Iron Deficiency in Young Rats Alters the Distribution of Vitamin A between Plasma and Liver and between Hepatic Retinol and Retinyl Esters

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Abstract:
We assessed whether iron deficiency alters the concentration of vitamin A (VA) in plasma or liver and the chemical distribution between hepatic unesterified and esterified retinol. Weanling male Sprague-Dawley rats ($n = 10$/group) were allocated to one of four diet groups: low iron (ID3, 3 mg of elemental iron/kg diet), marginal iron (ID15, 15 mg/kg), control diet food-restricted to the 103 group (FR, 35 mg/kg), and control diet ad libitum consumption (AD, 35 mg/kg). Both ID3 and FR rats grew less than AD and 1015 rats. At the end of 5.5 wk, plasma retinol concentrations of the 1D3 and FR rats were reduced >40% compared to ID15 and AD rats [Kruskal-Wallis test (K-W), $P < 0.0042$]. Paradoxically, the hepatic VA concentration was greater in FR rats, with accumulation of more retinyl esters and retinol compared to the other dietary groups. Concentrations of hepatic retinyl esters and retinol did not differ among the other groups, but the molar ratio of hepatic retinyl esters to retinal was greater in ID3 rats ($20.1 \pm 1.4$) compared to ID15 rats ($13.8 \pm 1.6$, $P = 0.02$), AD ($11.3 \pm 2.1$, $P < 0.0042$) and FR ($9.5 \pm 1.1$, $P < 0.0042$). Iron deficiency may cause changes in liver and plasma VA that are refractory to VA intake, and thus a benefit may be derived from combining iron and VA supplements during nutrition interventions.

KEY WORDS: hemoglobin, hepatic retinyl esters, linear regression, plasma retinol, rat

Article:
Iron-deficiency anemia and vitamin A (VA)$^2$ deficiency share several features. They are the two major nutritional deficiencies; high prevalences are confined to geographically similar areas, and growing children and women of reproductive age are the groups most vulnerable to both deficiencies, which may occur simultaneously (Trowbridge et al. 1993, Underwood and Arthur 1996, Yip 1994). Nonetheless, their association does not seem to depend entirely on sharing the same socio-economic risk factors.

VA deficiency is a potential cause of iron deficiency (Sommer and West 1996). Hodges et al. (1978) demonstrated a moderate degree of iron-deficiency anemia (mean hemoglobin of 118.70 g/L) during experimentally-induced VA deficiency in humans. This anemia developed in male subjects with retinol concentrations $\leq 0.35$ μmol/L and impaired dark adaptation (i.e., VA deficiency), despite a daily intake of 18-19 mg of elemental iron. Another salient feature of this anemia was a reduction of serum iron concentration, which occurred without a corresponding increase in total iron-binding capacity that was refractory to daily, therapeutic doses of oral iron. This anemia resolved only after the subjects were treated with VA or $\beta$-carotene (Hodges et al. 1978).

Evidence from observational and experimental studies of iron-deficient populations suggests that VA utilization may be reduced during iron deficiency. Molla et al. (1993) screened a population with a high prevalence of iron-deficiency anemia for VA deficiency. They found three times as many children with VA deficiency (i.e., serum retinol $<0.35$ μmol/L), or with sub-clinical VA deficiency (i.e., serum retinol $<0.70$ μmol/L) among children with low hemoglobin than among children with normal hemoglobin. In an experimental study of children with anemia, Mejia and Chew (1988) observed that iron supplementation alone increased serum retinol
compared to placebo-treated children. Although these differences were not significant, it is noteworthy that children receiving elemental iron, alone or in combination with VA supplements, had higher serum retinol concentrations than their respective controls. Experimental studies in anemic rats have shown reduced plasma retinol concentrations even though the rats were fed a VA-rich diet and VA accumulated within the liver (Amine et al. 1970, Staab et al. 1984).

Our objectives in the present studies were to demonstrate that iron deficiency is associated with a reduction of plasma retinol and to determine the possible role of growth retardation in this reduction. Evidence from these experiments suggests that hepatic VA metabolism is altered and that very likely the mobilization of VA from liver is impaired by iron deficiency.

MATERIALS AND METHODS

**Materials.** Reagent-grade chemicals were obtained from Fisher Scientific, Pittsburgh, PA, or Sigma Chemical, St. Louis, MO.

**Animals and experimental design.** Forty 21-d-old male Sprague-Dawley rats were randomly assigned to four different dietary groups. The diets consisted of a nutritionally complete formula, AIN 93G (Reeves et al. 1993) modified to contain three different concentrations of elemental iron as iron sulfate. These diets were a low-iron diet (ID3), containing 3 mg iron/kg of diet, and a marginally iron-deficient diet (ID15), containing 15 mg iron/kg. The 15 mg/kg iron concentration was chosen because it was shown to maintain normal hemoglobin concentrations and growth rates of rats, while reducing liver iron stores (Borel et al. 1991). Additionally, two control groups were included. One group had free access to the control diet containing 35 mg iron/kg diet [ad libitum (AD) group], and a second group was fed the control diet in an amount equal to that of the ID3 group [food-restricted (FR) group]. To control for growth retardation caused by low iron intake, food intakes and body weights were monitored every 2 d. The food intake of FR rats was restricted, beginning on d 9 of the experiment and every 2 d thereafter, to an amount of diet equal to the average consumed by the ID3 group in the previous 2 d. The other dietary groups consumed their respective diets ad libitum for the entire 5.5 wk of experimentation. Rats were housed individually in plastic cages with free access to distilled water in a room maintained at 22°C with a 12 h light 12 h dark cycle. During the period of experimentation, tail-blood samples were ob. rained periodically to monitor the rats’ iron and VA status. At the end of the experiment, rats were killed by CO₂ asphyxiation, and blood samples were collected from the vena cava into heparinized syringes, Plasma was later separated by centrifugation and stored under argon at -20°C. The livers were excised, blotted, and immediately frozen in liquid nitrogen for storage at -80°C until they could be processed (Furr et al. 1994). All experimental protocols were in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Pennsylvania State University.

**Pilot study.** Prior to the experiment described above, a pilot study was conducted to determine whether iron deficiency is associated with changes in plasma and liver VA concentrations and to determine the time when these changes occur. Tissue samples were obtained from rats that were part of a study of iron deficiency and brain iron distribution (Erickson et al. 1997). Briefly, five rats per diet group were fed one of three different levels of iron in a semipurified (AIN 76A) diet from weaning (21 d of age) through 5 wk of experimentation. These diet groups were low iron group, fed 3 mg of elemental iron as iron sulfate per kg diet; iron-replete group, fed 3 mg of iron/kg diet for 2 wk and then transferred to diet containing 15 mg iron/kg diet for the remainder of the study; control group, fed 35 mg of iron/kg diet ad libitum-consumption. Plasma and liver samples were obtained when rats were ~50 d old and were analyzed for retinol, retinal-binding protein (RBP), transthreitin (TTR), and total proteins in plasma and for RBP, retinol, and retinyl esters in liver samples.

**Tissue VA analysis.** Unesterified (retinol) and esterified retinol (retinyl esters) were determined by HPLC using trimethylmethoxy, phenyl-retinol as an internal standard (Ross 1986). Plasma and liver retinol and liver retinyl esters were extracted with ethanol and partitioned into hexanes from nonsaponified samples. The concentration of total retinyl esters is expressed as the molar equivalent of retinol. In addition, the molar ratio of hepatic retinyl esters to unesterified retinol was calculated to assess the effect of iron deficiency on the distribution of...
VA in the liver. Because of differences in body and liver weights among the treatment groups, the total mass (content) of plasma retinol was calculated by estimating blood volume as 0.056 of body weight (Hultin et al. 1996) and estimating plasma volume from blood volume based on the experimentally determined hematocrit. The masses of unesterified and esterified retinol in liver were corrected for differences in liver and body weight by expressing the amounts per g of body weight.

**Hemoglobin and liver nonheme iron.** Hemoglobin concentration was measured colorimetrically by the cyanmethemoglobin method (Sigma Chemical, procedure No. 525). Hepatic iron was measured by the ferrozine reagent method as described by Erickson et al. (1997).

**Statistical analysis.** Values are given as the mean ± SEM. Be. cause variances were not homogenous among the dietary groups, statistical comparisons were made with a nonparametric test, the Kruskal-Wallis one-way ANOVA, using Dunn's post-hoc adjustment; a two-tailed value of $q(0.05/k(k - 1)] = 0.0042$ was used as the P-value to test for significance (Rosner 1986); when variances were homogenous by Cochran's and Bartlet’s test, the differences among groups were assessed with a one-way ANOVA test, adjusting for multiple comparisons with the mean least significant difference procedure (Rosner 1986). Simple linear regression analysis (Weisberg 1985) was used to assess the association between hemoglobin and the molar ratio of hepatic retinyl esters to retinol.

**RESULTS**

**Pilot study.** Rats fed the low-iron diet had significantly lower hemoglobin concentrations and plasma and liver iron concentrations compared to controls or to iron-replete rats (Erickson et al. 1997). Concomitantly, mean plasma retinol, RBP, and TTR concentrations in iron-deficient rats were 68 (P = 0.12), 66 (P = 0.04), and 83% (P = 0.04), respectively, of controls and iron-replete rats (ANOVA). However, plasma total protein concentration did not differ among the groups (ANOVA, $P = 0.80$). Hepatic VA was greater (ANOVA, $P = 0.06$) in iron-deficient rats (166.6 ± 42.9 nmol/g) than in controls (122.3 ± 11.2 nmol/g) and iron-replete rats (136.9 ± 15.6 nmol/g). The accumulation of hepatic VA was due to an increase in retinyl esters concentration, which equaled 143% of the control rats (ANOVA, $P = 0.03$). In contrast, hepatic RBP did not differ among the groups (ANOVA, $P = 0.80$).

**Growth rate and hematological variables.** The rate of weight gain slowed after 2 wk in the 1D3 group, and remained significantly lower than that of the marginally iron-deficient group (ID15) or AD control group after d 20 (Fig. 1). The growth curve of food-restricted controls was similar to that of the 1D3 group.
At the end of the study, the body and liver weights of AD and 1D15 rats were not different from each other and were greater than those of FR and 1D3 rats, which did not differ (Table 1). Hemoglobin concentration was significantly lower in 1D3 rats than in FR and AD groups \( (P < 0.0042) \). Although hemoglobin concentration of ID15 rats was approximately four times that of ID3 rats, the difference was not significant \( (P = 0.02) \). The hematocrit was significantly lower in 1D3 rats than in any of the other dietary groups.

### Table 1

<table>
<thead>
<tr>
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<th>ID3</th>
<th>ID15</th>
<th>FR</th>
<th>AD</th>
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<tbody>
<tr>
<td>Body weight, g</td>
<td>133 ± 2.4a</td>
<td>246 ± 4.4a</td>
<td>146 ± 2.4b</td>
<td>266.6 ± 5.4a</td>
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<tr>
<td>Liver weight, g</td>
<td>4.4 ± 0.1b</td>
<td>8.8 ± 0.2a</td>
<td>4.2 ± 0.2b</td>
<td>9.0 ± 0.3a</td>
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<tr>
<td>Hemoglobin, g/L</td>
<td>34.0 ± 1.0p</td>
<td>129.8 ± 3.0p</td>
<td>169.0 ± 5.0p</td>
<td>165.0 ± 4.0p</td>
</tr>
<tr>
<td>Hematocrit, volume fraction</td>
<td>0.14 ± 0.01(b)</td>
<td>0.41 ± 0.001a</td>
<td>0.43 ± 0.004a</td>
<td>0.45 ± 0.014a</td>
</tr>
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*Values are mean ± SEM, n = 10. Different superscript letters in a row indicate significant differences. A nonparametric Kruskal-Wallis one-way ANOVA was used for statistical comparisons among the groups with the Dunn’s adjustment and a two-tailed \( a (0.05/k(k-1)) = 0.0042 \) as the significant \( P \) value.

*Groups are ID3: 3 mg of elemental iron as iron sulfate/kg diet; ID15: 15 mg of iron; FR: food-restricted 36 mg of iron; AD: Ad libitum consumption control 36 mg of iron.*

**Distribution of VA in plasma and liver.** The plasma retinol concentration of [D3 rats was lower than in 1D15 and AD rats (Kruskal-Wallis test, \( P < 0.0042 \)), but not compared to FR rats (Fig. 2). Differences in the plasma
total content (i.e., concentration x plasma volume) of retinol were similar except that the content in FR rats was significantly lower (80%) than in AD and ID15 rats (data not shown; Kruskal-Wallis test, \( P < 0.0042 \)). Hepatic retinol and retinyl ester concentrations were significantly higher in FR rats than in any other group (Fig. 2B and C). This elevation persisted after adjusting hepatic retinol and retinyl ester concentrations for differences in body and liver weights (data not shown). Although hepatic retinol and retinyl ester concentrations of ID3 rats did not differ significantly from AD or ID15 rats, the molar ratio of hepatic retinyl esters to retinol differed among the groups. The molar ratio of hepatic retinyl esters to retinol was greater in ID3 rats (20.1 ± 1.4) compared to ID15 rats (13.8 ± 1.6, \( P = 0.02 \)), AD (11.3 ± 2.1, \( P < 0.0042 \)), and FR (9.5 ± 1.1, \( P < 0.0042 \)). Hepatic nonheme iron concentrations (Fig. 2D) and contents (not shown) of ID3 and ID15 rats were significantly lower than those of FR or AD rats (Kruskal-Wallis test, \( P < 0.0042 \)).

**Hepatic retinyl ester accumulation.** To assess whether the chemical shift of hepatic VA was affected by the severity of iron deficiency, and remained congruous in both the pilot and main studies, a regression analysis of hemoglobin concentration versus the molar ratio of retinyl esters to retinol in rats from both studies was conducted. An inverse association between hemoglobin concentration and the molar ratio was found (Fig. 3; \( r^2 = 0.65, P < 0.001 \)).

**DISCUSSION**

Numerous studies have examined the effect of VA deficiency on hemoglobin concentrations; however, studies directly assessing the effect of iron deficiency on plasma retinol are few or nonexistent. Because of this, the simultaneous reductions of plasma hemoglobin and retinol concentrations have been interpreted as being due to VA deficiency (Sommer and West 1996). In the pilot study, iron-deficient rats receiving a VA-adequate diet had lower than normal plasma retinol and RBP concentrations. These reductions were not associated with plasma total proteins nor with hepatic RBP concentrations, which did not differ among the dietary groups. In addition, iron-deficient rats accumulated more hepatic retinyl esters compared to rats in the other dietary groups. These results suggest that the reduction of plasma retinol with a subsequent increase in hepatic VA were due to iron-deficiency anemia.

Previously, others had observed in rats reduced plasma retinol concentrations and increased hepatic VA during experimentally-induced iron deficiency. However, they dismissed these findings as indirectly resulting from hemodilution, reduced food intake, and reduced absorption of dietary VA. For example, Amine et al. (1970) noted low plasma retinol concentrations in rats consuming a diet with 3 mg elemental iron/kg diet and 1.5 mg VA/kg diet compared to rats consuming the same diet with 250 mg elemental iron/kg diet and 1.5 mg VA/kg
diet. Similarly, Staab et al. (1984) observed that rats consuming 3 mg elemental iron/kg diet and 12.0 mg VA/kg diet had plasma retinal concentrations that were 76% of controls that consumed more iron and less VA (33 mg iron/kg diet and 1.2 mg VA/kg diet). These findings are important because they showed that the reduction of plasma retinol concentration was a function of iron intake and not of VA intake. Staab et al. (1984) considered the reduction of retinol to be the result of hemodilution, although they observed in another two groups of rats that hepatic VA of low iron/lows VA rats was four times greater than the hepatic VA of high iron/lows VA rats.

However, hemodilution did not seem to explain the reduction of plasma retinal. Blood and plasma volume were calculated from body weights, hematocrit values were calculated as reported by Staab et al. (1984). Plasma total content of retinol, its mean, and SEM were estimated from the concentration of plasma retinal and its coefficient of variation. The plasma content of retinol was 20.6 ± 1.0 nmol in low iron/high VA rats versus 21.6 ± 0.7 nmol (mean ± SE) in normal iron/normal VA rats (t-test, P = 0.40). However, iron-deficient rats consumed 10 times more VA than controls. Blood volume, plasma volume, and plasma content of retinal were also calculated from the data reported by Amine et al. (1970); the plasma content of retinol was 6.2 ± 0.6 nmol in iron-deficient rats versus 13.4 ± 0.9 nmol in iron-sufficient control rats (c-test, P < 0.01). The data from the present pilot study and that recalculated from the studies of Staab et al. (1984) and Amine et al. (1970) provide strong evidence that the most likely cause of low plasma retinal during iron deficiency is an accumulation of hepatic VA and not hemodilution. However, the experimental designs of these three studies did not control for the possible confounding factor because Rechcigl et al. (1962) showed that growth retardation impairs the utilization of VA.

In the main study present herein, a food-restricted group was included to assess the independent effect of growth retardation on plasma and hepatic VA. In addition, an iron-deficient group without growth retardation (1015 group) was included to control for the independent effect of iron deficiency. To adjust for differences in plasma volume, liver and body weights among the groups; the concentrations of plasma and hepatic VA were expressed as content in the case of plasma retinol and as mass per g of body weight for hepatic VA.

Food-restricted rats grew at the same rate as rats fed a diet with very low iron (3 mg/kg diet, Fig. 1), but their liver iron concentrations were higher (Fig. 2). On the other hand, 1015 rats had liver iron concentrations that did not differ from 103 rats even though their growth was higher than that of 103 rats (Fig. 1). These differences demonstrate that the experimental design successfully produced four different outcomes: AD rats with normal growth and normal liver iron; IDI5 rats with normal growth and reduced liver iron; FR rats with growth retardation and increased liver iron; and 1D3 rats with growth retardation and reduced liver iron. In this setting, the independent effects of growth rate versus iron status on VA distribution could be evaluated.

Plasma retinol expressed as concentration or total content was significantly lower in both the 1D3 and FR groups compared to the other two groups (Fig. 2), indicating that hemodilution was not responsible for the reduction of retinol. This reduction occurred even though 1D3 rats had hepatic VA stores that were not different from either AD or 1015 rats. Additionally, FR rats had a significantly greater hepatic VA mass than any other dietary group. Thus, it is very unlikely that the reduction in plasma retinal contents of 103 and FR rats was due to reduced intake or poor absorption of dietary VA. In the pilot study, hepatic RBP concentration did not differ between iron-deficient rats and their controls. Accordingly, Perozzi et al. (1989) showed that the abundance of RBP mRNA in liver is not reduced by protein deficiency. Based on these data, we contend that the reduction of circulating retinal during iron deficiency and in growth-impaired, food-restricted rats without iron deficiency was not the result of a reduction in the hepatic synthesis of RBP. Smith and Brown (1974) showed that food-restricted control rats had hepatic RBP concentrations that did not differ from rats with free access to food (t-test, P = 0.30).

Both retinal and retinyl ester concentrations were elevated in the livers of FR rats. Previous studies showed an increase of hepatic total VA in experimentally-induced growth retardation (Rechcigl et al. 1962), but did not examine the distribution of retinal versus retinyl esters. Because 80-90% of hepatic retinal is esterified, an
In summary, we have shown that iron deficiency causes a reduction of plasma retinal and an accumulation of hepatic retinyl ester concentration. The message emphasized by the regression analysis is that a reduction in hemoglobin concentration is associated with a chemical shift of hepatic retinal to retinyl esters. This is of practical importance to public health practitioners because it stresses the negative effect of iron-deficiency anemia on the mobilization of hepatic VA, and thus, it suggests a benefit from combining iron and VA supplements to enhance the utilization of both nutrients. Whether these results are applicable to human cases of iron or VA deficiency may be a matter of the rate of progression and severity of nutrient deficiency. The more rapid rate of growth and compressed life span of rats compared to humans contributes to the rat's greater susceptibility to iron deficiency (Dallman 1986). Nonetheless, in a recent study in Mexico, children with no apparent infections but with iron-deficiency anemia had their serum retinal concentrations increased by 27% after iron supplementation alone (Personal communication, Dr. J. L. Rosado, National Institute of Nutrition, Mexico).
Abbreviations used: AD, ad libitum-consumption controls; CAB, cellular retinol-binding protein; FR, Food-restricted controls; 1015, 15 mg of iron/kg of diet group; ID3, low Iron diet group, 3 mg of elemental iron/kg of diet; RBP, retinol binding protein; TTR, transthyretin; VA, vitamin A.

LITERATURE CITED


