Do Nonesterified Fatty Acids Displace Thyroxin from Its Plasma Binding Sites in Severe Nonthyroidal Illnesses?

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Severe nonthyroidal illnesses have been associated with increases in nonesterified fatty acids (NEFA) and the dialyzable fraction of thyroxin (T4) in plasma. We have further investigated their possible relationship in severe nonthyroidal illnesses as well as in induced in vivo and in vitro situations involving increased NEFA. We demonstrate that there is no relationship between NEFA and the dialyzable fraction of T4, either in severe nonthyroidal illnesses or in the other situations, unless plasma NEFA concentrations exceed 5 mmol/L. In normal persons or 1.7 mmol/L in nontyroidal illnesses, and that this concentration was not reached in the patients we studied, with one exception. We conclude that NEFA are unlikely to contribute to an inhibition of the binding of T4 to the binding proteins that might be present in plasma of patients with severe nonthyroidal illnesses unless their NEFA concentrations are very high.

Nonesterified fatty acids (NEFA) have long been suspected of being able to displace thyroxin (T4) from the plasma T4-binding proteins, thus causing an increase in the unbound fraction of T4 (1–3). This increase can be measured as an increase in the dialyzable fraction of T4 (DFT4) in the plasma (4). It has been suggested that NEFA in low concentrations displace T4 from albumin and at higher concentrations from both albumin and thyroxin-binding globulin (TBG). Furthermore, it is claimed (4) that, in severe nonthyroidal illnesses, NEFA concentrations are sufficiently increased to cause such an increase in DFT4. However, this claim has been challenged (5).

Therefore, we have measured total NEFA concentrations and eight individual NEFA (myristic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidonic acids) in patients with severe nonthyroidal illnesses and assessed the relationship of each to DFT4. We also studied this relationship during prolonged fasting and in other situations with plasma NEFA concentrations increased, both in vivo and in vitro.

Materials and Methods

We studied nine patients, admitted to the Intensive Therapy Unit with various diagnoses.4 None was known to have thyroid disease or severe head injuries, nor had any received massive blood transfusion. A drug record was kept particularly to identify those drugs that might affect either the hypothalamus–pituitary–thyroid axis or the binding of T4 to binding proteins. Blood was sampled, with lithium heparin as anticoagulant (125 int. units/10 mL), at least daily from four to 28 consecutive days. In vivo increases in NEFA were studied in two situations.

In one, a volunteer fasted for 27 h, taking nothing but fluids, including coffee without cream or sugar. Blood was sampled and placed in lithium heparin tubes at 0, 2, 20, 24, and 27 h into the fast and at 21 h after the end of the fast.

The second study involved a patient undergoing a thyro- liberin (thyrotropin-releasing factor) test, during which 200 µg was administered, along with heparin to maintain the patency of the indwelling needle. Eight NEFA and DFT4 were measured in all samples. Results on the before-heparin sample were compared with those of blood sampled at various time periods up to 2 h after thyroliberin.

Aliquots of a normal plasma sample were stored at 4 °C and at −20 °C for as long as six months. At various times after this storage was begun, an aliquot stored at each temperature was immediately assayed for DFT4 and NEFA.

Oleic acid (99% pure), purchased from Altech Associates, Inc., Deerfield, IL, was dissolved in isopropanol and stored in the dark at 4 °C in a glass-stoppered volumetric flask. An aliquot was methylated and gas-chromatographed to check its purity. Oleic acid was added to plasma from a normal person and a person with severe nonthyroidal illnesses by the method of Mendel et al. (5); i.e., an aliquot of a solution in isopropanol was evaporated under nitrogen and the residue was taken up at 37 °C in an appropriate amount of serum and further dilutions made. The oleic acid was in fact dissolved, as shown by total NEFA measurements in the resulting solutions. Oleic acid in respective initial concentrations of 1, 2, 4, 6, 8, and 10 nmol/L was added to aliquots of normal plasma (albumin concentration 652 µmol/L, T4-binding prealbumin (TBPA) 5.19 µmol/L, TBG 277 nmol/L). DFT4 was measured in each aliquot, NEFA in the 1-, 8-, and 10-nmol/L samples. Similarly, oleic acid was also added to aliquots of plasma from a patient with severe nonthyroidal illness (albumin concentration 355 µmol/L, TBPA 1.1 µmol/L, and TBG 115 nmol/L) in concentrations of 0.17, 0.22, 0.26, 0.58, 0.71, 1.0, and 6.4 nmol/L.

We measured NEFA concentrations in plasma by gas chromatography, using modifications of the method of MacGee and Allen (6), as follows. Mix a 400-µL aliquot of plasma with 50 µL of pentadecanoic acid (as internal standard), 0.5 mL of 1 mol/L phosphoric acid, 5 mL of hexane, and 1.5 mL of methanol, and shake the mixture vigorously. Wash the hexane extract twice with 0.1 mol/L phosphoric acid, then re-extract into 10 µL of a 0.2 mol/L aqueous solution of (m-trifluoromethylphenyl)trimethylammonium hydroxide and inject a 2-µL aliquot onto a 100/120 Supelcoport column with 5% DEGS-PS coating.
The initial temperature is 128 °C, the final temperature 205 °C, the rate of temperature increase 6 °C/min. Our analytical recovery was 98–102%.

Total T₄ and TBG were measured by in-house direct radioimmunoassays (in the TBG RIA, ¹²⁵I-labeled TBG, rabbit anti-human 1st antibody, and sheep anti-rabbit 2nd antibody are used; TBPA by rocket immunoelectrophoresis; and albumin by the bromcresol green dye-binding method (in the Technicon SMAC). DFT₄ was measured by equilibrium dialysis (7).

Except for the storage experiment, NEFA was measured within 24 h of sampling the blood. Plasma specimens were stored at −20 °C and thawed on the day DFT₄ was to be measured.

Results

Patients with severe nonthyroidal illness: The pooled observations on the nine patients totaled 97 (Table 1). The actual values for plasma NEFA ranged from 0.16 to 2.48 mmol/L and for DFT₄ from 0.015% to 0.042%. Correlations between total NEFA or any of the eight individual NEFA and DFT₄ results were not significant except in two patients, where there was a significant correlation between stearic acid and DFT₄. In one patient there were instances of high DFT₄ with supranormal NEFA, preceded by even higher DFT₄ with unchanged or diminished NEFA. In five patients, high DFT₄ with supranormal NEFA was followed by even higher DFT₄, with unchanged or decreased NEFA. All nine patients showed normal DFT₄ co-existing with supranormal NEFA.

We also calculated the ratios of each of the eight NEFA to each of the three thyroid hormone-binding proteins and looked for a correlation between DFT₄ and the molar ratio between individual NEFAs, and individual thyroid hormone-binding proteins. No consistent correlations were found between DFT₄ and the various ratios, although in one patient there were correlations between DFT₄ and the ratios of each of the NEFA to TBG.

Effect of fasting: During the 27-h fast, although NEFA increased from 0.23 to 1.4 mmol/L, DFT₄ was unchanged (Figure 1).

Effect of heparin: In the thyroliberin test, infusion of heparin caused an increase in NEFA from 1.9 to 5.4 mmol/L within 2 or 3 min, and this was accompanied by an increase in DFT₄ from 0.008% to 0.053%, after which both NEFA and DFT₄ rapidly subsided (Figure 2).

The correlation constants (r and P values) between NEFA and DFT₄ for individual NEFA in each patient are available on request from M.L.W. or from the Editorial Office of this journal.

A table of the various correlations is available on request from M.L.W. or from the Editorial Office of this journal.

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Table 1. Summary of Pooled Results for Plasma T₄, DFT₄, T₄-Binding Proteins, and NEFA in Nine Patients with Severe Nonthyroidal Illnesses, Compared with Reference Intervals for Normal Subjects

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>Units</th>
<th>Mean</th>
<th>SD</th>
<th>Actual range</th>
<th>Reference Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total T₃</td>
<td>84</td>
<td>mmol/L</td>
<td>0.777</td>
<td>0.489</td>
<td>&lt;0.2–1.9</td>
<td>1.5–2.5</td>
</tr>
<tr>
<td>Total T₄</td>
<td>88</td>
<td>mmol/L</td>
<td>81.18</td>
<td>29.92</td>
<td>28–147</td>
<td>74–152</td>
</tr>
<tr>
<td>TBG</td>
<td>84</td>
<td>mmol/L</td>
<td>312</td>
<td>81</td>
<td>154–538</td>
<td>154–431</td>
</tr>
<tr>
<td>TBPA</td>
<td>92</td>
<td>μmol/L</td>
<td>3.554</td>
<td>1.994</td>
<td>0.556–8.519</td>
<td>3.519–7.22</td>
</tr>
<tr>
<td>Albumin</td>
<td>93</td>
<td>μmol/L</td>
<td>472</td>
<td>61.3</td>
<td>362–652</td>
<td>435–725</td>
</tr>
<tr>
<td>DFT₄</td>
<td>97</td>
<td>%</td>
<td>0.0248</td>
<td>0.0058</td>
<td>0.015–0.042</td>
<td>0.009–0.025</td>
</tr>
<tr>
<td>NEFA</td>
<td>93</td>
<td>mmol/L</td>
<td>0.771</td>
<td>0.42</td>
<td>0.16–2.48</td>
<td>&lt;0.3</td>
</tr>
</tbody>
</table>

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Fig. 1. Effects of fasting on plasma NEFA and % DFT₄ in a normal subject. Times shown are from the beginning of the fast. Vertical arrows indicate beginning and end of fast, respectively.

Effect of storage: At −20 °C, NEFA did not increase appreciably in concentration, whereas at 4 °C NEFA increased from an initial value of 0.4 mmol/L to 2.1 mmol/L. However, there was no discernible effect on DFT₄ at either temperature.

Effect of oleic acid addition: Increasing oleic acid concentrations by as much as 4 mmol/L in the normal plasma had a negligible effect on DFT₄. Addition of 6 mmol of oleic acid per liter was associated with an increase in DFT₄ from 0.017% to 0.024%. With additions of 8 and 10 mmol of oleic acid per liter, DFT₄ increased to 0.060% and 0.070%, respectively (Figure 3). By the criterion of Bregengard et al. (8)—i.e., an increase in DFT₄ >20% is regarded as statistically significant—increasing oleic acid concentrations by 5.25 mmol per liter in normal plasma was associated with a significant increase in DFT₄ (Figure 3). In plasma from a patient with severe nonthyroidal illness, there was a 20% increase in DFT₄ at 0.26 mmol of added oleic acid per liter (Figure 3).

Discussion

We measured eight of the NEFA most commonly found in plasma, thus including both saturated and unsaturated
predominantly 60

varieties rather than restricting the study to unsaturated NEFA. This decision was partly based on the demonstration by Chopra et al. (4) of significantly increased concentrations of palmitic and stearic acids in serum from patients with thyroid hormone-binding inhibitor (THBI) activity. Incidentally, the concentrations in plasma of unsaturated NEFA—linoleic, linolenic, and arachidonic acids—were not significantly different between THBI-positive patients with nonthyroidal illnesses and normal subjects (4). Chopra et al. (3) also reported that lauric acid (saturated NEFA) possesses substantial THBI activity.

With regard to the effects of the number of double bonds in the various NEFA on the binding of $T_4$, Benvena et al. (9) found the order of inhibitory potency to be arachidonic acid > linoleic acid > oleic acid (the number of double bonds being four, two, and one, respectively). These results accord with those of Herrmann et al. (10). However, when Herrmann et al. analyzed the fatty acid pattern of THBI-positive sera, and calculated the correlations between THBI activity and concentrations of four unsaturated fatty acids, they found a negative correlation between arachidonic acid and THBI activity, no correlations with linoleic or linolenic acids, and a significant positive correlation with oleic acid. Therefore, they concluded that, of all the unsaturated fatty acids, oleic acid and not arachidonic acid predominantly regulates the THBI activity in serum. In assessing the inhibitory potencies of individual NEFA and the possible influence of double bonds and chain length, Bregengard et al. (8) compared four unsaturated NEFA of equal chain length and increasing number of double bonds (18:0, 18:1, 18:2, 18:3) and found no differences in their effects on the free fractions of $T_4$, except for the 18:1, suggesting that the number of double bonds was without any significance. However, the inhibitory potencies increased with increasing chain length (i.e., 20:4 was more potent than the mean of 18:0, 18:1, 18:2, and 18:3, and both were more potent than the mean of 16:0 and 16:1). These findings confirmed the need to study commonly occurring NEFA of both saturated and unsaturated varieties.

All severe nonthyroidal illness patients studied had very low plasma $T_4$ concentrations. Plasma total $T_4$ concentrations ranged from 28 to 147 nmol/L and overlapped the laboratory reference interval for normal individuals of 74–152 nmol/L (Table 1). Many of the TBPA and albumin values are below the lower reference intervals (Table 1). Thus the patients were clinically and biochemically typical of severe nonthyroidal illness, and the expected increases in $DFT_4$ percentages (4) were found, the mean $DFT_4$ being 0.025%, with an actual range of 0.015% to 0.042% (reference interval 0.009–0.025%).

The NEFA values were also increased, the mean being 0.77 mmol/L and actual range 0.16 to 2.48, compared with an expected range of <0.3 mmol/L in nonfasting patients. However, no overall statistical relationship was found between the results for any individual NEFA and $DFT_4$. Herrmann et al. (10) found a significantly high negative correlation ($r = 0.843, P < 0.001$) between serum oleic acid concentrations and THBI activities in 27 patients with nonthyroidal illnesses. Serum total NEFA concentrations (10) were even lower than ours (0.485 mmol/L vs 0.771 mmol/L). However, we measured $DFT_4$ not THBI activity. It seems that NEFA exhibit THBI activity in the competitive ligand binding assay of Chopra et al. (3), in which the ratio of either extracts of the test serum (which contain NEFA) to serum containing $T_4$-binding proteins is very high. However, it does not necessarily follow that NEFA have an effect on free $T_4$ or free $T_4$ fraction in vivo.

The lack of relationship between NEFA and $DFT_4$ values in the plasma of patients with severe nonthyroidal illnesses is consistent with our supplementary studies involving increased plasma NEFA concentrations, which suggest that NEFA concentrations must reach 5 mmol/L before any effect on $DFT_4$ is seen in normal plasma.

In the storage study the highest NEFA concentration was 2.1 mmol/L, and $DFT_4$ was unchanged. During the 24-h period, NEFA concentrations reached 1.4 mmol/L and $DFT_4$ was unchanged. By contrast, in the thyrokinin study the highest NEFA concentration reached—5.4 mmol/L due to heparin administration—was accompanied by a $DFT_4$ increase from 0.008% to 0.053%, a value well above the upper reference interval. The NEFA and $DFT_4$ concentrations peaked simultaneously within 10 min of heparin administration, and both subsided to near normal within 1 h (Figure 2).

The study on enriching normal plasma with oleic acid...
also suggests that the threshold of effect of oleic acid on DFT₄ in normal plasma is between 4 and 6 mmol of oleic acid per liter (Figure 3). When the oleic acid concentration was increased to over 6 mmol/L, the DFT₄ values increased progressively, reaching 0.070% at 10 mmol of oleic acid per liter. Mendel et al. (5) had similarly demonstrated either no or little effect on DFT₄ until the total NEFA exceeded 3 mmol/L with oleic acid enrichment of pooled normal human serum. The plasma from the patient with severe nonthyroidal illness contained an endogenous NEFA concentration of 1.43 mmol/L and the addition of a further 0.26 mmol/L as oleic acid resulted in a significant (20%) increase in DFT₄.

Hence, in the plasma of patients with severe nonthyroidal illnesses, NEFA effects on DFT₄ become evident at concentrations of approximately 1.7 compared with 6 mmol/L for normal plasma, confirming the studies of Brengengard et al. (8) and Mendel et al. (5), the former finding positive effects of NEFA on DFT₄ at total NEFA concentrations of 1.65 mmol/L in severe nonthyroidal illness sera. Mendel et al. (5) demonstrated an albumin dependence of the NEFA-induced DFT₄ increase, wherein "albumin-free" serum showed an effect at 1 mmol of added oleic acid per liter, whereas after enrichment with albumin greater oleic acid concentrations were required to increase DFT₄. From this and other experiments, Mendel et al. (5) concluded that the molar NEFA:albumin ratio in the serum must exceed 5:1 before NEFA causes a significant increase in DFT₄ in both normal individuals and patients with severe nonthyroidal illnesses. In the study of Mendel et al. (5), this occurs when the total NEFA concentration, after oleic acid addition, is 1.9 mmol/L. The sample from severe nonthyroidal illness we selected had very low T₄-binding protein concentration; hence, lower NEFA concentrations than required for normal plasma displaced T₄ from the binding proteins.

Returning to the patient with severe nonthyroidal illness in the present study, we noted that albumin concentrations tend to be low, ranging from 362 μmol/L to 652 μmol/L, and the highest NEFA-to-albumin ratio encountered was 4:1. Thus it is unlikely that the increased NEFA concentrations encountered in these patients, even with moderately low plasma albumin, will inhibit the binding of T₄ to its binding proteins and increase DFT₄. In only two plasma samples (from the same patient) out of 93 were plasma NEFA values >1.7 mmol/L, and even in these two the DFT₄ was lower than in other samples with NEFA less than 1.7 mmol/L. Therefore it would seem unlikely that NEFA concentrations commonly encountered in severe nonthyroidal illnesses will inhibit the binding of T₄ in the serum or plasma, although this is contrary to the claim of Chopra and coworkers (4, 11). However, this claim has been extensively discussed and countered by Mendel et al. (5).

Other factors may affect the relationship between NEFA and T₄ binding to the various binders in plasma of patients with severe nonthyroidal illnesses. For example, moderately low TBP concentrations could contribute to either a direct or a NEFA-mediated increase in DFT₄. Also, the role of drugs with T₄-displacing potential must be considered (12). One patient in our series was treated with phenytoin and one with furosemide for a short time. However, both had DFT₄ values similar to those of the other seven patients not being treated with such drugs.

In summary, we conclude that, although some substance or substances that inhibit binding of T₄ in plasma may be present in the plasma of patients with severe nonthyroidal illnesses, it is unlikely that the NEFA concentrations are sufficiently great to contribute substantially to this inhibition, even though such an effect would be enhanced by decreased concentrations of T₄ binders such as albumin and TGPA. However, the possibility that the NEFA:albumin ratio might occasionally be increased sufficiently in severe nonthyroidal illnesses to contribute to T₄ binding inhibition cannot be completely excluded if, for example, very high doses of drugs are used that displace T₄ and have the potential for enhancing the inhibitory effect of NEFA on T₄ binding and so increasing DFT₄.

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References