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Influence of Nonesterified Fatty Acids and Lysolecithins on Thyroxine Binding to Thyroxine-Binding Globulin and Transthyretin

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ABSTRACT

The hydrolysis of lecithin by phospholipase produces equimolar amounts of nonesterified fatty acids (NEFAs) and lysolecithin. In this study, we have evaluated the effect of lysolecithins and NEFAs on thyroid hormone binding by examining their interactions with thyroxine-binding globulin (TBG)(serum 1:10,000 dilution) and purified transthyretin (TTR). Unsaturated NEFAs (palmitoleic, oleic, linoleic, linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acid) inhibited [^{125}I]T_4 binding to TBG. Their affinities, relative to unlabeled T_4, ranged from 0.005 to 0.0016%, except for oleic acid with relative affinity of <0.0005%. Saturated NEFAs, lauric, myristic, palmitic, and stearic acid were inactive. After purification by high-performance liquid chromatography, 1-oleoyl and 2-oleoyl lysolecithin displaced [^{125}I]T_4 from TBG with an affinity of 0.0006 and 0.0005%, respectively. On a molar basis, this affinity was approximately 10-fold lower than arachidonic acid, the most potent NEFA in inhibiting T_4 binding to TBG in this assay system. Of all the NEFAs tested, only arachidonic acid inhibited [^{125}I]T_4 binding to TTR, with an affinity relative to unlabeled T_4 of 0.49%. 1-Oleoyl, 1-palmitoyl, and 1-stearoyl lysolecithin were without effect on TTR binding. The T_4-displacing effects of NEFAs are markedly attenuated by their extensive binding to albumin. Using purified [^{14}C]NEFA preparations and heptane partitioning, the mean unbound percentages of linoleic, eicosapentaenoic, and docosahexaenoic acid in undiluted normal human serum were 0.00099, 0.0050, and 0.0042%, respectively (n = 3). In view of the very high degree of albumin binding of NEFAs, studies in diluted serum will grossly overestimate their competitor potency. The affinities of lysolecithins for the T_4 binding sites of TBG and TTR are lower than those of NEFAs and depend on the fatty acid component. Lysolecithins are unlikely to influence plasma protein binding of T_4 during critical illness.

INTRODUCTION

SEVERAL NONESTERIFIED FATTY ACIDS (NEFAs) have been shown to inhibit plasma protein binding of T_4 in healthy volunteers after prolonged exercise (1) and to inhibit hepatocyte T_4 uptake (2,3), which may impair deiodination of T_4 in patients with critical illness (2) and euthyroid obese subjects during calorie restriction (3). These studies suggest that NEFAs may be modulators of thyroid hormone delivery *in vivo*.

Lysolecithin and NEFA are produced in equimolar amounts from the hydrolysis of lecithin by phospholipase (4). Marked elevation of phospholipase A_2 has been reported in the sera of patients with a variety of critical illnesses, including acute pancreatitis, infections, multiple injuries, and rheumatoid arthritis (5–7). It is not yet known whether lysolecithins may also directly modulate the binding of T_4 to plasma proteins.

While the effects of palmitic, stearic, oleic, linoleic, linolenic, and arachidonic acid on T_4 binding to human TBG have been reported (8,9), information on other long-chain NEFAs is lacking. In addition, no data are available on the interaction of NEFAs with T_4 binding to human transthyretin (TTR), but oleic acid, arachidonic acid, and docosahexaenoic acid have been shown to interact with rat and mouse TTR (10,11). The main effect of competitor interactions with the binding of T_4 to TBG will result in the alteration of serum levels of T_3 and T_4. Interaction with TTR binding in humans will be more subtle because the proportion of circulating T_4 bound to TTR is relatively small (about 20%), and any displaced T_4 would rapidly

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equilibrate with the unoccupied sites on TBG and albumin. However, interaction of a competitor with TTR may be important in individuals with complete TBG deficiency, in nonprimate species in which TTR is the predominant T_4-binding protein (12), and in tissues such as the choroid plexus where TTR has a postulated T_4-transport function (13).

Heparin-induced alterations of $[^{125}I]T_4$ and drug binding have previously been reported (14–16). In these studies, the authors were unable to reproduce the alteration of binding by *in vitro* addition of concentrations of NEFAs equivalent to those generated by intravenous heparin administration (15,16). Such observations suggest that some unidentified factors in the serum could be responsible for altered binding of these ligands. Because lysolecithins are candidates for these effects, we have studied the interaction of lysolecithins with T_4 in comparison to that of NEFAs.

We have previously emphasized the importance of studying the interaction of competitors with T_4 binding in undiluted serum, in order to avoid dilution-related over- or underestimates of competitor potency (17). However, such studies preclude analysis of competitor interaction at specific T_4 binding sites on TBG and TTR. In this study, we have compared the competitive potency of numerous saturated and unsaturated NEFAs and lysolecithins for the binding of $[^{125}I]T_4$ to TBG and TTR. Using a TBG-specific system of normal human serum at very high dilution (1:10,000) and an isolated preparation of human TTR, we have identified marked differences in the hierarchy of competition by NEFAs and lysolecithins for T_4 binding to TBG and TTR.

MATERIALS AND METHODS

Materials

NEFAs and lysolecithins were purchased from Sigma (St. Louis, MO). The serum pool used for the binding study was from hospital outpatients who were not taking any drug known to influence the serum concentration or the binding of thyroid hormones or NEFAs. Concentrations of albumin, total NEFA, and total T_4 in this serum pool were 41 g/L (0.62 m*M*), 0.7 m*M*, and 95 n*M*, respectively.

NEFAs (highest concentration 0.1 m*M*) were dissolved in 5% ethanol. Lysolecithins (highest concentration 10 m*M*) were dissolved in 50% ethanol. After dilution with buffer, the final concentration of ethanol in the assay mixture was ≤0.5%, a concentration that did not interfere in the binding reaction.

TTR was isolated from pooled plasma from normal human subjects by a modification of the method of Dwulet and Benson (18) as previously described (19).

Purification of 1-acyl lysolecithins

Our initial findings suggested that commercial 1-acyl lysolecithin preparations were highly potent in displacing T_4 (20), but further evaluation showed a high level of phospholipase contamination. Hence, these phospholipids were purified using high-performance liquid chromatography (HPLC) according to the method of Gross and Sobel (21), using a SCX Radial-Pak cartridge (10 × 0.8 cm; Waters Associates), a mobile phase consisting of acetonitrile:methanol:water (300:100:70, v/v/v), and a flow rate of 2 mL/min. The absorbence of 1-acyl lysolecithin was monitored at 203 nm. One milligram of 1-acyl lysolecithin, dissolved in 20 μL of chloroform:methanol (2:1, v/v), was injected with each run. Preliminary experiments established that the retention time of 1-oleoyl, 1-palmitoyl, and 1-stearoyl lysolecithin was approximately 12.0 min. Therefore, during routine purification, effluent between 10.0 and 14.5 min was collected. The effluent was evaporated in a rotary evaporator and the residue transferred to a screw-capped vial. The percentage yield for the purification was 70% for 1-palmitoyl lysolecithin and 100% for 1-oleoyl and 1-stearoyl lysolecithin.

Preparation of 2-oleoyl lysolecithin

2-Oleoyl lysolecithin was prepared according to the method of Subbaiah et al. (22) by incubating 10 mg of di-oleoyl lysolecithin with 10^6 units of lipase suspension (Sigma L4384, Type XI from *Rhizopus arrhizus*). The mixture was incubated at 37°C for 75 min. At the end of incubation, the mixture was extracted by the method of Nishijima et al. (23) using 2 mL methanol and 8 mL petroleum ether:ethyl ether (1:1, v/v). The phospholipid containing the 2-oleoyl lysolecithin was purified by a modified HPLC procedure described by Creer and Gross (24) using a C18 μ-Bendapak cartridge (Waters Associates) with methanol:water (85:15, v/v) as mobile phase and a flow rate of 1.0 mL/min. In a typical preparation, the yield of 2-oleoyl lysolecithins was approximately 60%.

$[^{125}I]T_4$ binding to TBG (1:10,000 serum dilution)

The interaction of various NEFAs and lysolecithins with $[^{125}I]T_4$ binding to TBG was assessed in normal human serum at 1:10,000 dilution. The concentration of added $[^{125}I]T_4$ (1500 μCi/μg; Amersham, Aylesbury, Buckinghamshire, U.K.) was approximately 2 p*M*, giving a total concentration of T_4 (endogenous plus tracer) of less than 20 p*M*. The displacement assay was performed by the addition of 0.1–100 μM NEFAs or lysolecithins to the reaction mixture. After overnight (16–18 h) incubation at 4°C, non-protein-bound $[^{125}I]T_4$ was separated by the addition of 50% volume of ice-cold dextran-charcoal with immediate centrifugation (4°C, 20 min at 2000 *g*). This method has been extensively validated as a specific assessment of T_4 binding to TBG: (i) lack of detectable T_4 binding (<1%) to other sites at 1:10,000 dilution of serum from males with hereditary total TBG deficiency; (ii) Scatchard analysis of T_4 binding by 1:10,000 diluted serum shows mean affinity and capacity corresponding to purified TBG; (iii) mixing of high TBG and zero TBG serum samples in various proportions shows uniform affinity with capacity proportional to the concentration of TBG; and (iv) an order of potency of iodothyronine inhibition of $[^{125}I]T_4$ binding that corresponds to known specificity of TBG (19,25–28). In the absence of binding proteins, oleic acid and arachidonic acid (0.1 m*M*) did not influence adsorption of $[^{125}I]T_4$ to dextran-charcoal (data not shown). Minimum detectable inhibition corresponded to an affinity 0.0005% that of unlabeled T_4.

$[^{125}I]T_4$ binding to transthyretin

Binding of $[^{125}I]T_4$ to isolated TTR was studied using $[^{125}I]T_4$ (0.1–0.3 n*M* T_4; 40–60 μCi/μg; Amersham, Aylesbury,

Buckinghamshire, U.K.) with unlabeled T_4, NEFA, or lysolecithin and diluted TTR solution (3.6–7.2 μg/mL) in Tris-Cl buffer, pH 7.4. After overnight incubation at 4°C, free and protein-bound [^{125}I]T_4 were separated by adsorption of free [^{125}I]T_4 to methyl-cellulose charcoal suspension at 0°C, as previously validated (19). These modifications minimize "stripping" of [^{125}I]T_4 bound to TTR by charcoal. Minimum detectable inhibition in this system corresponded to an affinity 0.19% that of unlabeled T_4.

[^{14}C]NEFAs binding in serum

Serum binding of ^{14}C-labeled linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid was measured using heptane partition (29,30), modified for use with serum by adjustment of serum pH to 7.4 and by preliminary purification of [^{14}C]-NEFAs (30). [^{14}C]NEFA (1 to 2 × 10^5 cpm/mL) was added to the heptane phase and incubated with diluted serum (1:16) with intermittent rotation at 37°C for 90 min. Provided that [^{14}C]NEFAs were purified before use, this method shows a linear relationship with the unbound fraction over a 4096-fold range of serum dilution (30). Hence, the unbound fraction was corrected for dilution and the results expressed for undiluted serum.

RESULTS

[^{125}I]T_4 binding to TBG

Effect of NEFA. The inhibition of [^{125}I]T_4 binding to TBG by seven unsaturated (palmitoleic, oleic, linoleic, linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acid) and four saturated (lauric, myristic, palmitic, and stearic acid) long-chain NEFAs was examined using serum diluted 1:10,000. The unsaturated NEFAs displaced [^{125}I]T_4 binding from TBG with an affinity at least four orders of magnitude lower than that of T_4 itself (Fig. 1). The four saturated NEFA were inactive at final concentration up to 10 μ*M* (data not shown).

The affinities of unsaturated NEFA, relative to T_4 are shown in Table 1. The two most potent unsaturated NEFAs in displacing [^{125}I]T_4 from TBG are arachidonic acid and docosahexaenoic acid, both with a relative affinity of 0.005%. There were, however, only minor differences in the relative affinities of unsaturated fatty acids for the T_4 binding site on TBG which ranged from 0.005 to 0.0016%, except for oleic acid, which has a relative affinity of <0.0005%.

In view of the very high degree of albumin binding of NEFAs (30), it was important to establish whether the estimates of relative affinity were influenced by albumin binding of added NEFA in the 1:10,000 diluted serum. Accordingly, T_4 displacement activity of linoleic acid and arachidonic acid was compared using normal serum and serum from a patient with total hereditary analbuminemia (a generous gift of Professor E. Kallee, Tuebingen, Germany). In view of the high TBG concentration (60 mg/L) found in this analbuminemic serum, the dilution was adjusted (1:30,000) to achieved comparable initial binding of T_4 by normal and analbuminemic serum. Results indicated that the relative affinities of these NEFAs for T_4 binding site of TBG were virtually identical using normal and analbuminemic sera, suggesting that the results in normal serum (1:10,000 dilution) are little influenced by residual albumin binding of NEFAs.

Effect of lysolecithins. Figure 2 shows the displacement of [^{125}I]T_4 binding from TBG by unlabeled T_4, 1-oleoyl, 2-oleoyl, 1-palmitoyl, and 1-stearoyl lysolecithins in comparison to the effect of arachidonic acid, the most potent NEFA in inhibiting T_4 binding to TBG in this assay system.

Both 1-oleoyl and 2-oleoyl lysolecithin displaced [^{125}I]T_4 from TBG, with similar competitive potency. Relative to unlabeled T_4, the affinities of 1-oleoyl and 2-oleoyl lysolecithin were 0.0006 and 0.0005%, respectively. On a molar basis, this affinity was about one order of magnitude less potent than arachidonic acid. Both 1-stearoyl and 1-palmitoyl lysolecithin were inactive (Table 1).

[^{125}I]T_4 binding to transthyretin

Effect of NEFAs. We investigated the ability of four unsaturated (oleic, linoleic, linolenic, and arachidonic acid) and three saturated (myristic, palmatic, and stearic acid) long-chain NEFAs to displace [^{125}I]T_4 from TTR. Of the NEFAs studied, only arachidonic acid showed demonstrable interaction with [^{125}I]T_4 binding to isolated TTR, with an affinity relative to unlabeled T_4 of 0.49%.

Effect of 1-acyl lysolecithins. Neither 1-oleoyl, 1-palmitoyl, nor 1-stearoyl lysolecithin (0.1–10 m*M*) showed any detectable displacement of [^{125}I]T_4 bound to isolated TTR.

[^{14}C]NEFAs binding in serum

The unbound percentages of linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid in normal human serum were 0.00099 ± 0.000035, 0.0050 ± 0.00011, and 0.0042 ± 0.00023, respectively (mean ± SD, n = 3), unbound fractions

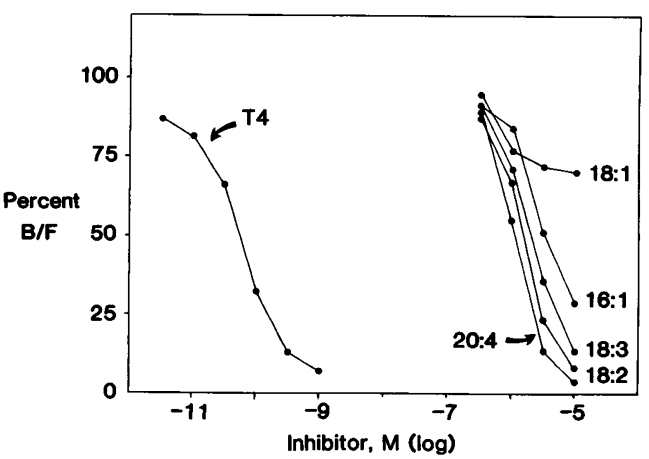

FIG. 1. Molar comparison of the effect of unlabeled T_4 and five unsaturated NEFA (palmitoleic acid 16:1; oleic acid 18:1; linoleic acid 18:2; linolenic acid 18:3; arachidonic acid 20:4) in inhibiting [^{125}I]T_4 binding to TBG (serum 1:10,000), Tris-Cl buffer, pH 7.4. Bound and free [^{125}I]T_4 were separated by charcoal adsorption. Each data point is the mean of 3–6 determinations in duplicate. The inhibition of [^{125}I]T_4 binding by eicosapentaenoic (20:5) and docosahexaenoic acid (22:6) is not shown because the curves overlapped with linoleic and arachidonic acid, respectively.

TABLE 1. RELATIVE AFFINITIES OF NEFA AND PHOSPHOLIPIDS FOR [^{125}I]T_4 BINDING TO TBG

Compound		*Concentration at 50% B/F[a] (M)*	*Relative affinity[b] (%)*
(T_4)		5.0×10^{-11}	100
NEFA			
Palmitoleic acid	16:1	3.2×10^{-6}	0.0016
Oleic acid	18:1	$>10^{-5}$	<0.0005
Linoleic acid	18:2	1.6×10^{-6}	0.0032
Linolenic acid	18:3	2.0×10^{-6}	0.0025
Arachidonic acid	20:4	1.0×10^{-6}	0.0050
Eicosapentaenoic acid	20:5	1.6×10^{-6}	0.0032
Decosahexaenoic acid	20:6	1.0×10^{-6}	0.0050
Phospholipids			
1-Oleoyl lysolecithin		8.3×10^{-6}	0.0006
2-Oleoyl lysolecithin		5.0×10^{-5}	0.0005

[a]Concentration obtained from Figure 1 and Figure 2.
[b]Expressed relative to T_4, taken as 100%; mean of 3–6 determinations in duplicate.

similar to those previously reported for myristic acid (0.0072%), palmitic acid (0.00067%), oleic acid (0.00090%), linolenic acid (0.0019%), and arachidonic acid (0.0020%) (30).

Estimates of the unbound concentration of the various NEFAs in the pooled serum (total NEFA concentration 700 μM) using the proportions of individual NEFAs previously published (31), ranged from 1.6 nM for oleic acid to 0.12 nM for linolenic acid.

DISCUSSION

This study demonstrates large differences between various NEFAs and phospholipids in their hierarchy of T_4 displacement from TBG and TTR. While all the unsaturated NEFAs tested interacted weakly with the binding of T_4 to TBG, there is a general lack of effect of the unsaturated NEFAs on the binding of T_4 to TTR (except arachidonic acid). With regard to T_4-TBG binding, it is important to note that even for the most potent NEFAs, arachidonic acid and decosahexaenoic acid, the potency relative to that of T_4 is only about 0.005%. This affinity is approximately 20-fold lower than furosemide, the most potent drug competitor for T_4-TBG binding so far described (19).

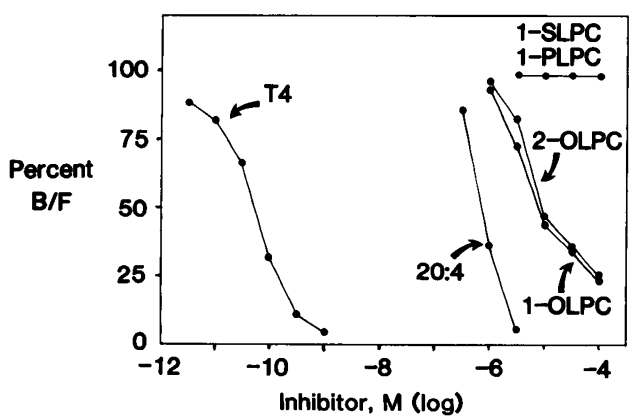

FIG. 2. Inhibition of [^{125}I]T_4 binding to TBG at serum dilution 1:10,000 by unlabeled T_4 and HPLC-purified 1-oleoyl (1-OLPC), 1-palmitoyl (1-PLPC), 1-stearoyl (1-SPLC), and 2-oleoyl (2-OLPC) lysolecithins, showing their activity relative to arachidonic acid (20:4), which is the most potent NEFA in inhibiting [^{125}I]T_4 binding to TBG. Bound and free [^{125}I]T_4 were separated by dextran-charcoal adsorption. Each point is the mean of two determinations in duplicate.

Of the four lysolecithins tested, only 1-oleoyl and 2-oleoyl lysolecithins inhibited the binding of [^{125}I]T_4 to TBG, with an affinity relative to unlabeled T_4 of about 0.0005%. On a molar basis, this affinity was about one order of magnitude less than arachidonic acid, the most potent unsaturated NEFA for T_4-TBG binding. Serum concentration of lysolecithins in normal human serum is between 0.2 and 0.3 mM (22), but this may increase during critical illness as a result of the marked elevation of the phospholipase A_2 levels (5–7). Given that the threshold concentration of unsaturated NEFAs for the displacement of [^{125}I]T_4 from plasma proteins in whole serum is between 1.5 and 2.0 mM (1,2), and taking into consideration the relative affinity of the lysolecithins for T_4-TBG binding, these findings suggest that lysolecithins are unlikely to influence the binding of T_4 to plasma proteins during critical illness.

The minute unbound fraction of linoleic acid, eicosapentaenoic acid, and docosahexaenoic acid in the present study, together with values for five other NEFAs previously reported (30), indicates almost complete binding of these lipids to serum proteins, principally to albumin (32). Such extensive binding limits the unbound concentrations, the physiologically important moiety that determines ligand interactions. However, despite the key role of albumin in determining the unbound concentration of NEFAs, numerous workers have attributed biological activities to various NEFAs, using systems either devoid of albumin or in diluted serum (33–38). The significance of these studies needs to be reassessed, taking heed of the unbound concentration of NEFAs at physiological albumin concentrations.

In conclusion, our study demonstrates widely varying affinities of various NEFAs and lysolecithins for TBG and TTR.

While unsaturated NEFA and 1-oleoyl and 2-oleoyl lysolecithins all showed interactions with TBG, only arachidonic acid interacted with T_4 binding to TTR. At normal concentrations, NEFAs are unlikely to influence the plasma binding of T_4 at physiological albumin concentrations, but marked increases in NEFAs concentrations might still influence T_4 binding *in vivo,* as, for example, during prolonged exercise (1) or when elevated NEFAs are associated with marked decreases in albumin concentrations (2,39,40). The weak interactions of lysolecithins with the binding of T_4 to TBG suggest that these compounds are unlikely to influence T_4 binding *in vivo.*

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