of GVAD arise from preweaning developmental changes. We compare the effect of this GVAD protocol in combination with a postweaning high-fat diet (HFD) or high-carbohydrate diet (LF12). Each is compared to an equivalent VA-sufficient combination. GVAD extensively decreased serum retinol and liver retinol, retinyl esters, and retinoid homeostasis genes (*Lrat, Cyp26b1* and *Cyp26a1*). These suppressions were each more effective with LF12 than with HFD. Postweaning initiation of VA deficiency with LF12 depleted liver retinoids, but serum retinol was unaffected. Liver retinoid depletion, therefore, precedes serum attenuation. Maternal LF12 decreased the obesity response to the HFD, which was further decreased by GVAD. LF12 fed to the mother and offspring extensively stimulated genes marking stellate activation (*Col1a1, Timp2* and *Cyp1b1*) and novel inflammation markers (*Ly6d*, *Trem2* and *Nupr1*). The GVAD with LF12 diet combination suppressed these responses. GVAD in combination with the HFD increased these same clusters. A further set of expression differences on the HFD when compared to a high-carbohydrate diet was prevented when GVAD was combined with HFD. Most of these GVAD gene changes match published effects from deletion of *Nr0b2/Shp*, a retinoid-responsive, nuclear co-repressor that modulates metabolic homeostasis. The stellate and inflammatory increases seen with the high-carbohydrate LF12 diet may represent postprandial responses. They depend on retinol and *Shp,* but the regulation reverses with an HFD.

Keywords: Retinoids; Vitamin A deficiency; Liver; Stellate cell activation; Small heterodimer partner (Nr0b2/Shp)

1. Introduction

Vitamin A (VA)/retinol is an essential lipophilic micronutrient that regulates development, reproduction and immunity. While a retinaldehyde isomer functions in the retina of the eye as a light sensor, retinol is largely active through conversion to retinoic acid (RA) by a set of selective alcohol and aldehyde dehydrogenases (*Rdh1, 10, 15*; *Aldh1a1–3*) [1]. RA activates transcription in the nucleus through a set of receptors, RAR forms (α, β, γ), that, like other nonsteroid receptors, function as heterodimers with retinoid X receptors which can be activated by 9-cis retinol [2]. Retinol may scavenge oxygen radicals and may have important functions as an antioxidant [3]. Retinol and RA play key spatiotemporal roles in controlling morphogenic aspects of embryogenesis and later in organogenesis [4].

Fetal development relies on maternal transplacental transfer of retinol [5], which decreases maternal liver retinyl ester (RE) content and disrupts serum retinol homeostasis between gestational day 9.5 and 15.5 in mice [6]. In rats, a postweaning VA-deficient diet (PWVAD) is sufficient to appreciably lower serum and liver retinoids and to change liver gene expression in accord with altered homeostasis [7,8]. However, mice exhibit stronger homeostatic control, possibly *via* coprophagy and recycling of retinoids, as well as conversion of provitamin A carotenoids in the diet [7,9–11]. Mice only show depletion of serum retinol when VA deficiency is initiated in the maternal diet at midgestation and maintained in the postweaning diet (GVAD). Attenuation of serum retinol concentration becomes extensive as mice approach maturity [10,12]. A liver decrease in RE stores is likely essential for serum retinol depletion; however, additional gene changes produced during fetal development may be required. We

Abbreviations: VA, vitamin A; RA, retinoic acid; RE, retinyl ester; PWVAD, postweaning initiation of vitamin-A-deficient diet; GVAD, gestational initiation of vitamin-A-deficient diet; VAD diet, vitamin-A-deficient diet; DIO, diet-induced obesity; HFD, high-fat diet; LFD, low-fat diet; LF12, novel low-fat diet with 12% kcal from fat; BD, breeder diet; E, embryonic day; PN, postnatal day; BW, body weight.

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* Corresponding author at: University of Wisconsin School of Medicine and Public Health, Department of Cell and Regenerative Biology, 1111 Highland Avenue, Madison, WI 53705. Tel.: +1 608 263 3975.
E-mail address: jefcoate@wisc.edu (C.R. Jefcoate).

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have developed a maternal diet that is particularly effective in causing this serum retinol depletion (VAD diet).

Dietary retinol is absorbed by intestinal epithelia and esterified by lecithin:retinol acyltransferase (LRAT) to RE, which is packaged in chylomicra with triglycerides, and transported through the lymphatic system. Triglycerides are deposited at target tissues, and the remaining chylomicron remnants are taken up by liver hepatocytes, which process RE for transport and storage [13–16]. Retinol binding protein 4 (RBP4), produced in the liver and adipose, sequesters retinol in the blood [17]. Holo-RBP4 complexes are internalized by binding with a plasma membrane receptor, STRA6, which then transfers the retinol to the intracellular binding protein RBP1 [18]. Recent work shows that hepatocytes predominantly express a related receptor, RBPR2, that functions in the same way as STRA6 [19].

The liver is the major storage site for RE, within specialized stellate cells that comprise less than 5 % of total liver cells and are located between endothelia and hepatocytes in the sinusoids [20]. The stellate cell is key to the progression of steatosis, wherein endothelia, hepatocytes, macrophages and platelets produce cytokines, which transform the quiescent stellate cell to an activated myofibroblast morphology [20]. Activation has been characterized by a loss of retinoid storage and increases in proliferation, contractility, matrix degradation and immune cell recruitment [21]. In the absence of stress, the liver contains an intermediate stellate cell type that retains RE in lipid droplets and expresses genes that are characteristic of myofibroblasts, including collagen 1a1 (*Col1a1*) and cytochrome P450 1b1 (*Cyp1b1*) [22]. We are particularly interested in *Cyp1b1* in the context of the effects of retinoids on obesity because this mRNA increases during adipogenesis [23] and is expressed during early development at sites of high retinol activity, suggesting regulatory cross talk [24].

The relationship between retinoids and metabolic homeostasis has been extensively studied with respect to catabolism and storage of lipids [8,15,25,26]. Retinoic acid administration can decrease adiposity, improve glucose tolerance [15] and reverse stellate cell activation [21]. Deletion of *Aldh1a1* allows accumulation of retinaldehyde, the precursor of RA, and prevents diet-induced obesity (DIO) [27]. While retinoids are largely antiadipogenic, supplementation of VA in a high-lipid-content diet increases adipose tissue weight [28]. A midgestation VA-deficient diet decreases fasting-mediated PPARα activation, concomitant with decreased fatty acid oxidation and increased triglyceride accumulation [25].

We have previously investigated the effects of a postweaning high-fat diet (60% kcal from fat, 20% kcal carbohydrate; HFD) compared to an approximately isocaloric low-fat diet (10% kcal fat, 70% kcal carbohydrate; LFD). DIO is recognized by increased weight gain and adipose deposition with the HFD relative to the LFD [29,30]. The DIO changes in males only appear at maturity (approximately diet week 5), at the same time that the GVAD protocol starts to decrease serum retinol.

Mice in which *Cyp1b1* is deleted exhibit suppressed DIO [29,30]. We have identified genes that responded to *Cyp1b1* deletion and the difference in diet, HFD *vs.* LFD [30], including gene clusters that did not respond to *Cyp1b1* deletion and also those that responded to the diet difference to the same extent in wild-type (WT) and *Cyp1b1*−/− mice, which we refer to as HF genes. While the genes that respond to *Cyp1b1* deletion are mostly controlled by endocrine mechanisms [30], we show here that HF genes are highly responsive to GVAD.

Similar to *Cyp1b1*−/− mice, GVAD is effective in blocking DIO. We hypothesize that VA deficiency from the GVAD protocol will affect the fetal/neonatal developmental regulation and imprint changes on liver metabolic homeostasis that are evident in adult offspring.

We have examined the selective responses produced by GVAD combined with an HFD (GVAD-HFD) and, here, provide evidence that the application of GVAD to a novel postweaning diet with low fat content (LF12) (GVAD-LF12) enhances the extent of serum retinol decreases in the offspring and changes their effects. Additionally, we examine the effects of changing the maternal diet from the standard breeder diet (BD) to the LF12 diet, which has an appreciably different fat content. Gene expression was effectively sorted into functionally related groups based on expression ratios between different treatment groups.

The effects of GVAD reveal that several diet-dependent gene clusters rely highly on retinol and show extensive stimulation by the LF12 diet in the maternal and postweaning periods. Evidence is presented that the high-carbohydrate content of the diet activates stellate cells and markers of a novel inflammatory response. We report a remarkable overlap between genes that respond to GVAD and those that respond to deletion of the co-repressor *Shp*/*Nr0b2*.

2. Materials and methods

2.1. Animal care and husbandry

Mice were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited University of Wisconsin Research Animal Resource Center facility. Mice were provided food and water *ad libitum* and were maintained in a controlled 12-h light/dark cycle environment. All protocols were approved by the School of Medicine and Public Health Animal Care and Use Committee (protocol number M00682). Nulliparous C57BL/6J (Jackson Labs, Bar Harbor, ME, USA) females (8–12 weeks of age) were time-mated, such that the presence of a vaginal plug was designated as embryonic day (E) 0.5. Prior to mating and until dietary administration, dams were maintained on standard chow BD (product number 2019; Harlan Teklad, Madison, WI, USA).

2.2. Dietary intervention

The initiation of diets occurred at either midgestation (E10.5) or weaning [postnatal day (PN) 21]. All postweaning diets were continued for 11 weeks. The detailed composition (and catalog numbers) of the diets is shown in Supplementary Table 1, and all dietary schemes are shown in Fig. 1. Midgestational initiation and maintenance on a VA-deficient diet (GVAD) or administration a VA-deficient diet only in the postweaning period (PWVAD) was used for decreased VA availability. The composition of the VAD diet had less fat (12% kcal) and more carbohydrate (70% kcal) (LF12) than the standard BD (Supplementary Table 1, Fig. 1).

The GVAD protocol was paired with a postweaning high-fat diet (GVAD-HFD) that otherwise had the same components as the control high-fat diet (HFD) protocol (BD-HFD) (Fig. 1A). The LF12 maternal diet was also used from midgestation to weaning prior to combination with either continued LF12 or HFD postweaning (LF12-LF12 and LF12-HFD) (Fig. 1B). The GVAD and PWVAD protocols were also examined with a postweaning LF12 diet (GVAD-LF12 and PWVAD-LF12) (Fig. 1C).

The VAD diets contained 220 IU/kg of retinyl palmitate as the source of VA, compared to the sufficient diet, which contained 24,000 IU/kg.

Mice were fasted for 4 h prior to euthanasia between 1 and 2 p.m. by CO_2 asphyxiation. Body mass is reported at the time of euthanasia. Blood was collected by cardiac puncture, and the serum was isolated by centrifugation at 14,000 rpm for 10 min and frozen at −80°C. Tissues were weighed, flash frozen and stored at −80°C.

2.3. Liver RNA isolation

Frozen liver was placed in RNAlater Ice according to manufacturer's instructions (Ambion, Foster City, CA, USA). Total RNA was isolated from 20-mg tissue using the RNeasy Mini Kit accompanied by Qiashredder columns (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA was spectrophotometrically measured for quantity and purity by A260/A280 and A260/A230 on a Nanodrop, followed by visual inspection by denaturing agarose gel electrophoresis.

2.4. Microarray analysis

All microarray analyses were performed with liver mRNA isolated from at least two mice in each treatment group that showed body weight (BW) and adipose characteristics close to the mean. Analyses were carried out on the Agilent Technologies 4×44k platforms. Each array includes 43,379 biological features, including replicates of 245 probes.

Samples were prepared according to the manufacturer's instructions for dual-color or one-color labeling, including sample preparation, hybridization and scanning. mRNA was submitted by J. Bushkofsky to Chris Bradfield's laboratory at the University of Wisconsin-Madison for dual-color labeling for GVAD-HFD and BD-HFD samples (Bushkofsky, unpublished results), such that competitive binding of each of the treatment samples (Cy3-labeled) was cohybridized with a pooled control sample, BD-HFD, of three biological replicates (Cy5-labeled). The Cy5 values were used as a measure