Vitamin A Repletion in Rats with Concurrent Vitamin A and Iodine Deficiency Affects Pituitary TSHβ Gene Expression and Reduces Thyroid Hyperstimulation and Thyroid Size

Ralf Biebinger, Myrtha Arnold, Wolfgang Langhans, Richard F. Hurrell, and Michael B. Zimmermann

Abstract

Concurrent vitamin A (VA) deficiency (VAD) and iodine deficiency (ID) are common in developing countries. VAD has effects on thyroid metabolism that may be dependent on iodine status. The aim of this study was to investigate the effect of VA supplementation (VAS) and/or dietary iodine repletion, alone and in combination, on the thyroid-pituitary axis in rats with concurrent VAD and ID. Weanling rats (n = 96) were fed diets deficient in VA and iodine or sufficient in both (control), for 30 d. Subsequently, deficient rats were repleted with iodine and/or single VAS or remained deficient for 10 d. Serum retinol (SR), thyroid hormones, serum thyrotropin (TSH), pituitary TSHβ mRNA expression level, and thyroid weight were measured. High-dose VAS restored SR concentrations to normal in both iodine-deficient and iodine-sufficient rats. Despite continuing VAD, provision of the iodine-sufficient diet entirely reversed the abnormalities of the pituitary-thyroid axis produced by VAD and ID. In iodine-sufficient rats, VAS had no discernible effects on the pituitary-thyroid axis; in iodine-deficient rats, VAS reduced pituitary production of TSH and thyroid stimulation but had no discernible effects on circulating thyroid hormone concentrations. Primary hypothyroidism in rats with concurrent VAD and ID does not reduce the efficacy of VAS, nor does VAD reduce the efficacy of dietary iodine to correct pituitary-thyroid axis dysfunction due to ID. In concurrent VAD and ID, VAS, independent of iodine repletion, reduces thyroid hyperstimulation and size, an effect likely mediated through the effects of VA on pituitary TSHβ gene expression.


Introduction

Vitamin A (VA) deficiency (VAD) and the iodine deficiency (ID) disorders (IDD) are major global public health problems, affecting >30% of the population worldwide (1). VAD is particularly common in Africa and Southeast Asia, where ~250 million preschool children are VA deficient (2). IDD affects one-third of school-age children worldwide and is the leading cause of preventable mental retardation (3). In many regions of the developing world, young women, infants, and children suffer from both VAD and IDD (4,5). Periodic high-dose oral VA supplementation (VAS) is the recommended strategy to control VAD in affected populations, many of whom are also iodine deficient. Conversely, many VA-deficient children in the developing world are consuming iodized salt.

VAD has multiple effects on thyroid metabolism that may be dependent on iodine status. In severely VA-deficient rats, thyroidal iodine uptake is decreased, thyroglobulin synthesis is impaired, and thyroid size is increased (6,7). Peripherally, VAD increases free and total circulating thyroid hormone (8–11) and binding of transthyretin to retinol-binding protein decreases VA turnover and enhances VA delivery (12,13). In the pituitary, both thyroxine (T4) and retinoic acid can suppress thyrotropin (TSH) synthesis, as both the thyroid hormone-activated thyroid receptor and the 9-cis-retinoic acid-activated retinoid X receptor (RXR) suppress transcription of the pituitary TSHβ gene by occupying half-sites on the promoter region of the gene (14,15).

In a recent study in Morocco, VAD in goitrous children was associated with increased TSH stimulation and thyroid size and...
reduced risk for hypothyroidism (5). VAS given with iodized salt reduced the goiter rate compared with iodized salt given alone (5). Therefore, the aim of this study was to investigate the effect of VAS and dietary iodine repletion, alone and in combination, on the thyroid-pituitary axis in rats with concurrent VAD and ID.

**Material and Methods**

**Rats and diets.** The Veterinary Office of the Department of Health of the Canton of Zurich gave the ethical approval for the study. Male weanling Sprague-Dawley rats (n = 96; Charles River) at 21 ± 3 d of age were housed individually in random order in stainless steel cages with grated stainless steel floors. The rats were kept under controlled conditions at 21°C and 55% humidity in a 12-h light-dark cycle. Rats consumed Millipore water (Milli-Q UF Plus, Millipore) ad libitum. The rat diets were prepared by Dyets following the AIN-93G purified rodent diet guidelines (16) and were based on L-amino-acids to avoid iodine contamination from casein as a major protein source.

**Study design.** During a depletion period of 30 d, rats (n = 72) consumed a VA- and iodine-deficient diet. Eight rats fed the VA- and iodine-deficient diet were matched with a pair-fed group (n = 8) consuming a VA- and iodine-sufficient diet. The control group (n = 16) consumed ad libitum a VA- and iodine-sufficient diet. After the 30-d depletion period, 8 control rats, the 8 rats from the VA- and iodine-deficient group (VAD + ID 30 d), and the pair-fed group were killed. The remaining VA- and iodine-deficient rats (n = 64) were randomly divided into 4 treatment and 4 pair-fed groups. The treatment groups received the following diets for 10 d: 1 group was fed a VA- and iodine-deficient diet (VAD + ID), one was fed a VA-deficient but iodine-sufficient diet (VAD + IS group), 1 was fed the VA- and iodine-deficient diet and received oral VAS (VAD + ID + VAS group), and one was fed a VA-deficient and iodine-sufficient diet and received oral VAS (VAD + IS + VAS group). Rats in the VAD + IS, VAD + ID, VAD + ID + VAS, and VAD + IS + VAS groups were individually matched to pair-fed rats fed the control diet (n = 8 in each of the 4 pair-fed groups). The rats in the 2 supplementation groups received 60,000 IU (18 mg) of retinol in the form of retinyl-palmitate via oral gavage. Figure 1 gives an overview of the study design.

All rats were handled daily to reduce stress at blood collection and killing. Food intake was recorded daily and body weight was measured 3 times/wk. Blood (750 μL) was collected by tail vein incision (17) and serum stored at −20°C. After the rats were killed by decapitation, the pituitaries were dissected within 60 s and stored in an experienced animal technician. Thyroids were weighed immediately after dissection. The pituitaries were dissected within 60 s and stored in RNalater solution (RNeasy Protect Mini kit, catalog no. 74104, Qiagen) at −80°C.

**Laboratory analysis.** Free and total T4 and triiodothyronine (FT4, TT4, FT3, and TT3) and serum TSH were measured using immunochemiluminescent assays (IMMULITE Buehlmann Laboratories AG). Serum retinol (SR) was measured with an optimized HPLC method (18,19) using retinyl-acetate as an internal standard and a commercially available reference material from the National Institute of Standards and Technology (SRM 986c) as an external standard. For measurement of TSHβ mRNA, total RNA extraction was done using the RNeasy Protect Mini kit, with samples stored after each working step at −80°C. To avoid DNA contamination, a DNA on column digestion was carried out during the total RNA extraction (RNase-free DNase set, catalog no. 79252, Qiagen). TSHβ mRNA expression levels were measured with SYBR-Green 1-step RT-qPCR (QuantiTect SYBR Green RT-PCR kit, catalog no. 204243, Qiagen) using an ABI Prism 7900 Thermocycler. The specific primer pairs were purchased from Qiagen. TSHβ mRNA expression levels were normalized with a housekeeping gene (Hrpt). For each rat, 5 replicates for the target gene and 3 replicates for the housekeeping gene were performed.

**Statistical analysis.** We used SPSS 13.0 and Microsoft Excel 2002 for data processing and analysis. Data were analyzed by 1-way ANOVA. Post-hoc comparisons were done using the Bonferroni test to detect significant differences among means. Differences were considered significant at P < 0.05. Values in the text are means ± SD.

**Results**

The pair-fed groups did not differ from the control group in body weight or in any of the measured biochemical variables (data not shown). Body weight did not differ among the groups and after 40 d was 322 ± 24 g. The measured biochemical variables in the control did not differ at 30 and 40 d (data not shown), so the 40-d control data were used for the analyses.

SR concentrations were lower in the VAD + ID 30 d, VAD + IS, and VAD + ID groups than in the 2 + VAS and control groups (Table 1, P < 0.001). Concentrations in the former 3 groups did not differ from one another, nor did concentrations in the latter 3 groups.

**Figure 1** Description of the general study design showing the groups of rats during depletion and repletion phases of the study.
Compared with the control group, serum TSH concentration (Fig. 2A) and thyroid weights (Fig. 2B) were greater in VAD/30 d and VAD+/ID (P < 0.01). Serum TSH (P < 0.001) and thyroid weights (P < 0.05) were greater in VAD+/ID compared with VAD+/ID 30 d. Serum TSH concentrations and thyroid weights of VAD+/IS and VAD+/IS+VAS were not significantly different from controls. Serum TSH (P < 0.001) and thyroid weights (P < 0.05) were greater in VAD+/ID compared with VAD+/ID+VAS. Serum TSH and thyroid weights did not differ between VAD+/ID+VAS and VAD+/ID 30 d.

Serum concentrations of TT3 and FT3 did not differ among any of the groups (Table 1). Serum TT4 and FT4 concentrations (Table 1) were lower in the iodine-deficient groups (VAD+/ID 30 d, VAD+/ID, and VAD+/ID+VAS) compared with the iodine-sufficient groups (control, VAD+/IS, VAD+/IS+VAS) (P < 0.001). Serum TT4 and FT4 concentrations did not differ among the iodine-sufficient or among the iodine-deficient groups. Among all groups, TT3 concentrations were not significantly different, with the exception that the VAD+/IS had significantly greater TT3 concentrations than VAD+/ID and VAD+/ID+VAS. FT3 concentrations were significantly lower in VAD+/ID compared with control (P < 0.05).

Relative expression levels for TSHβ mRNA were higher in the iodine-deficient groups compared with the iodine-sufficient groups (P < 0.001). Expression did not differ between the VAD+/ID+VAS and VAD+/ID groups.

### Discussion

At the end of the 30-d depletion period, combined dietary deficiency of VA and iodine resulted in: 1) moderate VAD, with SR concentrations reduced by ≈35%; and 2) primary hypothyroidism, with increases in serum TSH, pituitary TSHβ mRNA expression and thyroid size, and a decrease in thyroid hormone concentrations. The main findings of the 10-d repletion experiment were: 1) a single high dose of VA was sufficient to return SR concentrations to normal, in both iodine-deficient and iodine-sufficient rats; 2) despite continuing VAD, provision of an iodine-sufficient diet entirely reversed the abnormalities of the pituitary-thyroid axis produced by VA and iodine depletion; 3) a high dose of VA in iodine-deficient rats had no discernible effects on the pituitary-thyroid axis; and 4) a high dose of VA in iodine-deficient rats slightly reduced pituitary production of TSH and reduced stimulation of the thyroid but had no discernible effects on circulating thyroid hormone concentrations.

First, the high dose of VA was sufficient to return SR concentrations to normal, regardless of iodine status. The normalization of SR suggests VA repletion, although we did not measure liver retinol concentration to confirm this. Thus, ID and primary hypothyroidism do not impair VA repletion with high doses of oral VA. These data suggest that high-dose oral VA treatment will likely be effective in humans, even in areas of ID and endemic goiter.

Second, provision of the iodine-sufficient diet entirely reversed the abnormalities of the pituitary-thyroid axis produced by VA and iodine depletion, regardless of VA status. These data differ somewhat from previous animal studies where severe VAD adversely affected the pituitary thyroid axis, even if iodine supply was adequate. These effects included reduced thyroid iodine uptake (20), impaired synthesis of thyroglobulin and coupling of iodotyrosine residues to form thyroid hormone (6), reduced hepatic conversion of T4 to T3 (6,21) and TT3, FT3, and FT4 (6,8). The differences between our findings and those of previous studies may be explained by differences in the severity of VAD. For example, Ingenbleek (6) used a depletion period twice as long as ours and SR concentrations in that depletion study were only 10% of those in the control group. Our

### Table 1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>VAD+/ID 30 d</th>
<th>VAD+/ID</th>
<th>VAD+/IS</th>
<th>VAD+/IS+VAS</th>
<th>VAD+/ID+VAS</th>
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<tbody>
<tr>
<td>SR, μmol/L</td>
<td>1.94 ± 0.34a</td>
<td>1.11 ± 0.08b</td>
<td>1.03 ± 0.20b</td>
<td>0.89 ± 0.28b</td>
<td>1.94 ± 0.31a</td>
<td>1.67 ± 0.16b</td>
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<tr>
<td>TT4, μg/L</td>
<td>0.67 ± 0.06a</td>
<td>0.12 ± 0.03b</td>
<td>0.13 ± 0.08b</td>
<td>0.85 ± 0.13b</td>
<td>0.70 ± 0.09b</td>
<td>0.17 ± 0.18b</td>
</tr>
<tr>
<td>FT4, ng/L</td>
<td>0.36 ± 0.07a</td>
<td>0.21 ± 0.06b</td>
<td>0.10 ± 0.02b</td>
<td>0.41 ± 0.12b</td>
<td>0.31 ± 0.03b</td>
<td>0.12 ± 0.07b</td>
</tr>
<tr>
<td>TT3, ng/L</td>
<td>12.31 ± 1.59ab</td>
<td>12.44 ± 2.24b</td>
<td>9.97 ± 2.29b</td>
<td>13.50 ± 1.21ab</td>
<td>12.65 ± 2.33b</td>
<td>10.08 ± 1.91b</td>
</tr>
<tr>
<td>FT3, ng/L</td>
<td>6.08 ± 1.54a</td>
<td>4.60 ± 1.12ab</td>
<td>4.10 ± 1.13ab</td>
<td>5.88 ± 0.93ab</td>
<td>6.05 ± 0.83ab</td>
<td>4.64 ± 1.50ab</td>
</tr>
<tr>
<td>TSHβ mRNA2</td>
<td>6.54 ± 2.66a</td>
<td>7.19 ± 1.43a</td>
<td>1.02 ± 0.20b</td>
<td>1.28 ± 0.25b</td>
<td>6.30 ± 1.26a</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 8. Means in a row with superscripts without a common letter differ, P < 0.05.

2 Expression of VAD+/ID 30 d is relative to control rats after 30 d.
data suggest iodized salt programs are likely to be effective in controlling IDD, even in areas of moderate VAD.

Third, high dose oral VA in the iodine sufficient rats had no discernible effects on the pituitary-thyroid axis. These data differ somewhat from previous animal studies where much higher doses of VA than used in our study were administered to iodine-sufficient rats and the pituitary thyroid axis was altered. Morley et al. (11) gave pharmacologic doses of retinyl palmitate to rats (45 mg as retinyl palmitate, 3-5×/wk) and showed a decrease in thyroid size and serum T3 and T4, an increase in thyroidal iodine uptake, and hepatic conversion of T4 to T3 but no alteration in basal TSH or its response to thyroid releasing hormone. Other studies giving very high doses of retinol or retinoic acid to rats (up to 12 mg/d for 40 d) have reported decreased pituitary TSH content and decreased serum TT3 and TT4 (22,23). Evidence of a similar effect in humans was suggested by the development of hypothyroidism after treatment with a synthetic retinoid that specifically binds to the RXR in patients with T-cell lymphoma (24). In this study, the dose of VA was chosen to provide ≈50 mg/kg body weight to the rats. Compared with the doses previously used, this dose is somewhat closer to but still higher than, that recommended for treating VAD in preschool children. For example, 3–4 y-old children weighing 13 kg and receiving an oral dose of 200,000 IU (60 mg) VA would receive a dose of 5 mg/kg body weight. Thus, these data suggest the high dose VAS in iodine-sufficient areas is unlikely to affect thyroid function.

Finally, a high dose of VA in the iodine-deficient rats reduced pituitary production of TSH and reduced stimulation of the thyroid (as reflected in reduced thyroid size) but had no discernible effects on circulating thyroid hormone concentrations. These data are consistent with a previous report in hypothyroid rats, where pharmacologic doses of retinoic acid reduced basal TSH secretion and TSH response to thyroid releasing hormone (25). There are several potential explanations for this effect. Control of TSH production by the pituitary depends on 2 main factors; the binding of the thyroid hormone receptor, which is activated by T3 and T4, and the binding of the RXR, which is activated by retinoic acid (14). Both receptors suppress transcription of the pituitary TSHβ gene by occupying half-sites on the promoter region of the gene; thus, VA status modulates TSH production (14,26,27). In this study, VAS may have suppressed TSHβ mRNA expression, decreased serum TSH concentrations, and reduced thyroid hyperstimulation. Reduced TSH stimulation might have been expected to reduce thyroid hormone production and thereby reduce circulating levels of thyroid hormone, but circulating levels of T3 did not fall. This implies that either the sensitivity of the thyroid to TSH improved with VA repletion or metabolism of circulating thyroid hormone was altered to maintain T3 levels. Previous studies provide data to support both of these mechanisms. For example, Ingenbleek (6) reported combined VA and ID impaired thyroid hormone synthesis by reducing radiiodine incorporation into thyroglobulin and increasing the (moniodothyronine+diiodothyronine)/(T3+T4) ratio in the thyroid; these adverse effects were reversed by VA treatment. On the other hand, Morley et al. (11) found high-dose VAS to iodine-sufficient rats increased hepatic conversion of T4 to T3. In this study, it is possible that the effects of VAS on circulating thyroid hormones would have been different if the dose and/or duration of the repletion period were increased.

In summary, in rats with concurrent moderate VAD and ID, primary hypothyroidism due to ID does not reduce the efficacy of high doses of oral VA to increase SR, nor does VAD reduce the efficacy of dietary iodine to correct pituitary-thyroid axis dysfunction due to ID. However, VAS has effects on the pituitary-thyroid axis that are dependent on the iodine status of the rats. Given alone, high-dose VAS in combined VAD and ID reduces thyroid stimulation by TSH and reduces risk for goiter.

Literature Cited
23. Drill VA. Interrelations between thyroid function and vitamin metabolism. Physiol Rev. 1943;23:355–79.


