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Decreased hepatic retinyl palmitate hydrolase activity in protein-deficient rats

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Abstract

28-day-old weanling rats were fed a diet containing 3% casein as the only source of protein for eight weeks to induce protein deficiency. When compared to control animals (fed a diet containing 25% casein), these rats had significantly lowered body (5.2-fold reduction) and liver (2.5-fold reduction) weights. The circulatory level of retinol (nmol per ml plasma) as well as retinol (nmol per g tissue) in the liver of these protein-deficient animals were also reduced significantly, although their liver concentration of retinyl palmitate (nmol per g tissue) was comparable to that of the control group. Assay of liver tissue for retinyl palmitate hydrolase activity revealed a 4-fold reduction (compared to that of control animals) of specific enzyme activity (nmol retinol formed per g protein per h). These findings suggest that severe protein deficiency results in a decreased hydrolysis of retinyl esters in the liver, which may be in part responsible for the reduced level of metabolically 'active' retinoids available for normal physiological functions.

Keywords

Retinyl palmitate hydrolase; Protein deficiency; (Rat liver)

Introduction

More than 95% of all retinoids located in the liver exists as metabolically 'inactive' retinyl esters stored in lipid droplets of fat-storing cells (nonparenchyma cells) [1-4]. Mobilization of hepatic retinoids requires the hydrolysis of these esters in order that retinol is released [5-9]. Freed retinol is then transported in the blood [10,11] to different tissues where it is oxidized to either retinal [12,13] or, where appropriate, (irreversibly) to retinoic acid [14]. Retinol, retinal and retinoic acid are considered metabolically 'active' species because they are all related to various physiological functions of vitamin A [2].

Diseases of the liver and protein-energy malnutrition have been reported to be associated with significant reductions of plasma levels of retinol and/ or serum retinol binding protein [15-17]. The decrease in the circulatory level of retinol resulting from these pathological conditions has been attributed to a reduction of retinol binding protein synthesis in the liver

[2]. However, the possibility that the reduction of circulatory retinol is the result of a decrease in the hydrolytic activity of hepatic retinyl esters has not been explored.

In the present study, weanling rats free from parasites and existing pathology were made protein deficient by feeding them a diet containing 3% casein as the only source of protein. Data presented here confirm that protein deficiency results in a significant reduction of the circulatory level (nmol per ml plasma) of retinol. However, protein deficiency did not change the liver concentration (nmol per g tissue) of retinyl palmitate. Instead, we noted a significant (5.5-fold) decrease in the level of (nmol per g tissue) retinol in the liver. This reduction of retinol is of such a magnitude that it cannot be attributable solely to a decreased retinol binding protein level. These observations suggest the possibility that protein deficiency gives rise to a decreased hydrolytic activity in the liver which reduces the release of retinol from retinyl esters. This mechanism can explain in part the reduced availability of retinol in protein-malnourished animals. Assays of the specific enzyme activity of retinyl palmitate hydrolase in liver tissues obtained from protein-malnourished animals indicated that indeed such a reduction in the hepatic retinyl palmitate hydrolase activity was associated with protein deficiency.

Materials and Methods

Male Sprague-Dawley rats were purchased from Charles Rivers Laboratories, Wilmington, MA. Diets were obtained from Ziegler Brothers, Gardner, PA [23].

21-day-old weanling rats were housed separately and fed a 25% casein diet for 1 week. Beginning on day 28 (postnatal), one group ($n = 7$) of animals was fed a 3% casein diet, as previously described by Glew et al. [23]. In contrast, a control group ($n = 5$) was fed an identical diet except that the casein content was increased to 25% at the expense of sucrose. Animals were weighed daily for the duration (8 weeks) of the experiment. At the end of the experiment, animals were decapitated, blood was collected and liver removed immediately following the cessation of blood flow. All tissues (blood and liver) were immediately placed at -80°C until further analysis. All samples were analyzed within 30 days from the time when they were removed from the animals. Tissue samples were thawed and kept on ice prior to extraction of retinoids and assay of hydrolase activity.

Extraction of retinoids

Liver retinoids were extracted by grinding 1 g portions of respective liver tissue in anhydrous sodium sulfate, followed by the additions of 2×6 ml of acetone (extraction efficiency: 95%). The acetone extract was dried under a stream of nitrogen, the residue was redissolved in a small volume (less than 1 ml) of 0.5% dioxane in *n*-hexane and then applied onto an alumina column (5% water deactivated, 2×1 cm). Retinyl esters were collected from this column by elution with 10 ml of 0.5% dioxane in *n*-hexane [19]. Retinol was then eluted from the column following the application of 10 ml of 10% dioxane in *n*-hexane.

Both retinyl esters and retinol fractions were subjected to separate analysis by (isocratic) high-performance liquid chromatography (see below). Retinoids in rat sera (0.2 ml aliquots) from control and protein-deficient animals were extracted with petroleum ether (2×1 ml)

after the addition of 0.2 ml ethanol to denature retinol binding protein (extraction efficiency: 95%; see Ref. 18). The petroleum ether extract was dried under a stream of nitrogen, the residue was redissolved in a small volume of 0.5% dioxane in *n*-hexane and then applied onto an alumina column as previously described. Retinol was eluted from this column following the application of 10 ml of 10% dioxane in *n*-hexane.

High-performance liquid chromatography (HPLC)

The retinyl ester fraction was dried under a stream of nitrogen. The residue was redissolved in 25 μ l of 0.2% dioxane in *n*-hexane and applied (by injection) onto a 5 μ M Ultrasphere HPLC column eluted with 0.2% dioxane in *n*-hexane. The rate of elution was 1 ml/min with detection at 340 nm. The retention time of all-*trans* retinyl palmitate was observed to be 15 min.

Retinol fractions collected from the alumina column were also analyzed isocratically by eluting with 10% dioxane in *n*-hexane at a flow rate of 1 ml/min. Under these conditions, the retention time of all-*trans* retinol was observed to be 12 min. Quantitation of retinol and all-*trans* retinyl palmitate was achieved by the comparison of integrated peak areas of tissue samples to those obtained by injection of known amounts of authentic standards. HPLC methods used in the present study have been described in detail elsewhere [18,19].

Enzyme assay

Hepatic retinyl palmitate hydrolase activity was measured according to the method of Cooper and Olson [9]. Briefly, 1 g of liver was homogenized in 5 ml of 0.05 M Tris-maleate buffer (pH 8.0). A 100 μ l aliquot was removed and transferred to a reaction mixture containing 0.5 mM retinyl palmitate, 2 mg/ml Triton X-100 and 100 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate) in a final volume of 0.5 ml. Incubations were 37 °C for 1 h. Reactions were terminated by the addition of 0.5 ml ethanol. Retinoids were extracted by petroleum ether, dried under a stream of nitrogen and analyzed as previously described.

Protein was determined according to the procedure described by Lowry et al. [20] using bovine serum albumin as a standard.

Results

The efficacy of the low protein diet (3% casein) was reflected in liver and total body weights of animals fed this diet in comparison to those fed the control diet (25% casein). The mean liver weight of rats fed the low protein diet (recorded at the end of the 8-week experimental period) was 7.0 g ($n = 7$) which was 2.5-times lower than the mean liver weight of control animals (17.3 g, $n = 5$). Likewise, protein-malnourished animals exhibited a mean body weight of 76.8 g ($n = 7$) which was 5.2-times lower than the mean body weight of control animals (402.1 g, $n = 5$). All animals prior to the study weighed approximately 100 g.

As shown in Fig. 1, a significantly lower level of all-*trans* retinol was observed in pooled plasma from protein-deficient animals when compared to control animals (compare peaks 2 in Fig. 1c and d). Based on triplicate determinations, control rats exhibited levels of

circulatory retinol (0.73 ± 0.08 nmol/ml serum) approximately one and a half times greater than that observed for protein-deficient animals (0.49 ± 0.08 nmol/ml serum).

The HPLC profile of hepatic retinyl esters showed only a single, major peak which co-eluted with authentic *all-trans* retinyl palmitate. Thus, retinyl esters in the rat liver were mainly *all-trans* retinyl palmitate, an observation which is in agreement with other investigators, indicating retinyl palmitate to be the major retinyl ester in the rat liver [3]. A small amount of 13-*cis* isomer (less than 5% of the *all-trans* isomer) was also observed in the chromatographic elutions but was not quantitated in the present study. Quantitative comparison showed that no significant difference was found between the hepatic levels of retinyl palmitate in the control and protein malnourished animals (Table I).

In distinct contrast, the metabolically 'active' retinol was significantly reduced in liver tissue of protein-deficient animals. HPLC profiles of retinol extracted from control and protein-malnourished liver tissue are shown in Fig. 1. Only one major isomer (peak 2 = *all-trans* retinol, Fig. 1 a and b, the minor peak 1 is 13-*cis* retinol) was observed. This material coeluted with authentic *all-trans* retinol, confirming the identity of this isomer. The quantity of *all-trans* retinol detected in control animals was approximately 5.5-times greater than that observed for the protein-deficient animals.

The activity of retinyl palmitate hydrolase was approximately 4-times greater in liver tissue of control animals (571 nmol retinol formed/ g protein per h, $n = 5$) than that of protein malnourished animals (169 nmol retinol formed/ g protein per h, $n = 7$, Table I). The large within-group variance observed here is in agreement with those reported by other investigators [6,7].

Discussion

The quantity of hepatic retinyl palmitate shown in the present study agrees well with the total amount of hepatic vitamin A reported by others using isotopic dilution and fluorometric means of analysis [3,21]. Although hepatic retinyl palmitate did not change in response to protein malnourishment, protein-deficient animals exhibited a large decrease (21.8 nmol/ g liver) in metabolically 'active' retinol. This change cannot arise solely from a reduction of retinol binding protein concentration in the liver tissue as Smith and coworkers estimated that the retinol binding protein concentration in the rat liver is about 120 μ g/g liver [22]. Assuming that these are all holoproteins, the concentration of retinol attributable to retinol binding protein is only 5.7 nmol of retinol/ g liver. (The molecular weight of retinol binding protein in the rat is 21 000 and one retinol binding protein binds stoichiometrically one molecule of retinol [10].)

Our estimation of retinyl palmitate hydrolase activity in the rat liver is also in good agreement with reported values of others [5,9]. As shown in Table I, the specific activity of hepatic retinyl palmitate hydrolase is reduced significantly in protein-malnourished animals. This 4-fold reduction may partly account for the large decrease of hepatic retinol in protein-deficient animals. Such a reduction of hepatic retinyl palmitate hydrolase activity, in combination with other factors such as a reduction of synthesis of retinol binding protein in

the liver, may give rise to the significantly depressed levels of circulatory retinol, thus impairing the mobilization of retinoids for metabolic and visual functions.

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Fig. 1.

HPLC profile of all-*trans* retinol (peak 2) extracted from liver (a and b) and blood plasma (c and d) of rats fed a control and a low protein diet. Fig. a indicates a typical profile of retinols in the liver of a rat fed the control diet. In comparison, b shows retinols in the liver of a rat fed the low protein diet. The amount of hepatic all-*trans* retinol from the control animal (a, peak 2, 3.10 nmol) was significantly higher than the amount of all-*trans* retinol from the animal fed the low protein diet (b, peak 2, 1.08 nmol). Retinoids in pooled sera of control and protein-malnourished animals were also extracted and analyzed by HPLC. c and d indicate that all-*trans* retinol in pooled sera of rats fed a control diet (peak 2, 0.15 nmol, c) was much higher than all-*trans* retinol in rats fed the low protein diet (peak 2, 0.08 nmol, d). Column: 5 μ M Ultrasphere silica; elution: 10% dioxane in *n*-hexane at 1 ml per min; detection: 340 nm; peak 1, 13-*cis* retinol; peak 2, all-*trans* retinol.

Table I

QUANTITATION OF ALL-*trans* RETINOL AND ALL-*trans* RETINYL PALMITATE (nmol PER g WET WEIGHT OF LIVER) AND THE SPECIFIC ACTIVITY OF RETINYL PALMITATE HYDROLASE (nmol OF RETINOL FORMED PER g PROTEIN PER h) IN THE LIVER OF CONTROL AND PROTEIN-MALNOURISHED ANIMALS

Means and standard deviations (<i>n</i> = number of observations) are shown in the table.		
	Control (<i>n</i> = 5)	Protein-malnourished (<i>n</i> = 7)
Retinyl palmitate	1125.17 ± 321.49	1126.97 ± 321.49
Retinol	26.62 ± 11.49	4.78 ± 1.45 *
Retinyl palmitate hydrolase activity	571.11 ± 296.28	169.29 ± 14.42 *

* Significantly different from the value in the control group (Student's *t*-test, *P* = 0.05).