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Embryonic phenotype, β-carotene and retinoid metabolism upon maternal supplementation of β-carotene in a mouse model of severe vitamin A deficiency

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ABSTRACT

We investigated the effect of β-carotene (bC) supplementation during pregnancy in a mouse model of severe vitamin A deficiency, i.e. *Lrat−/−Rbp−/−* dams maintained on a vitamin A-deficient diet during gestation. bC, a provitamin A carotenoid, can be enzymatically cleaved to form vitamin A for use by the developing embryo. We found that an acute supplementation (13.5 days *post coitum*, dpc) of bC to *Lrat−/−Rbp−/−* dams on a vitamin A-deficient diet activated transcriptional mechanisms in the developing tissues to maximize the utilization of bC provided to the dams. Nevertheless, these regulatory mechanisms are inefficient under this regimen, as the embryonic phenotype was not improved. We further investigated the effect of a repeated supplementation of bC during a crucial developmental period (6.5–9.5 dpc) on the above-mentioned mouse model. This treatment improved the embryonic abnormalities, as 40% of the embryos showed a normal phenotype. In addition, analysis of retinoic acid-responsive genes, such as *Cyp26a1* in these embryos suggests that bC cleavage results in the production of retinoic acid which then can be used by the embryo. Taken together, these *in vivo* studies show that bC can be used as a source of vitamin A for severely vitamin A-deficient mammalian embryos.

Introduction

Adequate maternal nutrition during pregnancy is critical for fetal health [1,2]. One such essential nutrient is vitamin A, of which maternal dietary intake must be carefully monitored, as deficient or excessive vitamin A intake can cause a range of birth defects [3]. Furthermore, a poor pre-pregnancy maternal vitamin A status (i.e. low stores of the fat-soluble vitamin in body tissues) reduces the availability of vitamin A to be mobilized from the maternal liver to the fetus during times of inadequate dietary intake of this nutrient, and thus increases the risk of fetal vitamin A deficiency (VAD[2]) [4].

VAD among women of child-bearing age is a serious global health problem. According to the World Health Organization, nearly 10 million pregnant women worldwide suffer from night blindness, and ~20 million have low serum retinol levels [5]. Even in more developed countries (e.g. UK), 30% of women between 19 and 34 years of age have reported vitamin A intakes below the recommended lower limit [6]. In many countries where VAD is prevalent, there is limited access to preformed vitamin A (retinol, retinyl esters, and retinoic acid) from meat and dairy products. On the other hand, plant products containing the vitamin A precursors (provitamin A carotenoids such as β-carotene [bC]) are more abundant [6]. Various human studies have reported neutral or positive maternal and fetal health outcomes upon vitamin A supplementation, but fewer studies have tested the effects of bC supplementation exclusively [7]. The Hohenheim consensus conference of 2009 recommended that some of the dietary vitamin A be obtained as bC (at least 6 mg/day in the case of low retinoid intake) [6], but it is unclear under what conditions bC alone can deliver adequate amounts of vitamin A. Thus, it is important to understand whether supplementation with carotenoids is sufficient to support normal embryogenesis, under conditions of both maternal *dietary* VAD and vitamin A-deficient *status*.

Ingested vitamin A (as retinyl esters) and bC (up to 45% in its intact form, with the remainder being converted to vitamin A [8]) are taken up by enterocytes, and enter the lymphatic system packaged in chylomicrons [9]. In the vascular endothelium, chylomicron hydrolysis by lipoprotein lipase (LPL) generates chylomicron remnants, which still contain bC and retinyl esters [10]. The majority (~75%) of retinyl esters in chylomicron remnants is taken up by the liver [11] and hydrolyzed to retinol [12], either to be stored

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[2] *Abbreviations used:* dpc, days *post coitum*; bC, β-carotene; WT, wild-type; LRAT, lecithin:retinol acyltransferase; RBP/RBP4, retinol-binding protein; L−/−R−/−, *Lrat−/−Rbp−/−*; VAD, vitamin A deficiency.

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in stellate cells after re-esterification by lecithin:retinol acyltransferase (LRAT) [13,14], or to be re-secreted bound to retinol-binding protein (RBP) [15]. The remaining 25% of retinyl esters from chylomicron remnants are taken up by peripheral tissues, including the placenta [11]. In addition, exchange of bC and vitamin A may also occur among lipoprotein particles (HDL, LDL, VLDL) in the bloodstream [16]. Tissue vitamin A needs are met either by uptake of retinoids and their precursors from circulating lipoproteins, or by uptake of RBP-retinol *via* STRA6, the cell-surface receptor for holo-RBP [17]. Most cells (including those of the placenta and embryo [4]), are capable of esterifying retinol *via* LRAT for storage [18]. Alternatively, cellular retinol can be reversibly oxidized to retinaldehyde *via* retinol dehydrogenases (e.g. RDH10 [19,20]). Retinaldehyde also can be generated by bC cleavage. Central cleavage by β-carotene-15,15′-oxygenase (CMO1) generates two molecules of retinaldehyde [21], while eccentric cleavage by β-carotene-9′,10′-oxygenase (CMO2) [22] followed by chain shorting of β-apo-carotenoids [23] ultimately can generate one molecule of retinaldehyde. The latter is irreversibly oxidized by retinaldehyde dehydrogenases (e.g. RALDH2) to retinoic acid [24], the biologically active vitamin A metabolite that regulates the transcription of hundreds of genes [25]. Retinoic acid can be converted to non-transcriptionally active metabolites by CYP26A1 [26].

Previous studies in mice have elucidated that maternal circulating bC can be taken up by embryos *via* the placenta, to support a large degree of normal embryogenesis in the absence of other vitamin A sources [27]. However, prior work has not demonstrated to what extent bC can contribute to embryonic vitamin A needs when the pregnant mother is vitamin A-deficient (i.e. by status). Our lab has generated and described a model of marginal VAD, the *Lrat*−/−*Rbp*−/− strain (L−/−R−/−). While phenotypically normal on a vitamin A-sufficient diet, these mice rapidly become vitamin A-deficient and generate highly malformed embryos when deprived of dietary vitamin A, due to their inability to store retinoids *via* LRAT or mobilize retinol *via* RBP [4]. Recently, we showed that on a vitamin A-sufficient diet, placental bC uptake was regulated by different mechanisms in L−/−R−/− and wild-type (WT) mice, due to the marginal vitamin A-deficient status of the L−/−R−/− dams [28]. These results indicated that the maternal vitamin A status affects bC uptake and metabolism in the developing tissues.

In the present study, we use WT and L−/−R−/− mice to investigate the effects of *dietary* VAD or a vitamin A-deficient *tissue status*, respectively, on the uptake and processing of bC by maternal and embryonic tissues as well as the extent to which such vitamin A-deficient tissues can generate retinoids from bC to support normal embryogenesis. Even severely vitamin A-deficient developing tissues respond to bC supplementation by maximizing proper utilization of the provitamin A as a source of retinoid to support embryogenesis. However, improvement of the embryonic malformations can be achieved only when bC is provided to the dams at early stages of development. This regimen is also effective at improving the maternal vitamin A status.

Materials and methods

Knockout Mice, nutritional manipulation, and β-carotene supplementation

Wild-type (WT) and *Lrat*−/−*Rbp*−/− (L−/−R−/−) double knockout mice [4,28] were used in the current study. All mice had a mixed genetic background (C57BL/6 x sv129), with the L−/−R−/− being a model of VAD [4]. Throughout the study, both water and diet were consumed *ad libitum*, and mice were maintained on a 12 h light/dark cycle from 7 a.m. to 7 p.m. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals [29] and were approved by the Rutgers University Institutional Committee on Animal Care.

Prior to nutritional manipulation, all mice were maintained on a non-purified diet copious in vitamin A (29 IU/g, Prolab Isopro RMH3000 5p75) but devoid of bC (trace to 2.6 μg/g). At 3 months of age, WT and L−/−R−/− females were mated with their respective males, and the presence of a vaginal plug was established as 0.5 days *post coitum* (dpc). Henceforward, dams were fed a purified vitamin A-deficient diet (Research Diets, <0.2 IU/g) until the time of sacrifice (14.5 dpc). Solutions of bC or its Vehicle (Veh) were prepared as previously described [28]. Briefly, 50 mg bC (Type I, Sigma Aldrich) was mixed into 5 mL vehicle (ethanol: Cremophor: PBS, 1:11:18) by vortexing, and the concentration of the resulting solution was determined by spectrophotometry at 450 nm. Due to poor solubility of bC, the final concentration varied from 2 to 5 mg/mL.

For the acute bC supplementation study, WT and L−/−R−/− pregnant dams were randomly assigned to the Veh or bC treatment groups, and were injected with 250 μL of the assigned solution intraperitoneally (IP) at 13.5 dpc. For the repeated bC supplementation study, L−/−R−/− pregnant dams were randomly assigned to be given an IP injection of Veh or bC daily from 6.5–9.5 dpc. The resulting dose of one injection of bC given to the pregnant dams was ~40 μg/g body weight. Regardless of treatment, all dams were sacrificed at 14.5 dpc by CO_2 inhalation between 9:30 and 11:30 a.m. Serum and tissues (livers, placentas, and embryos) were collected, frozen, and stored at −80 °C until further processing.

Hplc

Retinoid (retinol and retinyl esters) and bC concentrations in maternal serum, liver, placenta and embryo were measured by reversed-phase HPLC analysis as described previously [4,30].

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR

RNA extraction, cDNA synthesis, and quantitative real-time RT-PCR (qRT-PCR) were performed on embryos and placentas as previously described [4]. Primer sequences were as published for β-*Actin*, *Stra6*, *Raldh2*, *Cyp26a1* [4], *Cmo1* [28], *Cmo2* [31], and *Rdh10* [32]. Changes in mRNA expression were analyzed by the ΔΔCT method.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (IBM SPSS Statistics, version 16). Normal distribution of data was assessed by the Shapiro–Wilk test. Normally distributed data were analyzed by Student's *t* test for comparisons of two groups, or by two-way ANOVA and *post hoc* analysis (least significant difference, LSD, for groups with equal variance; Tamhane's for groups with unequal variance) for comparisons of genotype and treatment effects, followed by Student's *t* test. Data that were not normally distributed were analyzed by the Mann–Whitney U test for comparisons of two groups, or by the Kruskal–Wallis test followed by Mann–Whitney U test for comparisons of three or more groups. $P < 0.05$ was considered significant. Data are presented as mean ± standard deviation (SD).

Results

We previously showed that the placental uptake of bC is enhanced in an established model of marginal VAD such as the L−/−R−/− mice fed a vitamin A-sufficient diet [4,28]. Here we employed the same strain to understand whether a severe maternal vitamin A-deficient status would affect the uptake and metabolism