Accumulation of retinol in the liver after prolonged hyporetinolemia in the vitamin A-sufficient rat

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Abstract We assessed the effects of prolonged reduction of plasma retinol concentrations (hyporetinolemia) on the distribution of tissue vitamin A (VA) and of its active compounds using a model of continuous recombinant human interleukin-6 (rhIL-6) infusion via osmotic minipumps in VA-sufficient male rats. Plasma retinol and retinol-binding protein (RBP) concentrations remained decreased and lower in rhIL-6-treated rats compared with controls from 7.5 h throughout 7 days of infusion ($P < 0.001$). This reduction was accompanied by a 68% increase in hepatic retinol concentration by 7 days ($P < 0.05$). Hepatic and renal retinyl palmitate and retinoic acid concentrations did not change, and renal megalin content remained unchanged; hepatic RBP concentrations were 41% lower in rhIL-6-treated rats compared with controls ($P < 0.05$). These results indicate that instead of being lost, retinol accumulated in the liver during inflammation and that hyporetinolemia was attributable to a decrease in the availability of hepatic RBP. A plausible consequence of the effect of rhIL-6-induced hyporetinolemia is that by 7 days tissues that are dependent on plasma retinol may become deprived of VA. ◼ These results have important implications in understanding the mechanism by which measles infection induces hyporetinolemia and VA deficiency of extrahepatic tissues.— Gieng, S. H., J. Raila, and F. J. Rosales. **Accumulation of retinol in the liver after prolonged hyporetinolemia in the vitamin A-sufficient rat.** *J. Lipid Res.* 2005. **46:** 641–649.

Supplementary key words megalin • retinoic acid • retinol-binding protein • retinyl esters

Vitamin A (VA), in its alcohol, aldehyde, and acid forms, is an essential fat-soluble micronutrient known to have multiple metabolic actions in animals, such as being a prosthetic group of the visual pigment, nuclear modulator of gene expression, carrier of mannosyl units in the synthesis of hydrophobic glycoproteins, and for the retinoylation of proteins (1). Moreover, plasma all-*trans*-retinol concentration (VA alcohol; retinol) is homeostatically regulated and has been suggested to be at the crossroads between stores and degradation of VA (2). Because of its intrinsic role in VA metabolism, changing the abundance of plasma retinol may have consequences for the distribution of VA (e.g., stores vs. degradation).

The immune response to infections causes an increase in plasma concentrations of C-reactive protein or α-1-acid glycoprotein (AGP) and a decrease of circulating retinol (3). This has been termed hyporetinolemia (hyporetinemia) (4). The pathogenesis of various infections, such as measles and malaria, is also accompanied by hyporetinolemia. Clinical consequences of this hyporetinolemia are an increase in morbidity (i.e., duration of hospitalization) and mortality of children in poor and rich nations (4, 5). In the case of measles infections, supplementation with VA reduces both morbidity and mortality (4). The correction of this hyporetinolemia has been suggested as a possible mechanism for improving the survival of children with measles. However, the association between VA and immune function suggests a more complex mechanism, such as an increased utilization of VA by immune cells, an enhancement of renal clearance of retinol, or an increase in the degradation of retinol through oxidation.

Thus, the goals of this research were to experimentally produce inflammation and prolonged hyporetinolemia in the rat and to examine the metabolic distribution of the major biological forms of VA. We have assessed the abundance of megalin, the renal proximal tubule protein responsible for the reabsorption of retinol-binding protein (RBP), which may be important for retinol homeostasis during disease states as well. In addition, we have assessed α-tocopherol as a proxy for oxidative stress, which may help in isolating the effects of inflammation from those of oxidative stress related to disease states. We hypothesize that the reduced availability of hepatic RBP can explain inflam-

mation-induced hyporetinolemia in a model of continuous infusion of recombinant human interleukin-6 (rhIL-6).

MATERIALS AND METHODS

Materials

Unless otherwise noted, chemical reagents were purchased from EMD Chemicals (Gibbstown, NJ), Fisher Chemicals (Pittsburgh, PA), Mallinckrodt Baker (Phillipsburg, NJ), Pharmco (Brookfield, CT), and Sigma (St. Louis, MO). rhIL-6 was purchased from Austral Biologicals (San Ramon, CA). Lipopolysaccharide (LPS) derived from *Psuedomonas aeruginosa* was purchased from Calbiochem (San Diego, CA). Solid-phase extraction (SPE) cartridges were purchased from Phenomenex (Torrance, CA). AGP single radial immunodiffusion (RID) kits were purchased from Tridelta (Greystones, Ireland). Osmotic minipumps were purchased from Alzet (Cupertino, CA). Trimethylmethoxyphenyl (TMMP)-retinol and TMMP-retinoic acid (acitretin) were generous gifts from Hoffmann-La Roche (Basel, Switzerland).

Antibodies

All antibodies and calibrators were from DakoCytomation California, Inc. (Carpinteria, CA) or as otherwise indicated. Anti-rat RBP antibody was a generous gift from Dr. A. C. Ross (6, 7). Megalin antibody was a generous gift from Prof. T. E. Willnow (Max-Delbrueck-Center for Molecular Medicine, Berlin, Germany). For immunoblotting and immunohistochemistry, rabbit anti-human RBP (Accurate Chemical and Scientific Corp., Westbury, NY; code H6808) and sheep anti-rabbit megalin were used. The binding of primary antibodies was visualized using peroxidase-conjugated goat anti-rabbit IgG (Dako; code P0448) or rabbit anti-sheep IgG (Dako; code Z0228).

Animals

Male pathogen-free Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Kingston, NY). All experimental procedures were approved by the Institutional Animal Care and Use Committee at Pennsylvania State University (University Park).

All animals were housed with a 12 h light/dark cycle at 22°C and provided diet ad libitum. Body and food weight measurements were taken daily. Blood samples were collected from the tail vein. At the end of each experiment, rats were killed by carbon dioxide asphyxiation. Blood was drawn from the inferior vena cava into heparinized syringes. Livers and kidneys were collected immediately by dissection and blotted and frozen at −80°C until analysis.

LPS- versus rhIL-6-induced hyporetinolemia

Seven week old rats (n = 15) weighing 223–275 g were housed in plastic cages and provided pelleted chow. Rats were injected intraperitoneally (0 h) with rhIL-6 (n = 6; 65 μg/kg), LPS (n = 5; 500 μg/kg), or PBS (control; n = 4). At times 0, 1.5, 3, and 5 h, rectal temperatures were determined as recommended by the manufacturer with a telethermometer and a rectal temperature probe for small animals (Yellow Spring Instrument Co., Inc., Yellow Springs, OH). Blood samples (200 μl) were collected at times 0, 6.5, 12, and 24 h.

Prolonged hyporetinolemia by rhIL-6

Six week old rats (n = 19) weighing 177–199 g were housed in individual hanging metal wire cages and provided a powdered chow in a feeding jar (Lab Diet 5001) containing 22 IU VA/g diet (8). Plasma and tissue samples were collected from three rats for baseline measurements. rhIL-6 (n = 8; 65 μg/kg/day) or PBS (n = 8) in 100 or 200 μl was continuously infused for 3 or 7 days, respectively, at 1 μl/h by osmotic minipumps implanted subcutaneously after induction of anesthesia with isoflurane. Blood was collected at baseline and at 7.5, 20.5, 44, and 68 h for the 3 day treated rats, whereas in the 7 day treated rats additional blood samples were collected at 95.25, 119.25, 140, and 164 h after pump implantation.

Blood and tissue collection

Whole blood was collected from the tail vein into heparinized tubes (20 U heparin/ml blood). After centrifugation (Sorvall RT6000B, H-1000B rotor) at 1,300 g for 15 min at 4°C, plasma was collected, purged with nitrogen gas, and stored at −80°C until analysis. Livers and one kidney per rat were excised, blotted, and frozen immediately in liquid nitrogen and stored at −80°C until processing and analysis. The other kidneys were stored in 4% formalin, fixed in paraffin wax, and sent for megalin analysis by immunohistochemistry.

Tissue retinoid and tocopherol extraction

Retinol and retinyl palmitate concentrations in plasma, liver, and kidney samples were determined using TMMP-retinol as an internal standard (9) after extraction with hexanes for plasma and a modified Folch procedure for livers and kidneys (10, 11). α-Tocopherol concentrations in plasma were determined using α-tocopherol acetate as an internal standard. For the Folch procedure, ∼1 g of tissue was minced and mixed with 20 ml of chloroform-methanol (2:1, v/v), put into the dark, and allowed to sit overnight. The next day (>12 h), samples were sequentially washed four times: 1× water, 2× 0.6% sodium chloride-methanol-chloroform (235:240:15, v/v/v) and water-methanol-chloroform (235:240:15, v/v/v), with a 10 min centrifugation procedure after washes 1 and 4 and a 2 min centrifugation procedure after washes 2 and 3 at 1,300 g. After each centrifugation, the top layer was aspirated by vacuum, leaving the bottom layer for the subsequent washes.

Tissue retinoic acid extraction

Retinoic acid concentrations in plasma, liver, and kidney samples were determined after normal-phase SPE using acitretin as an internal standard after the Folch purification procedure. SPE columns were NH_2 (55 μm, 70 Å) and 500 mg/3 ml in volume and weight. The columns were first conditioned with 2 ml of dichlormethane-2-propanol (1:1, v/v) and then with 2 × 2 ml of 100% hexanes. After conditioning, the samples were loaded onto the column. The column was then washed with 2 × 2 ml of chloroform-2-propanol (2:1, v/v). The sample was eluted with 2 × 2 ml of diethyl ether containing 3% acetic acid. Solvent was forced through the column by negative pressure using a vacuum system (Phenomenex; 12 position vacuum manifold) at a rate of 1–2 ml/min for the conditioning and washing steps. Loading and eluting of the sample were accomplished by gravity. After collection of the samples, they were dried under nitrogen gas, reconstituted in 2 × 100 μl of methanol-acetic acid (100:2, v/v), and 25 μl was injected into the HPLC apparatus.

HPLC analysis

Analyses were conducted under fluorescent lights shaded with ultraviolet light-blocking film (CLCH; Sydlin, Lancaster, PA). A Hewlett-Packard 1100 series HPLC apparatus was used for all-*trans*-retinol, α-tocopherol, retinyl palmitate, and retinoic acid analyses. The reversed-phase system included a Zorbax Eclipse XDB-C8 (5 μm; 4.6 × 150 mm) with a C8 guard column (Agilent Technologies, Wilmington, DE) and a multiple wavelength ultraviolet light detector. Retinol and retinyl palmitate were detected

at 325 nm, α-tocopherol at 292 nm, and retinoic acid at 350 nm. Coefficient of variation within run was 3.2%. Coefficient of variation between run was 2.1%. The accuracy of the retinol and α-tocopherol measurements was confirmed by extracting and measuring pooled human plasma samples (Standard Reference Material 986c) from the National Institute of Standards and Technology (Gaithersburg, MD). Recovery of retinoids was based on TMMP-retinol and acitretin. Overall retinol recovery was 99.3 ± 8.9%. Overall retinoic acid recovery was 120 ± 12.5%. The conditions for retinol, α-tocopherol, and retinyl palmitate analyses were a modification of the procedure by Green et al. (12). Briefly, methanol-water (90:10, v/v) was run through the column for 5.25 min, with a subsequent 0.5 min linear gradient to methanol-water (95:5), and maintained for the next 7.75 min. Next, a 1 min linear gradient to 100% methanol was performed and maintained for 7.5 min. The original conditions were reverted to and maintained for 5 min before the next injection. The flow rate was 1 ml/min, and the column temperature was maintained at 25°C. The conditions for retinoic acid analysis were a modification of the procedure by Tang and Russell (13). Briefly, for the first 30 min, a linear gradient from 100% methanol-water (3:1, v/v) containing 10 mM ammonium acetate to 100% methanol-dichloromethane (4:1, v/v) was performed and sustained for 5 min thereafter. Second, the solvents were reversed in a linear gradient for the next 5 min. Finally, the original conditions were maintained for 10 min before the next injection. The flow rate was 0.8 ml/min, and the column temperature was maintained at 25°C.

Plasma AGP and RBP analysis

Plasma AGP concentrations were determined by single RID using a commercial kit (Tridelta). Plasma RBP determination was based on a modified method of Mancini, Carbonara, and Heremans (14). Briefly, 5 μl samples in duplicate were randomly added to precut wells of an agar gel containing anti-rat AGP or RBP antibody. Plates were kept in a humidified box and allowed to incubate at room temperature (25°C) for 24 h. Rings were read with a RID ruler, and AGP concentrations were based on a curve of calibrators supplied with the kit after a diameter-squared transformation. RBP concentrations were based on relative concentrations to pooled control rat plasma, and data were expressed as arbitrary units.

Western blot analysis

Cross-reactivity between the human RBP antibody and rat RBP was tested by use of Western blot analysis, as described elsewhere (15). For SDS-PAGE immunoblot analysis of hepatic RBP, ∼1 g of liver from rhIL-6-treated or control rats was homogenized in 3 volumes of 0.25 M sucrose. To this homogenate, an equal volume of 1% Triton X-100 was added to solubilize the microsomes and vortexed for 15 s. Aliquots of these liver samples were subjected to 12% SDS-PAGE using polyacrylamide minigels (Mini Protean II; Bio-Rad, Hercules, CA). After SDS-PAGE, the proteins were electroblotted onto a nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad). Blots were blocked with 5% nonfat milk in TBS containing 0.1% Tween 20 (TBST). The membrane was then incubated with cross-reacting rabbit anti-human RBP at 4°C overnight. After washing in 0.3% TBST, the blots were incubated with peroxidase-coupled goat anti-rabbit IgG for 1 h at room temperature. After a final wash with 0.3% TBST, antibody binding was visualized using 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide in 0.1 M imidazole buffer (pH 7.1). Band intensity was read with a scanner (CanoScan FB 620P) and analyzed with the NIH Imager program (ImageJ). The obtained areas under the curve of immunoreactive RBP bands were corrected for total liver protein concentration, which were analyzed by the method of Bradford (Bio Safe Coomassie; Bio-Rad).

Immunohistochemistry

For indirect peroxidase immunostaining of megalin, kidney slides from rhIL-6-treated or control rats were deparaffinized, rehydrated in a decreased series of alcohol to water, and exposed for 30 min in 0.5% hydrogen peroxide in methanol to deactivate endogenous peroxidases. Nonspecific antibody binding was blocked for 30 min in TBS (pH 7.6) containing 5% BSA. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) by heating in a microwave oven (750 W for 12 min) to improve the immunoreactivity. The primary sheep anti-megalin antibody was diluted 1:30,000 in 1% BSA in TBS and incubated overnight at 4°C. After the overnight incubations at 4°C, the sections were incubated with peroxidase-coupled rabbit anti-sheep IgG (1:100 in 1% BSA in TBS) for 30 min. The antigen-antibody binding sites were visualized by incubating the sections in a solution of DAB containing 0.01% hydrogen peroxide in 0.1 M imidazole buffer (pH 7.1). This provided the substrate for a peroxidase reaction; the presence of brown staining during the subsequent examination revealed any immunoreactive substances in the sections. Counterstaining was performed with Mayers hematoxylin. Negative controls, which included the omission of the primary antibodies, revealed no significant labeling. The sections were examined and photographed with an Olympus BX-51 microscope equipped with a SPOT II RT camera (Diagnostics Instruments, Sterling Heights, MI). All tissue samples were evaluated by two investigators without prior knowledge of the group to which the rats belonged.

Statistical analysis

Values are represented as mean ± standard deviation. Significance between groups was calculated using Student's *t*-test at a two-tailed level of $P \leq 0.05$. Relative values were calculated as V_1/V_0.

RESULTS

rhIL-6 decreases plasma retinol concentrations

A single intraperitoneal dose of rhIL-6 caused a 25% decrease in plasma retinol concentrations by 6.5 h relative to baseline concentrations ($P < 0.05$), reaching −42% by 12 h ($P < 0.05$) and returning to −10% by 24 h ($P = 0.24$), whereas saline-treated controls showed nonsignificant changes in plasma retinol concentrations during the experimental period (**Fig. 1**). It is noteworthy that the relative reduction in plasma retinol concentrations at 12 h was significantly different from that at 6.5 and 24 h, which did not differ from each other, suggesting that the effect of rhIL-6 was still present at 24 h. In LPS-treated rats, plasma retinol concentrations were reduced significantly by 24 h compared with control and rhIL-6 treatments. It is important to note that the reduction in plasma retinol concentrations caused by rhIL-6 by 12 h or LPS by 24 h did not differ from each other (−42.2 ± 5.4% vs. −33.5 ± 16.1%, respectively; $P = 0.29$). However, rhIL-6 did not cause an increase in rectal temperatures (data not shown).

Continuous rhIL-6 infusion induces inflammation

Plasma AGP concentrations were higher in rhIL-6-treated rats compared with controls by 21 h ($P < 0.01$), reaching a maximum by 68 h, 20 times higher than baseline concentrations, and remained higher than controls for up to 7 days ($P < 0.001$), confirming inflammation. However, the increases in plasma AGP concentrations were not ac-
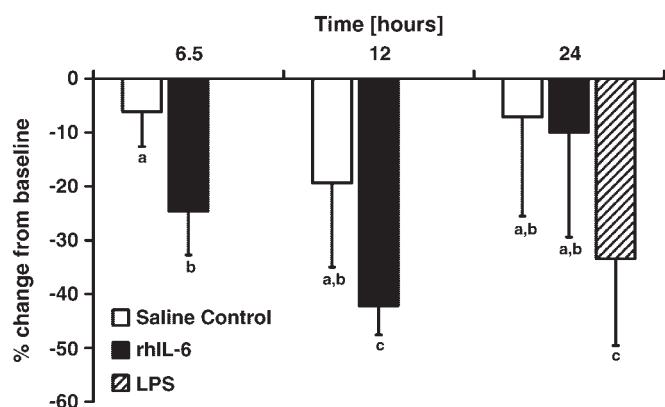
Fig. 1. A single intraperitoneal dose of recombinant human interleukin-6 (rhIL-6) induced hyporetinolemia. Like lipopolysaccharide (LPS), rhIL-6 administered in a single intraperitoneal dose induced hyporetinolemia. The reduction of plasma retinol concentrations that occurred in rhIL-6-treated rats at 12 h was comparable to that in LPS-treated rats at 24 h. Control (PBS), n = 4; rhIL-6, n = 5; LPS, n = 6. The percentage change was calculated for each rat. Bars marked by different letters represent significance with $P < 0.05$. Error bars indicate SD.

Fig. 2. Prolonged hyporetinolemia. Continuous rhIL-6 infusion in rats induced hyporetinolemia for the duration of the experiment (7 days). Plasma retinol concentrations decreased 80% from baseline values within 2 days of rhIL-6 treatment and remained significantly decreased and lower than controls for up to 7 days. Control (PBS), n = 4; rhIL-6, n = 4. * $P < 0.05$, *** $P < 0.001$. Error bars indicate SD.

companied by an increase in plasma α-tocopherol concentrations or changes in rates of body weight gain and food intake (**Table 1**). The rates of body weight gain for these rats consuming a normal chow diet were within the age- and strain-specific ranges indicated by the breeders (Charles River Breeding Laboratories). Although α-tocopherol concentrations in rhIL-6-treated rats differed from those of controls at baseline and 7 days, these concentrations remained unchanged relative to baseline concentrations based on the slope of the linear function describing these concentrations over time for each group (rhIL-6 rats, 0.016 ± 0.024 μM/day vs. control rats, 0.006 ± 0.008 μM/day; $P = 0.14$).

Inflammation-induced hyporetinolemia

The inflammatory response to continuous infusion with rhIL-6 was associated with a reduction in retinol. Plasma retinol concentrations were lower than in controls by 7.5 h ($P < 0.05$), decreasing close to 80% by 68 h, and remained lower than in controls for up to 7 days ($P < 0.001$) (**Fig. 2**).
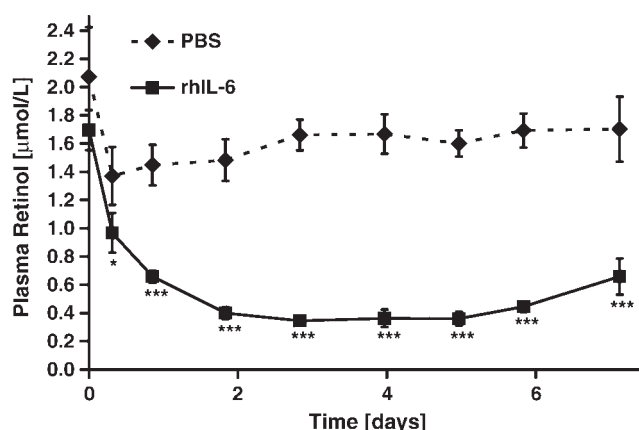
Although plasma retinol palmitate concentrations were analyzed, they were not detected during the experimental period. Plasma retinoic acid concentrations measured at 7 days did not differ between the groups (**Table 2**), and their values were comparable to those previously reported (16).

Hepatic and renal concentrations of retinoids during inflammation

A time-dependent response in the distribution of retinoids in the liver during inflammation was observed. Three days after minipumps were implanted, hepatic concentrations of retinol did not differ in rhIL-6-treated rats compared with controls or with baseline concentrations. However, by 7 days, hepatic retinol concentrations were higher in rhIL-6-treated rats compared with controls or with baseline concentrations ($P < 0.05$) (**Fig. 3A**). This represented a 68% increase in hepatic retinol. In the kidneys, retinol concentrations were significantly lower than in controls at 3 and 7 days (Fig. 3B). In contrast, hepatic and renal retinol

TABLE 1. Changes in inflammation and nutritional status during infusion with rhIL-6

Variable	Treatment	Baseline	Day 1	Day 3	Day 7
Plasma α-1-acid glycoprotein (g/l)	PBS	0.08 ± 0.04	0.20 ± 0.09	0.20 ± 0.09	0.13 ± 0.02
	rhIL-6	0.08 ± 0.05	0.41 ± 0.10^a	1.57 ± 0.13^a	0.77 ± 0.18^a
Plasma α-tocopherol (μM)	PBS	20.06 ± 0.87	22.49 ± 0.40	20.12 ± 5.51	21.15 ± 4.05
	rhIL-6	17.07 ± 1.31^a	21.46 ± 4.39	18.26 ± 2.12	14.43 ± 3.31^a
Food intake (g/kg/day)	PBS	NM	101.48 ± 18.70	106.17 ± 1.09	98.24 ± 8.81
	rhIL-6	NM	125.86 ± 10.39	103.78 ± 9.45	117.3 ± 13.89
Body weight (g)	PBS	201.55 ± 6.11	212.65 ± 7.55	234.60 ± 5.37	266.20 ± 6.45
	rhIL-6	186.38 ± 8.00	192.3 ± 11.58	218.55 ± 17.17	239.05 ± 18.60

NM, not measured; rhIL-6, recombinant human interleukin-6. Vitamin A-sufficient male rats weighing ∼200 g were infused with rhIL-6 via minipumps for 7 days with blood sampling as indicated. Values represent means ± SD. Control (PBS), n = 4; rhIL-6, n = 4.

^{a} $P < 0.05$ relative to PBS control.

TABLE 2. Tissue retinyl palmitate and retinoic acid concentrations

Variable	Treatment	Retinyl Palmitate			Retinoic Acid	
		Baseline	Day 3	Day 7	Day 3	Day 7
		nmol/g			*ng/ml or ng/g*	
Plasma	PBS	ND	ND	ND	NM	0.61 ± 0.06
	rhIL-6	ND	ND	ND	NM	0.64 ± 0.15
Liver	PBS	613.68 ± 44.76	676.54 ± 57.78	694.93 ± 70.04	4.58 ± 0.96	5.21 ± 0.82
	rhIL-6	613.68 ± 44.76	665.01 ± 11.93	708.10 ± 43.67	4.97 ± 0.70	5.29 ± 0.33
Kidney	PBS	5.05 ± 3.24	2.43 ± 1.34	2.84 ± 1.25	1.87 ± 0.37	2.18 ± 0.27
	rhIL-6	5.05 ± 3.24	2.97 ± 1.17	2.25 ± 0.32	1.93 ± 0.31	2.02 ± 0.15

ND, not detected; NM, not measured. Vitamin A-sufficient male rats weighing ∼200 g were infused with rhIL-6 via minipumps for 3 or 7 days, at which point they were killed and tissues were collected. Values represent means ± SD. Baseline, n = 3; control (PBS), n = 4; rhIL-6, n = 4.

palmitate and retinoic acid concentrations did not differ between groups or from baseline concentrations (Table 2).

Plasma and hepatic RBP concentrations and renal megalin abundance during inflammation

Concomitant with changes in plasma retinol concentrations, plasma RBP concentrations were lower in rhIL-6-treated rats compared with controls (**Fig. 4**). However, plasma RBP concentrations decreased faster than retinol concentrations. In rhIL-6-treated rats, RBP concentrations decreased to 20% of baseline within 21 h versus 44 h for retinol (Fig. 2). In addition, Western blot analysis of hepatic RBP showed that rhIL-6-treated rats had 41% reduced concentrations compared with controls at 7 days (**Fig. 5**). Megalin expression in kidneys of the rhIL-6-treated or control rats showed strong immunoreactivity within the apical membrane of the proximal convoluted and proximal straight tubular cells (**Fig. 6**), indicating no differences in the expression of megalin between the treatment groups.

DISCUSSION

In previous publications (4, 5, 17–21), the terms hyporetinemia and hyporetinolemia have been used interchangeably to describe the reduction in circulating retinol during inflammation. Etymologically, the term hyporetinolemia is specific in referring to retinol, whereas hyporetinemia implies a reduction in VA including retinol, retinyl esters, and retinoic acid. The present results indicated
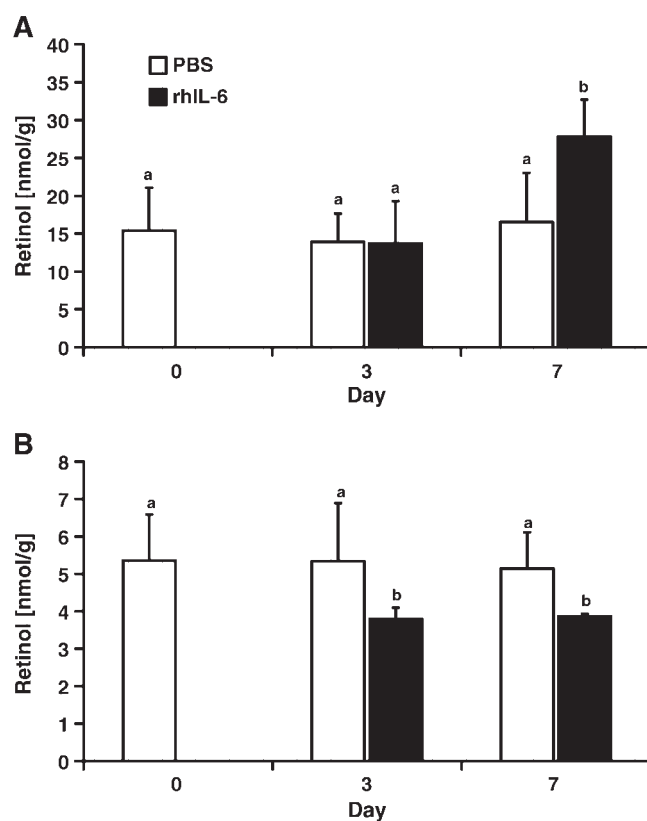

Fig. 3. Hepatic accumulation of retinol with a reduction in renal retinol by 7 days of hyporetinolemia. A: In the liver, unesterified retinol concentrations were significantly higher in rhIL-6-treated rats compared with controls or baseline concentrations. B: Renal retinol was significantly lower in rhIL-6-treated relative to control rats at days 3 and 7. Significance for the reduction in renal retinol was based on a one-tailed *t*-test. Baseline, n = 3; control (PBS), n = 4; rhIL-6, n = 4. Bars marked by different letters represent significance with $P < 0.05$. Error bars indicate SD.
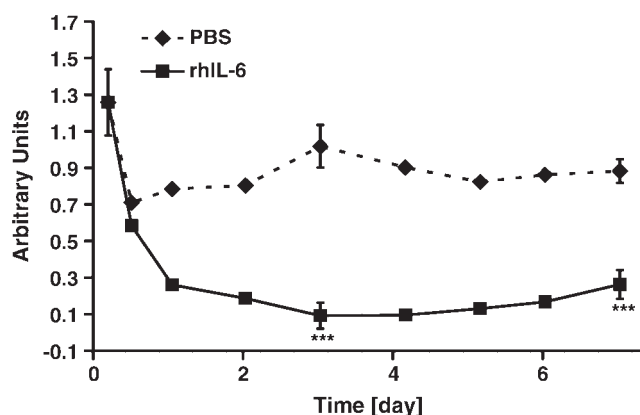

Fig. 4. rhIL-6-induced hyporetinolemia was associated with prolonged reduction of plasma retinol-binding protein (RBP). Plasma RBP decreased and was significantly lower in rhIL-6-treated rats compared with control rats. Because of the limited amount of plasma available, samples for all time points, except at baseline, 3, and 7 days, were pooled. Each pooled sample represented four samples. Data are displayed as arbitrary units relative to baseline. On days 0, 3, and 7, n = 4. *** $P < 0.001$. Error bars indicate SD.
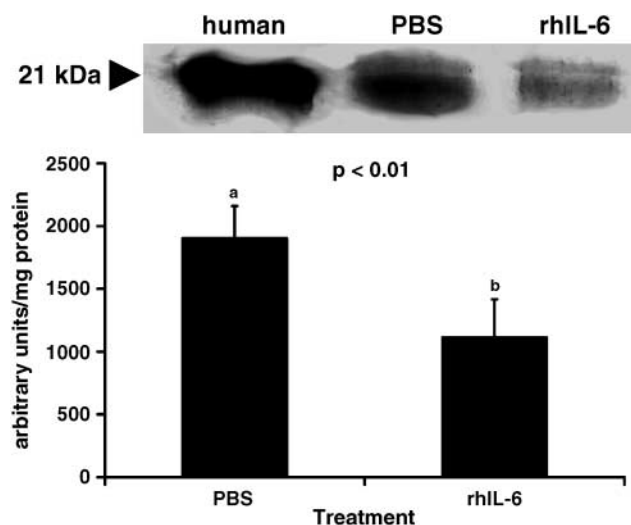
Fig. 5. Hepatic RBP was significantly lower by 7 days. Western blots of hepatic RBP showed that rats treated with rhIL-6 had 41% reduced concentrations relative to controls ($P < 0.05$). Control (PBS), n = 4; rhIL-6, n = 4. Results are expressed as arbitrary units normalized for total protein in the samples. Bars marked by different letters represent significance with $P < 0.01$. Error bars indicate SD.

Fig. 6. Megalin abundance in rhIL-6 and control rats. Each panel is a representative slide from each group. These sections are from rats after 7 days of treatment. Megalin expression in kidneys of the rhIL-6-treated (A) or control (B) rats showed strong immunoreactivity within the apical membrane of the proximal convoluted and proximal straight tubular cells, indicating no differences in the expression of megalin between the treatment groups. G, glomerulus.

that a reduction of other retinoids did not occur together with inflammation. Therefore, the use of the term hyporetinolemia is recommended to accurately describe the changes of plasma retinol during inflammation.

Hyporetinolemia was successfully induced with rhIL-6 in VA-sufficient rats. In the first experiment, a single intraperitoneal injection of rhIL-6 reduced plasma retinol concentrations within 6–12 h. The ensuing hyporetinolemia was comparable in magnitude to that induced by LPS. Previously, we had demonstrated that LPS reduces plasma retinol concentrations within 12–24 h after an intraperitoneal injection (17); thus, in the present study, we measured LPS-induced hyporetinolemia by 24 h. Comparing the time effects across studies, the results from the present experiment indicated that rhIL-6 induced hyporetinolemia earlier than LPS. The inflammatory response to LPS results in the synthesis of inflammatory cytokines such as IL-1β, tumor necrosis factor-α, and IL-6 (22). These events and their time line may explain the earlier induction of hyporetinolemia with rhIL-6 compared with LPS. IL-6 is an important cytokine because it is necessary and sufficient to induce or inhibit the synthesis of acute-phase proteins of inflammation (23), and it is responsible for the transition from acute to chronic inflammation (24). In addition, rhIL-6 does not induce tolerance, whereas repeated doses of LPS induce an endotoxin tolerance effect (25) that leads to a reduction of the synthesis of proinflammatory cytokines (26). In this regard, rhIL-6 administered continuously in nonhuman primates and rats successfully produces and sustains an inflammatory response (27, 28). Measles virus infections are characterized by severe hyporetinolemia in children (4). Also, measles virus causes the synthesis and secretion of IL-6 (22), leading to high circulating IL-6 concentrations (29). For
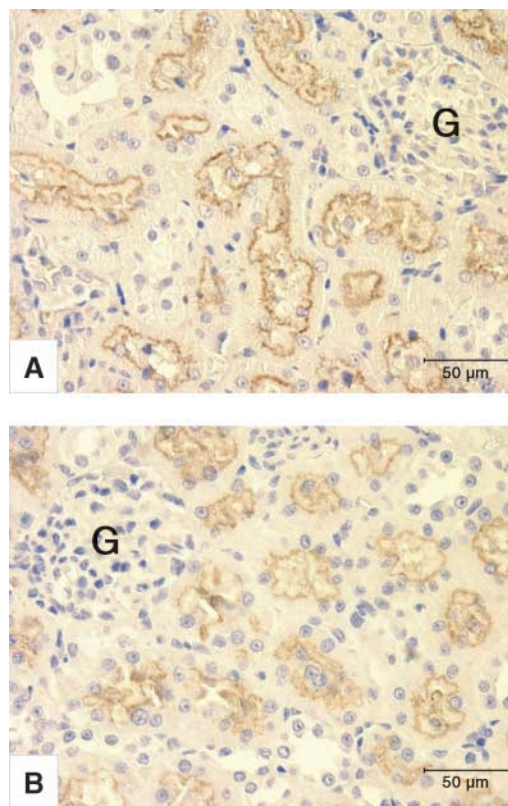
these reasons and to examine the consequences of prolonged hyporetinolemia on the distribution of VA, a model of continuous infusion of rhIL-6 was developed.

In the second experiment, a continuous infusion of rhIL-6 precipitated hyporetinolemia within 7 h, and plasma retinol concentrations remained low for the rest of the experimental period (7 days). The infused dose provided 13 μg of rhIL-6 per rat per day, and the total amount was calculated to last for 7 days. This daily dose was equivalent to that used in previous studies to induce hypoferremia without deleterious effects to experimental animals (30). Even larger doses would not have led to pathological changes in the liver or kidney (31). In the present study, the continuous infusion of rhIL-6 was characterized by a significant increase in plasma AGP concentrations, a marker of inflammation. However, this dosage did not affect rates of body weight gain or food intake of rhIL-6-treated compared with control rats. Moreover, plasma α-tocopherol concentrations in rhIL-6-treated rats were not affected, indicating no apparent oxidative stress. This model allowed for examination of the consequences of prolonged hyporetinolemia on retinol homeostasis. Retinol concentrations are tightly controlled, and they reflect a balance between hepatic stores of VA as retinyl esters and its oxidative me-

tabolism into active forms such as retinoic acid (2, 32). We speculated that by setting the homeostatic set point of retinol to a low concentration, as in prolonged hyporetinolemia, it would affect the metabolic distribution of retinol among its storage and active forms.

Alongside hyporetinolemia, there was a decrease in plasma RBP concentrations, mirroring the reduction in retinol (Fig. 2), and this was accompanied by a decrease in hepatic RBP concentrations that persisted for up to 7 days when it was measured (Fig. 5). Previously, it was demonstrated that LPS-induced hyporetinolemia is the result of a reduction in hepatic RBP of both its mRNA and protein concentrations (17). The rapid onset of hyporetinolemia coupled with reductions of plasma and hepatic RBP concentrations indicated that a decrease in the availability of RBP in the liver was the mechanism leading to and maintaining hyporetinolemia induced by rhIL-6. In this regard, the half-life of rat RBP is ∼6 h (33), corroborating that RBP is likely involved in this mechanism, because hyporetinolemia ensued rapidly after inducing inflammation with rhIL-6. It is interesting that plasma RBP concentrations decreased more rapidly compared with plasma retinol concentrations during inflammation. This is in contrast to VA deficiency (34), in which plasma retinol concentrations decreased more rapidly compared with RBP. Therefore, during inflammation, plasma RBP concentrations decrease more rapidly than plasma retinol concentrations, supporting the underlying mechanism of a reduced synthesis and secretion of hepatic RBP during inflammation. Other mechanisms have been proposed to explain inflammation-induced hyporetinolemia, including an increased utilization of VA subsequent to its immune-potentiating role during infections (35) and increased glomerular filtration followed by an impaired reabsorption in the proximal tubule, leading to retinol and RBP being excreted in urine (36). However, none of these mechanisms appears to be the case in this study.

We assessed these mechanisms and the effects of decreasing the homeostatic set point of retinol by measuring the concentrations and distributions of VA, including retinol in its ester and alcohol forms and retinoic acid, in the liver, plasma, and kidneys, as well as the abundance of megalin in the kidneys, which is responsible for the tubular reabsorption of retinol bound to RBP (37). There were no differences in retinol palmitate or retinoic acid concentrations in the liver, kidneys, and plasma between rhIL-6-treated rats and controls. This indicated that although plasma retinol is at the crossroads between being stored and being oxidized as part of the metabolism of VA (2), its reduction, prolonged for 7 days, did not affect this balance. In addition, these findings indicated that no VA was being lost during prolonged hyporetinolemia, because hepatic retinol concentrations increased by 7 days. Rather, the increase in hepatic retinol concentrations suggested some degree of accumulation of VA in the liver secondary to a reduction in RBP concentrations, although there were no differences in hepatic retinol concentrations at 3 days between rhIL-6-treated rats and controls. In mice lacking RBP, there is a lag of 6–8 h between the reduction of labeled retinol in plasma and its subsequent accumula-

tion in the liver after an oral dose of labeled retinol (38). However, the turnover time for plasma retinol is 1.9 h (39). Thus, it takes some time for the reduction of plasma retinol to be associated with an increase in hepatic VA. Moreover, plasma retinol is small compared with hepatic retinol (e.g., <5%), and although 48% of plasma retinol is recycled back into the liver (39), it is very unlikely that this small amount makes a substantial contribution to hepatic retinol, especially in VA-sufficient rats. Therefore, we believe that the accumulation of hepatic retinol by 7 days is mainly attributable to VA from the diet, although it may include some from retinol being recycled from plasma.

In contrast, renal retinol concentrations were lower in rhIL-6-treated rats compared with controls at 3 days and remained lower at the end of the study. This suggested that renal retinol concentrations reflected either hyporetinolemia or a reduced capacity of tubular reabsorption. However, the abundance of megalin was not altered by rhIL-6 treatment, nor were there any changes in renal retinoic acid or retinol palmitate concentrations. Thus, renal retinol concentrations reflected mainly the reduction in plasma retinol concentrations as previously demonstrated (17).

Studies using knockout mouse models for specific proteins involved in VA metabolism have demonstrated that a lack of these proteins affects the metabolic distribution of specific retinoids. In RBP knockout mice (38, 40), at 3 weeks and 5 months, plasma retinol concentrations decline; however, only at 5 months do hepatic retinol concentrations increase, indicating that although plasma retinol is low, hepatic retinol accumulates. This phenomenon still occurs when knockout mice receive a diet low in VA (40). These results show striking similarity to the results in the present study, in which there was an accumulation of hepatic retinol by the end of the study. In contrast, genetic inactivation of cellular retinol-binding protein-I causes a 50-fold reduction in hepatic retinyl esters (41), whereas inactivation of transthyretin (TTR) increases hepatic RBP concentrations relative to wild-type animals (42). Therefore, the reduction of hepatic RBP either by genetic manipulation or induced by inflammation decreases the mobilization and secretion of hepatic retinol. However, hyporetinolemia did not cause any metabolic modifications in the distribution of retinyl esters or retinoic acid in the liver, indicating that although the retinol homeostatic set point was decreased, it did not affect its storage as an ester or its further oxidation into retinoic acid.

In summary, these results indicated that instead of being lost, retinol accumulated in the liver during inflammation and that the most plausible mechanism was through a reduction in hepatic RBP. In both types of inflammation studied, either induced with LPS for 24 h (17) or with rhIL-6 for 7 days, hyporetinolemia was significantly associated with a reduction of plasma and hepatic RBP concentrations. Moreover, because this mechanism and its consequences in accumulating hepatic retinol are similar to those demonstrated in mice with genetic inactivation of hepatic RBP, it is reasonable to infer that VA deficiency of extrahepatic tissues may occur during inflammation as observed in RBP knockout mice. In these mice, retinal func-

tional alterations are common (38). This is relevant in explaining the morbidity associated with measles infections. In marginally VA-deficient children with measles infections, there are eye and respiratory alterations similar to those described as part of the VA deficiency disorders (43). However, when these children receive large doses of VA, 200,000 IU daily for 2 days, these signs are resolved and their survival is enhanced (4). Our model would predict that in measles-induced inflammation, reduced mobilization of hepatic retinol impairs the distribution of VA to extrahepatic tissues. In this regard, renal tissue showed a significant reduction of unesterified retinol during inflammation, which suggests that extrahepatic tissues that depend on retinol as a source of VA will be affected. Thus, providing VA supplements to children with measles infection could alleviate this deficiency by making VA available, probably as retinyl esters or retinol bound to lipoproteins.ᴵᴸ

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