Retinoic Acid Modulates Hepatic Iron Homeostasis in Rats by Attenuating the RNA-Binding Activity of Iron Regulatory Proteins 1,2

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Abstract

Vitamin A deficiency has been widely associated with perturbations of iron homeostasis, a consequence that can be reversed by retinoid supplementation. Despite the numerous studies that demonstrate an interaction between these 2 nutrients, the mechanistic basis for this relation has not been well characterized. Because iron regulatory proteins (IRP) have been established as central regulators of iron homeostasis, we investigated the potential role of IRP in the regulation of iron homeostasis under conditions of vitamin A deficiency and supplementation with all-trans-retinoic acid (atRA). Rats were fed a control diet or a diet deficient in either vitamin A or iron or both micronutrients. Four parallel groups of rats were supplemented with atRA daily (30 μmol/kg body weight) during the final week of this study. As expected, iron-deficient (−Fe) rats exhibited a decrease in hepatic nonheme iron levels and a subsequent increase in IRP RNA-binding activity, resulting in diminished ferritin abundance. Interestingly, atRA supplementation inhibited the increase in IRP RNA-binding activity in −Fe rats to a level that was not significantly (P = 0.139) different from control values, and it partially restored ferritin abundance. This inhibition of IRP RNA-binding activity by atRA supplementation was also associated with a 40% reduction in transferrin receptor abundance. Taken together, these results indicate that IRP represent a mechanistic link between vitamin A and the regulation of iron homeostasis, a key finding toward further understanding this important nutrient-nutrient interaction.  J. Nutr. 137: 2686–2690, 2007.

Introduction

Vitamin A and iron deficiency are major nutritional issues that collectively affect billions of people worldwide. It has been known for over 80 y that there is an association between these 2 essential nutrients (1). Numerous human and animal studies have consistently shown that an interaction exists between the metabolism of vitamin A and iron, including the suggestion that vitamin A status plays a role in the regulation of iron homeostasis (2–16). Moreover, supplementation with vitamin A or all-trans-retinoic acid (atRA) 3 was found to restore normal iron homeostasis in part by regulating the abundance of such key proteins as transferrin receptor (TfR) and ferritin (17–24). However, the mechanistic basis for the influence of vitamin A on iron homeostasis was not completely understood.

Iron homeostasis is regulated through various cellular proteins, including those involved in transport (transferrin), cellular uptake (TfR), and storage (ferritin). These proteins dictate the cellular usage of iron, ensuring that adequate iron is available for cellular needs, as well as preventing iron toxicity. Consequently, regulating the abundance of these proteins in response to changes in intracellular iron concentrations is critical for maintaining homeostasis. The central proteins that post-transcriptionally regulate the translation of TfR and ferritin are the iron regulatory proteins (IRP),IRP1 and IRP2, which are present in the cytosol (25,26). IRP bind to iron responsive elements (IRE): stem-loop structures located on the 3′- and 5′-untranslated region of TfR and ferritin mRNA, respectively. For ferritin, the IRE-IRP complex formation results in a decrease in ferritin synthesis, because of inhibition of translation, whereas IRP binding to multiple IRE on the TfR mRNA stabilizes the message, thereby enhancing translation. Activation of IRP-IRE binding is inversely related to intracellular iron concentrations, thus elevating TfR abundance when iron concentrations are low, and increasing ferritin synthesis under high iron conditions.

Because IRP play a central role in regulating iron homeostasis, identifying and understanding the factors that regulate their activation and/or inactivation is critical. This study provides

1 Supported in part by the Cancer Research and Prevention Foundation and the Center for Designing Food to Improve Nutrition, Iowa State University.
2 Author disclosures: S. E. Schroeder, M. B. Reddy, and K. L. Schalinske, no conflicts of interest.
3 Abbreviations used: −A, vitamin A–deficient; atRA, all-trans-retinoic acid; −Fe, iron-deficient; −Fe/−A, iron- and vitamin A–deficient; IRE, iron responsive element; IRP, iron regulatory protein; TfR, transferrin receptor.
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0022-3166/07 $8.00 © 2007 American Society for Nutrition.
evidence that vitamin A status, and atRA in particular, represents an additional nutritional factor that can modulate the RNA-binding function of hepatic IRP. These novel findings advance our understanding of the interaction between vitamin A and iron homeostasis.

**Materials and Methods**

**Chemicals.** Reagents were obtained from the following companies: atRA, Sigma-Aldrich; Calbiochem; polyclonal ferritin antibody, Boehringer Mannheim; HepG2 cells, ATCC; cell culture reagents, Gibco; hemoglobin kit, Wako Diagnostics; goat anti-rabbit IgG horse-radish peroxidase, Southern Biotechnology; Riboprobe System T7 for radiolabeled IRE synthesis, Promega; polyclonal TIR antibody, BD Pharmingen; [α-32P]-UTP, PerkinElmer Life and Analytical Sciences; and Western blotting detection reagents, Amersham Pharmacia. The ferritin IRE cDNA plasmid was kindly provided by R. S. Eisenstein, University of Wisconsin, Madison. All other chemicals were analytical grade.

**Experimental design.** All rat experiments were approved by and conducted in accordance with Iowa State University Laboratory Animal Resources Guidelines. Forty-eight male Sprague-Dawley (Harlan Sprague-Dawley) rats (35–50 g) were housed in plastic cages in a room with a 12-h light:dark cycle. The rats were given food and water ad libitum. Following an acclimation period with a semipurified control diet (27), the rats were randomly assigned to 1 of 2 treatment groups (24 rats per group): control or vitamin A–deficient (−A). The rats were fed either the control diet or the same dietary mixture minus the retinyl palmitate in the vitamin mix (Harlan Teklad, TD 94161). At wk 4, half of the rats in the control and −A groups were placed on an iron-deficient (−Fe) diet (Harlan Teklad, TD 81062) for the remaining 4 wk of the study (8 wk total), resulting in 4 groups of rats (n = 12/group): control, −A, −Fe, or both iron- and vitamin A–deficient (−Fe/−A). At wk 8, half of the rats in each of the 4 diet groups (n = 6) were administered an oral daily dose of atRA (30 μmol/kg body weight) or vehicle (corn oil) using a positive displacement pipette. Preliminary experiments with varying doses of atRA demonstrated that 30 μmol/kg body weight had the greatest impact on IRP function without signs of toxicity (28). Following a 7-d treatment period with atRA, all rats were anesthetized, and blood samples were collected with heparinized needles via cardiac puncture. Whole blood samples were immediately used for hematocrit determination, and the remaining blood was stored at 4°C for subsequent hemoglobin analysis. A 1-g portion of the liver was homogenized in 5 volumes of buffer containing 10 mmol/L HEPES (pH 7.5), 40 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 1 mmol/L dithiothreitol, and 0.32 mol/L sucrose with protease inhibitors (0.1 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 1 mmol/L dithiothreitol, and 0.5% Nonidet P-40). The cell lysate samples were preincubated in the presence of 3% 2-mercaptoethanol for 30 min at 4°C to separate the soluble from the insoluble protein fraction. After washing, the pellets were resuspended in 5 volumes of buffer containing 10 mmol/L HEPES (pH 7.5), 10 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 50 mmol/L β-glycerophosphate, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 2 mmol/L benzamidine, 100 mg/L leupeptin and pepstatin, 250 mg/L soybean trypsin inhibitor, 0.2 mmol/L phenylmethylsulfonyl fluoride, 24 mg/mL P-nitroanilinobenzonate, and 0.5% Nonidet P-40. The cell lysate was centrifuged at 16,000 × g for 10 min at 4°C, and the supernatant was stored at −70°C. For both tissue extracts and cell lysates, the total soluble protein concentration was determined using a commercial reagent (Coomassie Plus, Pierce) based on the method of Bradford (29).

**Ferritin and TfR abundance.** Supernatant samples from each treatment group were subjected to electrophoresis and immunoblotting as described previously (7,30,31). In brief, a 10–20% SDS-polyacrylamide gradient gel was used to separate ferritin and TfR. Proteins were transferred electrophoretically to nitrocellulose paper and incubated separately overnight with a 1:5,000 dilution of each primary polyclonal ferritin and TfR antibody. Each membrane was washed and incubated with goat anti-rabbit IgG horseradish peroxidase secondary antibody for 1 h at room temperature followed by chemiluminescent detection. SigmaGel software (SPSS) was used for densitometric analysis of the film.

**Analysis of IRE.** IRE RNA-binding activity was determined utilizing an electrophoretic mobility shift assay (32,33). To determine endogenous IRE RNA-binding activity, 1 mmol/L 32P-IRE was incubated with tissue lysate (0.5 μg protein) for 10 min at room temperature, and aliquots were immediately loaded onto a 4% nondenaturing gel for separation of bound and unbound IRE. To determine total IRP RNA-binding activity, lysate samples were preincubated in the presence of 3% 2-mercaptoethanol for 10 min at room temperature prior to the initiation of the IRE binding reaction. During preliminary studies, we determined that this level of reductant was the optimal concentration to completely convert inactive IRP to the active RNA-binding form. Gels were dried and exposed to film, and bands were excised for subsequent liquid scintillation counting to quantify the bound and unbound IRE.

**Determination of iron and vitamin A status.** Hematocrit capillary tubes were filled with whole blood, sealed, and stored at 4°C for <2 h prior to standard analysis by centrifugation. Whole blood samples were used to determine hemoglobin concentrations using a commercial kit (Hemoglobin B Test, Wako Pure Chemical) based on established methods (34,35). Hepatic nonheme iron content was measured using the colorimetric assay described by Reddy and Cook (36). Frozen liver samples were analyzed for vitamin A content by the HPLC method described previously (37).

**Statistical analysis.** All statistical analyses were accomplished using SigmaStat software (SPSS). Mean values from treatment groups were expected, rats fed an −Fe diet exhibited only a slight decrease in hematocrit and no consistent change in hemoglobin concentration, liver nonheme iron concentration was depleted by >50% for all 4 −Fe groups (Table 1). Neither a lack of vitamin A in the diet nor supplementation with atRA had an effect on any of the indices of iron status. Similarly, rats fed the −A diet exhibited a severe depletion in hepatic vitamin A stores (Table 2). Interestingly, iron deficiency alone resulted in the accumulation (40–65%) of vitamin A in the liver. Taken together, these nutritional indices and consistent growth across treatment groups indicate that rats were in the relatively early stages of deficiency.

**Results**

**Nutritional status of rats.** As expected, the rats were moderately deficient in iron and vitamin A. Rats did not differ in weight gain over the experimental period regardless of treatment group (data not shown), supporting the evaluation that they were moderately deficient in vitamin A and iron. Although rats receiving an −Fe diet exhibited only a slight decrease in hematocrit and no consistent change in hemoglobin concentration, liver nonheme iron concentration was depleted by >50% for all 4 −Fe groups (Table 1). Neither a lack of vitamin A in the diet nor supplementation with atRA had an effect on any of the indices of iron status. Similarly, rats fed the −A diet exhibited a severe depletion in hepatic vitamin A stores (Table 2). Interestingly, iron deficiency alone resulted in the accumulation (40–65%) of vitamin A in the liver. Taken together, these nutritional indices and consistent growth across treatment groups indicate that rats were in the relatively early stages of deficiency.

**Diminished hepatic ferritin abundance by iron deficiency was partially restored by atRA supplementation.** As expected, rats fed an −Fe diet for 5 wk exhibited hepatic ferritin levels that were virtually undetectable (Fig. 1A). However, atRA supplementation increased ferritin abundance in −Fe rats
as well as in the iron-adequate groups, regardless of vitamin A status. The ability of atRA to increase ferritin abundance was also demonstrated in HepG2 cells in a dose-dependent manner (Fig. 1B), where ferritin levels were ~2-fold greater at the highest atRA dose (50 μmol/L).

Supplementation with atRA decreased TfR abundance in the liver. Neither a lack of vitamin A nor a lack of iron had a significant effect on hepatic TfR abundance. However, −Fe rats treated with atRA exhibited a significant decrease in hepatic TfR abundance compared with control values (Fig. 2). Taken together, rats receiving atRA exhibited a 40% decrease in TfR abundance (P = 0.028).

Elevated IRP RNA-binding activity in rats fed an −Fe diet was diminished by atRA supplementation. Regardless of vitamin A status, iron deficiency significantly increased the RNA-binding activity of IRP, as would be expected (Fig. 3). However, atRA supplementation maintained hepatic IRP RNA-binding activity in both −Fe groups at a level that was not significantly (P = 0.139) different from control values. In support of this finding, IRP RNA-binding activity was also diminished in HepG2 cells treated with 50 μmol/L atRA, a dose that increased ferritin abundance (Fig. 1B), and in HepG2 cells treated with the iron chelator, desferrioxamine (data not shown).

Discussion

An interaction between vitamin A and iron has been recognized for some time in both human and animal studies (2–24). To date, it is clear that these 2 essential nutrients interact to modulate each other’s normal function and metabolism; however, little is known about the mechanistic basis for this relation. This study demonstrated that regulation of IRP by vitamin A status and atRA supplementation might in part represent a mechanistic basis for this nutrient-nutrient interaction. Understanding the mechanistic relation between vitamin A and iron is critical because, worldwide, they represent the 2 most prevalent nutritional deficiencies.

We showed that many of the expected changes owing to iron deficiency were reversed by treatment with atRA. Both the rat and cell culture studies demonstrated that atRA has the ability to increase ferritin abundance, a finding that was attributed at least in part to decreasing IRP RNA-binding activity in −Fe rats. This was supported by our finding that atRA clearly decreased TfR abundance. Thus, it appears that atRA can modulate IRP RNA-binding activity, resulting in the divergent regulation of ferritin and TfR. It is also interesting that iron deficiency resulted in a significant increase in hepatic vitamin A content, confirming the observations reported by others (6,16).

Most of our findings are in agreement with earlier studies that examined this vitamin A and iron interaction. However, a key and novel aspect to our study design was to focus on the early events, such as altered IRP function, that might result in changes in iron homeostasis, as well as provide a supplemental source of vitamin A in the form of atRA, to determine its effectiveness at preventing changes resulting from a deficiency of vitamin A and/ or iron. In earlier studies with marginal vitamin A deficiency and iron restriction, researchers found that a lack of iron significantly reduced growth, hematocrit, hemoglobin, and liver iron, whereas liver TfR, ferritin, and retinol concentrations were significantly reduced growth, hematocrit, hemoglobin, and liver iron, whereas liver TfR, ferritin, and retinol concentrations were elevated. (16). Overall, vitamin A deficiency alone had little effect on iron homeostasis. In contrast, Kelleher and Lomondal (7) demonstrated that vitamin A deficiency resulted in a decrease in hepatic iron and TfR, whereas ferritin was unaffected. Because the translational regulation of key proteins involved in iron homeostasis is mediated by IRP, our results extended this earlier research by focusing on a potential mechanistic basis for the relation between vitamin A status and iron homeostasis.

### TABLE 2

<table>
<thead>
<tr>
<th>Liver vitamin A, nmol/g</th>
<th>Control</th>
<th>−A</th>
<th>−Fe</th>
<th>−Fe/−A</th>
<th>P-values</th>
<th>Diet</th>
<th>atRA</th>
<th>Diet × atRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>−atRA</td>
<td>198 ± 37</td>
<td>0.6 ± 0.3</td>
<td>327 ± 23</td>
<td>0.2 ± 0.1</td>
<td>&lt;0.001</td>
<td>0.902</td>
<td>0.237</td>
<td></td>
</tr>
<tr>
<td>+atRA</td>
<td>219 ± 25</td>
<td>0.5 ± 0.4</td>
<td>307 ± 49</td>
<td>0.6 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (n = 6). Values without a common letter differ, P < 0.05.
Moreover, the changes reported here with IRP RNA-binding activity are consistent with the known divergent regulation of hepatic TfR and ferritin. The differences noted in these studies (7,16), as well as others (6,18,22,24), likely reside in the overall experimental design, including the animal/cell culture model, tissue/cell type, degree of deficiency, and length of experimental period. Our goal was to focus on creating a hepatic loss of vitamin A and iron but minimize any subsequent anomalies in growth that occurred as the deficiencies became more severe. By designing the study to focus on the early events involved in the interaction between vitamin A and iron, our ability to determine the initial basis for altered regulation of iron homeostasis was enhanced.

Although it is clear that atRA appears to modulate the RNA-binding activity of IRP, much remains to be explored about how this is accomplished. Typically, atRA exerts its action at the transcriptional level through the activation of nuclear retinoic acid receptors and the subsequent binding to retinoic acid response elements as a means to induce gene expression. Because the total (i.e., 2-mercaptoethanol-inducible) IRP RNA-binding activity was not altered across treatment groups, nor was there a discernible change in IRP1 abundance as determined by Western blotting (data not shown), it did not appear that changes in the expression of IRP was a factor in this study. Moreover, it did not appear that atRA significantly increased the hepatic nonheme iron concentrations in Fe rats as a means to diminish IRP RNA-binding activity. Nonetheless, we cannot rule out the possibility that atRA regulates the expression of other proteins that may play a role in regulating IRP function. Besides intracellular iron concentrations, there are many factors that can activate and inactivate IRP, including oxidative stress, hypoxia, and phosphorylation (32,33,38–40). Likewise, it is plausible that the increase in ferritin abundance by atRA may reflect both a decrease in IRP function (translational control) and transcriptional regulation. This was shown to be a viable mechanism for the increase in ferritin expression by atRA in neuronal tissue/cells (24). Lastly, in this study, we have not determined the relative contribution of IRP1 and IRP2 to the observed changes in RNA-binding activity. IRP1 and IRP2 both regulate IRE-dependent translation of proteins involved in iron homeostasis, although they exhibit key differences in their response to changes in intracellular iron concentrations. Based on our preliminary studies using an IRP1 antibody and performing supershift assays to separate IRP1- and IRP2-dependent IRE binding, it appears that most of the IRP activity we reported here is IRP1, as would be expected for hepatic tissue (data not shown). Future studies in our laboratory will be directed at more fully understanding the mechanistic basis for modulation of IRP function by atRA, either directly or indirectly.

In summary, we found that vitamin A status and atRA modulated iron homeostasis through an IRP-dependent mechanism. To our knowledge, this is the first report demonstrating that atRA is a signal to decrease the RNA-binding activity of IRP, thereby altering the abundance of key proteins involved in iron homeostasis. This new knowledge represents an important mechanistic observation that will be essential in further understanding the metabolic interaction between vitamin A and iron.
Acknowledgment
We thank Dr. Sherry Tanumihardjo, University of Wisconsin, Madison, for the hepatic vitamin A analysis.

Literature Cited