Serum iodothyronine concentrations in intestinally decontaminated rats treated with a 5'-deiodinase type I inhibitor 6-anilino-2-thiouracil

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Enteric bacteria have been postulated to have a role in thyroid economy by promoting the hydrolysis of thyroid hormone conjugates of biliary origin, thus permitting the absorption and recycling of thyroxine (T₄) and triiodothyronine (T₃). An enterohepatic circulation of T₄ might be more pronounced under conditions in which type I iodothyronine deiodinase activity (5'D-I) is inhibited, because this augments the accumulation of T₃ sulfate conjugates in bile. This residual of increased gut reabsorption of T₃ might explain, at least in part, the failure of serum T₃ values to decrease appreciably when marked reductions in peripheral 5'D-I activity are induced by selenium deficiency or 6-anilino-2-thiouracil (ATU) administration. Thus, studies were performed to determine the effect of intestinal decontamination, in the absence and in the presence of 5'D-I inhibition, on plasma T₄ and T₃ concentrations. Groups of adult male rats received either enteric antibiotics or no antibiotics for 12 days and then, in half of the rats in each group, treatment for 10 days with ATU, a 5'D-I inhibitor that does not affect thyroid hormone synthesis. The activity of intestinal arylsulfatase and arylsulfotransferase, enzymes that catalyze hydrolysis of thyroid hormone conjugates, was reduced markedly by approximately 87% in rats that received antibiotics, regardless of whether or not they also received ATU. The ATU treatment markedly inhibited the activity of the ATU-resistant conjugate in the intestinal lumen. In non-antibiotic-treated rats, control 399 ± 32 U/mg protein (mean ± SEM); ATU = 152 ± 17; antibiotics = 351 ± 29; antibiotics + ATU = 130 ± 10; p < 0.01) and significantly increased plasma T₄ and T₃ sulfate (T₄S, T₃S) concentrations (control: T₄S = 2.8 ± 0.4 and T₃S = 6.7 ± 1.3 ng/dl; ATU: T₄S = 6.2 ± 1.4 and T₃S = 10.6 ± 2.1 ng/dl; antibiotics: T₄S = 1.8 ± 0.2 and T₃S = 3.6 ± 1.0 ng/dl; antibiotics + ATU: T₄S = 6.8 ± 0.7 and T₃S = 9.7 ± 1.8 ng/dl; p < 0.05). The ATU treatment was associated with a significant increase in plasma T₄ and T₃ concentrations but did not affect plasma T₃ concentrations, and intestinal decontamination did not alter these ATU-associated effects on circulating thyroid hormones. These results suggest that anaerobic enteric bacteria in the rat do not have an important role in recycling of thyroid hormones, either under normal conditions or in circumstances where 5'D-I activity is markedly reduced, and that increased gut absorption of T₃ from T₃S cannot explain the near-normal serum T₃ values found when peripheral 5'D-I activity is markedly decreased.

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Thyroxine (T₄) is secreted by the thyroid and is considered to be prohormone. It is metabolized by outer or phenolic ring (5') deiodination (5'D-I) to 3,5,3'-triiodothyronine (T₃), the biologically active form of the thyroid hormone, and to the biologically inactive diiodothyronine 3,3',5'-triiodothyronine (reverse T₃, rT₃) (1, 2) by inner ring or 5-deiodination. In humans, a relatively large fraction of the daily T₃ production is derived from peripheral conversion of T₄ to T₃ (3). Triiodothyronine is also generated from circulating T₄ in the rat but the fraction that is produced by this pathway is less than in humans, because secreted T₃ is a major source of serum T₃ in the rat (3). It has been shown in vitro and in vivo that T₃ is metabolized by three major pathways: glucuronidation, sulfation and direct deiodination (4). While T₃ glucuronide (T₃G) is a relatively stable conjugate and is excreted into the bile, T₃ sulfates (T₃S) are rapidly deiodinated, primarily in the liver, by 5'D-I (4, 5). In normal rats, T₃S is found in the bile only in small amounts. However, in thyroidectomized rats or rats treated with propylthiouracil (PTU), resulting in decreased 5'D-I activity, T₃S, measured by specific RIAs, accumulates in the bile and plasma (5–8). Both T₃G and T₃S are hydrolyzed in the gut by glucuronidases and sulfatases, enzymes produced by intestinal
bacteria (9–11). Hydrolysis of T₃ and T₄ conjugates is probably initiated at the level of the cecum and absorption of T₃ and T₄ occurs only in their unconjugated forms (12). Arylsulfotransferases and arylsulfatases are the principle intestinal bacterial enzymes that metabolize phenolic sulfate esters in humans and rats (13, 14).

De Herder et al. have suggested that intestinal microflora play an important role in the enterohepatic circulation of T₃ in the rat (11). However, the importance of this process to overall thyroid hormone economy has not been established fully. The present study was designed to determine the role that gut flora might have in the enterohepatic recycling of conjugated thyroid hormones to serum T₃ in normal rats and in rats treated with 6-anilino-2-thiouracil (ATU); ATU is a PTU analog that, like PTU, inhibits 5'D-I in the liver but does not affect thyroid hormone synthesis (15). Furthermore, we have suggested the possibility that hydrolysis of T₃S in the gut, generating T₃, with subsequent absorption across the gut mucosa, may play a role in maintaining near-normal serum T₃ values when 5'D-I activity is inhibited (selenium deficiency, ATU administration) with subsequent elevations in T₃S (3).

Materials and methods

Animals and diet

Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used and fed 5008 rat purina chow throughout two experiments having the same design. Rats were housed in stainless-steel cages and water was available ad libitum. Intestinal decontamination was carried out by treatment of the rats with antibiotics in the drinking water (1 g of ampicillin, 1 g of neomycin and 1 g of polymixin B per liter) (11). The rats were divided into four groups and each group consisted of 10 rats. The first group (A) received no specific treatment. Rats in this group were fed rat purina chow and received tapwater throughout the 22-day experiment. The second group (B) received antibiotics in the drinking water. The third group (C) was given tapwater and 0.1% ATU was added to the diet during the last 10 days of the experiment. The fourth group (D) received antibiotics in the drinking water and 0.1% ATU was added to the diet for the last 10 days of the experiment. All rats were sacrificed by decapitation on day 22. Feces were collected on day 12 of the experiment for bacterial colony counts. Blood, feces, thyroid and liver were collected on the day of sacrifice for measurement of 5'D-I activity, serum hormone values and fecal sulfatase activities. Portions of livers were homogenized immediately in 4 vols (w/v) of 250 mmol/l sucrose, 20 mmol/l HEPEs buffer (pH 7.0), 1 mmol/l EDTA and 1 mmol/l DTT. Feces were homogenized in 10 vols (w/v) of 0.01 mmol/l TRIS·HCl (pH 7.0), centrifuged at 1000 g for 10 min and the resulting supernatant fluid was used for enzymatic assay. All tissue and fecal samples and sera were stored at −20°C until assayed.

Materials

Ampicillin, neomycin and polymixin B were supplied by Sigma Chemical Co. (St Louis, MO); [125I]rT₃ was obtained from NEN (Boston, MA) and ATU was synthesized as described previously (16).

Enzyme assays

Liver and thyroid 5'D-I activity was measured by the release of 125I from 10 µmol/l rT₃ in the presence of 20 mmol/l DTT at 37°C (17). All samples were run in duplicate and results were expressed as U/mg protein: 1 unit of enzyme activity represents the release of 1 pmol/min.

The relationship between liver 5'D-I inhibition by 0.1% ATU in the diet and the substrate concentration employed for measuring liver 5'D-I was tested. The enzyme assay conditions were otherwise identical to that described above. Inhibition of 5'D-I activity was slightly but not significantly greater when rT₃ substrate concentrations of 1 µmol/l or 2 nmol/l were employed than when the rT₃ substrate concentration was 10 µmol/l (percentage inhibitions (mean ± SEM) of 64.2 ± 0.8, 64.3 ± 1.3 and 60.2 ± 1.6 for rT₃ substrate concentrations of 2 nmol/l, 1 µmol/l and 10 µmol/l, respectively: N = 8).

Arylsulfotransferase activity was assayed as follows: 0.1 ml of the enzyme solution was added to the assay mixture containing 0.29 ml of 20 mmol/l tyramine, 30 µl of PNS (p-nitrophenyl sulfate) and 0.21 ml of 0.1 mol/l TRIS·HCl (pH 8.0) and the mixture was incubated at 37°C 1 h. The reaction was stopped by the addition of 1 mol/l NaOH (0.4) and absorbance at 405 nm was measured. Arylsulfatase activity was measured under the same conditions without the addition of tyramine. Control samples were measured under the same assay conditions, except that the substrate was added after stopping the reaction (13, 14). Results were expressed as U/g wet feces and 1 unit of enzymatic activity represents the amount required to catalyze the formation of 1 µmol p-nitrophenol/h at 37°C. Protein was measured by the method of Bradford (18).

Hormone assays

Serum TSH was measured by RIA using materials obtained from the National Pituitary Agency, NIH (Bethesda, MD). Serum T₄ and T₃ concentrations were determined by specific RIAs. Serum rT₃ was measured by RIA using materials from Serono-Baker Diagnostics (Polymedico, Cortlandt Manor, NY). Serum T₃S and T₄S were measured by specific RIAs (19). All hormones were run in duplicate, in the same assay and in random order.

Statistics

The results are presented as means ± SEM. Statistical significance of differences between the groups (p < 0.05)
Results

The results of Experiment 1 are shown in the figures, except that Figure 3 shows the pooled results for experiments 1 and 2 for the serum $T_3$S concentrations. Nearly identical results were obtained in Experiments 1 and 2, except where noted.

Body and thyroid weights (Table 1)

During 22 days of the experiments neither antibiotics nor ATU treatment had an effect on body or thyroid weights.

Effects of intestinal decontamination alone and in combination with ATU treatment on hepatic and thyroid 5’D-I activity

Bacterial colony counts in the feces of rats collected on day 12 were decreased significantly only in the rats that received antibiotics (groups B and D). Arylsulfatase and arylsulfotransferase activities in feces were decreased markedly in the antibiotic-treated groups ($p < 0.001$) (Fig. 1).

The groups of rats that received ATU in the diet (groups C and D) had a marked decrease in hepatic 5’D-I activity ($p < 0.001$) (Fig. 2). This decrease ranged from 63 to 70% in the ATU groups compared to the groups that did not receive ATU. Thyroid 5’D-I activity was measured only in the second experiment and was decreased significantly in the groups that received ATU ($p < 0.001$) (Fig. 2).

Effects of intestinal decontamination alone or in combination with ATU treatment on serum thyroid hormone concentrations

The ATU treatment had a significant effect in elevating serum $T_3$S and $T_4$S concentrations, whereas antibiotic treatment did not influence these iodothyronines ($p < 0.05$) (Fig. 3). Serum $T_4$ and $rT_3$ concentrations were elevated significantly in the ATU-treated groups. The combination of ATU treatment and antibiotics was not consistently associated with a different serum $T_4$ concentration than ATU alone, but serum $rT_3$ concentrations were significantly higher in the group that received ATU and antibiotics compared to the group that received ATU alone (Fig. 4).

There was little variability in mean serum $T_3$ concentrations among the four groups in either experiment. In one experiment, however, serum $T_3$ concentrations were slightly but significantly higher in the antibiotic-treated group compared to the other three

Table 1. Effects of intestinal decontamination alone or in combination with 6-anilino-2-thioracil (ATU) treatment on body and thyroid weights.

<table>
<thead>
<tr>
<th>Group</th>
<th>Rx</th>
<th>Thyroid weight (mg/100 g BW)</th>
<th>Body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>A</td>
<td>Control</td>
<td>6.2 ± 0.3</td>
<td>229 ± 5</td>
</tr>
<tr>
<td>B</td>
<td>Antibiotic</td>
<td>6.1 ± 0.5</td>
<td>233 ± 1</td>
</tr>
<tr>
<td>C</td>
<td>ATU</td>
<td>6.8 ± 0.3</td>
<td>244 ± 5</td>
</tr>
<tr>
<td>D</td>
<td>Antibiotics + ATU</td>
<td>6.6 ± 0.3</td>
<td>236 ± 5</td>
</tr>
</tbody>
</table>

*Values are means ± SEM. Rx =.

![Fig. 1. Effects of intestinal decontamination alone and in combination with 6-anilino-2-thioracil (ATU) treatment on arylsulfatase and arylsulfotransferase activities in the feces. Group A is the control group, group B received antibiotics in the drinking water, group C received ATU treatment for the last 10 days of the experiment and group D received antibiotics in the drinking water and ATU treatment for the last 10 days of the experiment.](image1)

![Fig. 2. Effects of intestinal decontamination alone and in combination with 6-anilino-2-thioracil (ATU) treatment on hepatic and thyroid 5'-deiodinase type 1 (5'D-I) activity. Group A is the control group, group B received antibiotics in the drinking water, group C received ATU treatment for the last 10 days of the experiment and group D received antibiotics in the drinking water and ATU treatment for the last 10 days of the experiment.](image2)
groups. In the other experiment, serum T₃ concentrations were similar in all four groups (Fig. 4).

Serum TSH concentrations were not affected by either ATU or antibiotic treatment (data not shown).

Discussion

The potential sources of circulating serum T₃ in the rat are direct secretion of T₁ from the thyroid by de novo synthesis of T₁ by moniodotyrosine and diiodotyrosine coupling, intrathyroidal and peripheral T₄ to T₁ deiodination by 5′D-I and intestinal reabsorption of T₁ from its conjugates excreted in the bile (3). The absolute contribution of peripheral T₄ to T₃ conversion to the circulating T₃ concentration has been difficult to determine but it is probably less than that of humans (3). It has been shown previously that a marked decrease in 5′D-I activity in the liver and kidney by more than 90% in selenium-deficient intact rats was associated with only a modest or no decrease in the serum T₁ concentration (20–22). In addition, in selenium-deficient, thyroidectomized, T₄-replaced rats the serum T₁ concentration was only decreased by 20% as compared to selenium-supplemented, thyroidectomized, T₄-replaced rats (23). These data are consistent with the finding that in mice lacking 5′D-I the serum T₁ concentration is normal (24, 25). Intrathyroidal 5′D-I deiodination of T₄ to T₃ does not appear to be a major source of circulating T₃, because serum T₁ concentrations were not decreased in the ATU-treated rats despite a 60% reduction in 5′D-I activity. However, Laurberg has reported that intrathyroidal deiodination of T₄ to T₃ accounts for a considerable portion of the T₃ secreted from the dog thyroid (26, 27) and we have also suggested that this pathway of T₃ generation in the thyroid appears to be important in the rat (23).

We have now studied the possible role of gut flora in maintaining near-normal serum T₃ concentration under conditions of a marked reduction in hepatic 5′D-I activity associated with an increase in serum T₃S values. Administration of ATU markedly inhibited hepatic 5′D-I activity, resulting in an increase in serum T₃S and T₄S concentrations. These findings agree with previous studies reporting that PTU or selenium deficiency, which both inhibit 5′D-I activity, increase serum T₃S concentrations due to decreased deiodination of T₃S (6, 22). This increase in the serum T₃S concentration most probably leads to increased availability of the T₃S as a substrate for intestinal bacterial hydrolysis, after its excretion into the gut through the bile. Subsequent desulfation to T₂ which could then be absorbed, might play a role in maintaining near-normal serum T₃ concentrations in spite of decreased T₄ to T₃ conversion. To further explore this hypothesis, rats were decontaminated intestinally with
antibiotics, essentially eliminating the sulfatase activity of gut flora. Administration of ATU induced a marked reduction in 5'D-I activity with little change in serum T₃ values. Serum T₃ concentrations were essentially unchanged in ATU-antibiotic treated rats, ruling against the possibility that increased availability of T₃ generated from T₃S in the gut by fecal sulfatase activities is likely, because these sulfatase enzymes were inhibited. If this pathway was important when 5'D-I activity was inhibited and T₃S increased, absence of gut sulfatase activity would have resulted in a decrease in serum T₃. Fecal glucuronidase activity was not measured in the present study, but was most probably also inhibited in the groups of rats that received antibiotics (11). Intestinal glucuronidase activity is not inhibited completely in decontaminated rats, however, probably because mucosal cells have glucuronidase activity (11). Thus, the present studies suggest that gut flora are likely to have little role in T₃ recycling but leave open the possibility that there is an enterohepatic circulating for T₃ that is mediated by the glucuronidase activity of gut mucosa. The explanation for why the combination of ATU and antibiotics (group D) in one experiment resulted in the highest serum rT₃ values remains unclear.

In conclusion, our results suggest that serum T₃ levels remain normal or near-normal even when peripheral and thyroidal 5'D-I activity is decreased significantly. In addition, the enteric bacterial hydrolysis of T₃S does not appear to play an important role in conserving serum T₃. Whether thyroid secretion of T₃ increases under conditions of 5'D-I inhibition, thus helping to maintain near-normal serum T₃ concentrations, remains to be determined.

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