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Inhibition of pancreatic lipase *in vitro* by the covalent inhibitor tetrahydrolipstatin

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Tetrahydrolipstatin inhibits pancreatic lipase from several species, including man, with comparable potency. The lipase is progressively inactivated through the formation of a long-lived covalent intermediate, probably with a 1:1 stoichiometry. The lipase substrate triolein and also a boronic acid derivative, which is presumed to be a transition-state-form inhibitor, retard the rate of inactivation. Therefore, in all probability, tetrahydrolipstatin reacts with pancreatic lipase at, or near, the substrate binding or active site. Tetrahydrolipstatin is a selective inhibitor of lipase; other hydrolases tested were at least a thousand times less potently inhibited.

INTRODUCTION

Pancreatic lipase is the key enzyme of dietary triacylglycerol absorption. It acts at the surface of emulsified lipid droplets [1]. Under physiological conditions triacylglycerol droplets are emulsified by bile salts [2] and colipase anchors and stabilizes lipase at the interphase [3]. A number of inhibitors of pancreatic lipase have been so far described; many are surfactants. Alkyl and aryl boronic acid derivatives are selective, reversible inhibitors of pancreatic lipase, and are supposed to act at the active centre [4]. Lipstatin, isolated from *Streptomyces toxytricini* [5], and its derivative tetrahydrolipstatin are the first selective irreversible inhibitors of lipase.

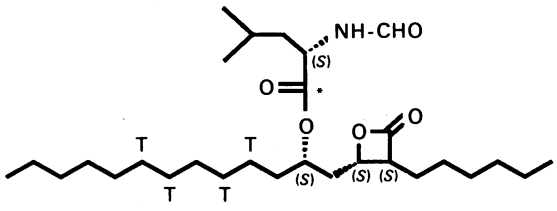
MATERIALS AND METHODS

Materials

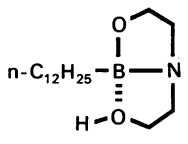
Tetrahydrolipstatin (M_r 496, see structure below) and [^{14}C]THL were obtained by chemical synthesis [6]. [^{3}H]THL was prepared from lipstatin, of microbial origin [7], by catalytic reduction with ^{3}H gas. The material obtained had a specific activity of 34 Ci/mmol and was diluted to 250 μCi/mmol for storage. It was repurified before use by t.l.c. (h.p.t.l.c silica gel plates from Merck, Darmstadt, Germany, developed with hexane:chloroform:dioxane, 1:1:0.22, by vol.). Ro 8-5263 (M_r 283.3) was synthesized by Professor Ramuz at F. Hoffmann–La Roche, Basle.

Trioleylglycerol ether was synthesized by a modification of the published method [8]. Porcine pancreatic lipase (EC 3.1.1.3) was purchased from Sigma (type VI-S, L 0382; lot 23-F-8095 was used in all but the experiment shown in Table 2, in which lot 55-F-8095 was used). An analysis performed by Professor B. Borgström (Lund, Sweden) showed that in comparison to his purified standard, only 28% of the protein of lot 23-F-8095 and only 14% of lot 55-F-8095 is active lipase (personal communication). Based on a total protein content of 70% and 66%, respectively, and an M_r of 50000, lot 23-F-8095 contains, maximally, 3.9 nmol, and lot 55-F-8095 1.85 nmol, of active lipase per mg. *N*-Terminal sequence analysis of lot 23-F-8095 showed that about 50% by weight of the protein corresponded to the sequence of lipase and the molar ratio of lipase to colipase was about 1.0:0.7. Human pancreatic juice was obtained from two patients after stimulation with cholecystokinin–pancreozymin. Intestinal fluid was recovered from four mice by rinsing the intestine with saline. α-Amylase (EC 3.2.1.1) from pig pancreas, esterase (EC 3.1.1.1) from pig liver, phospholipase A_2 (EC 3.1.1.4) from pig pancreas, chymotrypsin (EC 3.4.21.1) from bovine pancreas and trypsin (EC 3.4.21.4) from bovine pancreas were purchased from Boehringer Mannheim.

Succinyl-L-phenylalanine 4-nitroanilide was from SERVA, Heidelberg, Germany. Phosphatidylcholine (Soyaphosphatid NC 100) was obtained from Natermann, Köln, Germany, and sodium salts of bile acids were purchased from Calbiochem. All other chemicals were from FLUKA, Buchs, Switzerland.



THL (T: ^{3}H label, *: ^{14}C label)



Ro 8-5263

Abbreviation used: THL, tetrahydrolipstatin.

Methods

All experiments were performed at least three times and representative single experiments are shown.

Pancreatic lipase activity

For the determination of lipase activity, the hydrolysis of triolein to fatty acids was followed at pH 8 for 10 min at room temperature using a recording pH-stat (Metrohm, Herisau, Switzerland, modified for a 100 μl syringe). The substrate emulsion (1.5 ml per assay) was prepared by ultrasonication of triolein (30 mg/ml) in a solution containing 1 mM-taurochenodeoxycholate, 9 mM-taurocholate, 0.1 mM-cholesterol, 1 mM-phosphatidylcholine, 15 mg of bovine serum albumin/ml. 2 mM-Tris/HCl, 100 mM-NaCl and 1 mM-$CaCl_2$ [a Branson (Danbury, CT, U.S.A.) Sonifier was used]. After addition of the test compound dissolved in 150 μl of dimethyl sulphoxide, or vehicle alone, the pH was adjusted to 8.0 and the reaction was started within 1 min by the addition of 15–20 μl of lipase (dissolved in saline/4% bovine serum albumin at a concentration of 70 μg/ml) or 100 μl of duodenal juice. A standard lipolytic activity was achieved by adding sufficient lipase to result in the liberation of 0.2–0.3 μmol of fatty acids/min per ml.

Activity of additional hydrolases

α-Amylase activity (1 unit in 1.5 ml final volume) was measured for 5 min at 30 °C with soluble starch as substrate according to Bernfeld [9].

Esterase activity was measured with ethyl butyrate as substrate and quantification of the liberated ethanol with alcohol dehydrogenase/NAD^+ by a combination of the methods described for these enzymes [10].

Phospholipase A_2 activity was measured at room temperature with egg yolk (which contains phosphatidylcholine as the main phospholipid) as substrate by titration of the liberated fatty acids with a recording pH-stat set to pH 8.0, essentially as described in [10].

Chymotrypsin activity was measured with succinyl-L-phenylalanine 4-nitroanilide as substrate [11].

Trypsin activity was measured with benzoylarginine *p*-nitroanilide as substrate [12].

In all assays bovine serum albumin, 25 mg/ml, was included. THL was added in dimethyl sulphoxide (10% final concentration) and the reaction was started immediately by the addition of the enzyme.

RESULTS AND DISCUSSION

Inhibition of lipase activity

Determination of lipase activity was based on the amount of oleic acid liberated from emulsified glycerol trioleate. The composition of the test emulsion was chosen to mimick as closely as possible the conditions *in vivo*. The concentration of bile acids (10 mM) approximated concentrations found in duodenal aspirates [13], and phospholipids, cholesterol and protein were also included. The titration curves of the liberated fatty acids approached a slightly sigmoidal shape (Fig. 1*a*); this was probably due to modulation of the interphase by the generated products [14]. Especially in the presence of calcium, the substrate surface can undergo profound changes in the course of the lipolytic process [15]. Nevertheless, an almost linear dependence of lipolytic activity on the amount of lipase added was obtained (not shown). The degree of lipase inhibition by THL largely depended on the time of incubation with the enzyme. As THL is insoluble in the absence of the substrate, preincubation with the enzyme is impractical. Therefore, inhibitory activity was calculated from the activity measured during the first 10 min of incubation after addition of enzyme to substrate plus inhibitor. The resulting dose–response curve (Fig. 1*b*) for THL is suitable for comparative quantification of the inhibitory activities.

For an equal initial lipolytic activity, 50% inhibition resulted from the addition of 0.11 μg of THL/ml to porcine pancreatic lipase, of 0.27 μg/ml to mouse intestinal fluid and of 0.12 μg/ml to human duodenal juice. Not only is the amount of THL necessary for half-maximal inhibition of lipase activity from the three species very similar, but also the dose–response curves parallel each other (not shown). The octanol/buffer, pH 7.5, partition coefficient of THL is greater than 1000. In accordance, in our test system [^{3}H]THL is recovered exclusively in the lipids after phase separation by ultracentrifugation or ultrafiltration through a 0.2 μm membrane filter. Therefore, the concentration of THL for a given degree of inhibition is dependent on the lipid phase present and the calculation of a K_i value is meaningless. The lipolytic activity of the intestinal content can largely be attributed to pancreatic lipase, but some lingual (gastric) lipase activity may also be present [16]. Since THL completely inhibits the lipolytic activity of intestinal fluid, lingual lipase, if present and active on the substrate used, is also inhibited.

Selectivity of lipase inhibition

Of the hydrolases evaluated, only liver esterase was slightly inhibited at the highest concentrations of THL which could be tested (Table 1). High selectivity of THL for lipase is apparent since the concentration of THL necessary to inhibit esterase was three orders of magnitude higher than for lipase. Esterase and lipase have overlapping substrate specificities and probably related enzymic mechanisms; therefore, a slight inhibition of esterase by THL is not surprising.

Formation of a covalent lipase–THL complex

Binding experiments with [^{3}H]THL incubated for 10 min with the triolein emulsion and with a 10–50-fold molar excess of lipase over THL showed that over 90% of the radioactivity remained bound to the protein even after denaturation by extraction with chloroform/methanol [17]. If bovine serum albumin was substituted for lipase and subjected to the same procedure, then less than 1% of the radioactivity was recovered in the protein precipitate. This excludes an unspecific reaction of THL with proteins. After incubation of lipase as above, the addition of 1 M-HCl for 24 h at room temperature prior to the extraction did not affect the binding of THL to the lipase; in contrast, treatment with 1 M-NaOH for 1 h led to complete release of the radioactivity into the chloroform phase. Over 90% of the radioactivity from [^{14}C]THL labelled in the leucine part of the molecule was

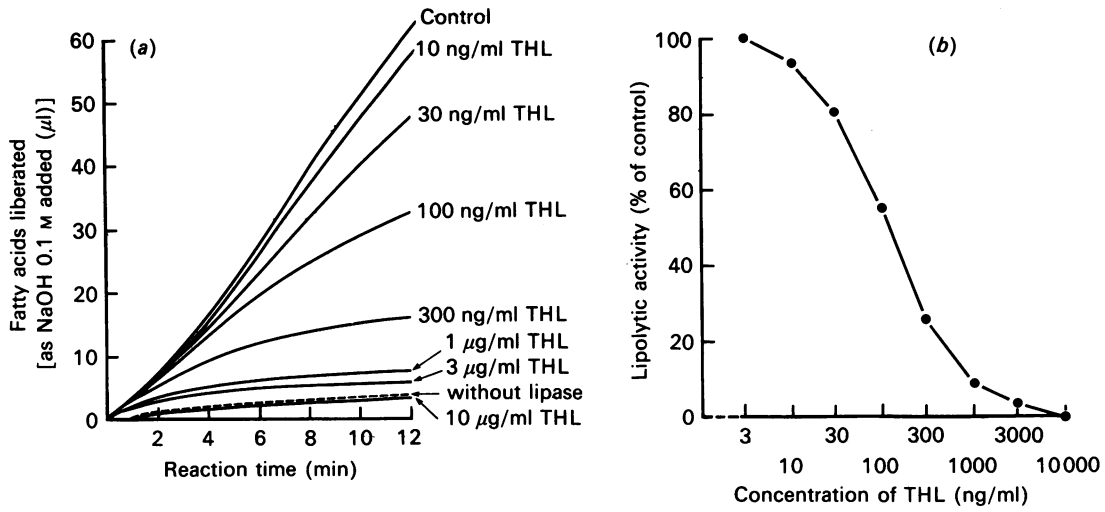


Fig. 1. Inhibition of pancreatic lipase by THL

(*a*) Titration curves for the release of oleic acid from triolein in the presence of different amounts of THL. (*b*) Inhibition of lipolytic activity as calculated from the amount of fatty acid released from $t = 0$ to $t = 10$ min compared with control at a given THL concentration. Data are derived from (*a*). For experimental details see under 'Materials and methods'.

also bound by lipase. In the presence of saturating amounts of [^{3}H]THL, incorporation of radioactivity by lipase was paralleled by loss of enzymic activity (Table 2). Because of the low percentage of active lipase in the available preparation, the stoichiometry of THL bound to lipase could not be exactly determined. Based on a content of 1.85 nmol of active lipase/mg of the preparation used in this experiment, it seems very probable that the binding of 1 molecule of THL per molecule of lipase is sufficient for complete inhibition of lipolytic activity. A stoichiometry close to 1:1 is supported by experimental results obtained both with an older lot (23-F-8095) of lipase from Sigma, which had twice the specific activity as compared with the lot shown in Table 2, and with a reference sample of purified porcine pancreatic lipase, a generous gift from Professor B. Borgström, Lund, Sweden.

Close analogues of THL that lack the β-lactone ring (e.g. the β-hydroxy acid analogue) are completely inactive (not shown). This suggests that the functional group of THL that is involved in covalent bond formation with lipase is the β-lactone. Since no reaction occurs with serum albumin and since the reaction stoichiometry with lipase is close to 1:1, it seems that this reaction takes place only at a specific microenvironment within the lipase molecule.

Table 1. Inhibition of various hydrolases by THL

The highest concentration of THL which could be obtained in the respective assay system was used and the residual enzyme activity at this concentration is shown.

Enzyme	Highest concn. of THL tested (μM)	Residual activity (%)
Trypsin	200	92
Chymotrypsin	200	79
Esterase (liver)	200	55
Phospholipase A_2	2000	100
Amylase	600	100

Rate of lipase inhibition

Lipase inhibition by THL is progressive with time and the rate of inactivation increases with increasing concentration of THL (see Fig. 2). After precipitation of lipase and removal of excess THL by extraction with acetone (67% final concentration, 0 °C), 50% reactivation occurs in buffer solution in 24 h. Hence, from a practical point of view, inhibition of pancreatic lipase by

Table 2. Binding of [^{3}H]THL to pancreatic lipase and loss of lipolytic activity

Crude porcine pancreatic lipase (500 μg) (Sigma no. L 0382, lot 55-F-8095) was preincubated in a final volume of 275 μl containing 125 μl of triolein emulsion (see under 'Methods') with the indicated amount of [^{3}H]THL (added in 25 μl of dimethyl sulphoxide) for 45 min at room temperature. Residual activity was measured by addition of 25 μl of the 1:10 diluted preincubation mixture to 1.5 ml of triolein emulsion. Unbound [^{3}H]THL was extracted with chloroform/methanol [17]. The calculated amount of active lipase in the preincubation mixture was 0.92 nmol (see under 'Materials').

Preincubation with [^{3}H]THL (nmol)	[^{3}H]THL bound to lipase (nmol)	Inhibition of lipolytic activity (%)
0.23	0.10	11
0.52	0.21	23
0.99	0.39	42
1.54	0.54	58
2.04	0.65	71
2.55	0.77	84
3.01	0.86	93

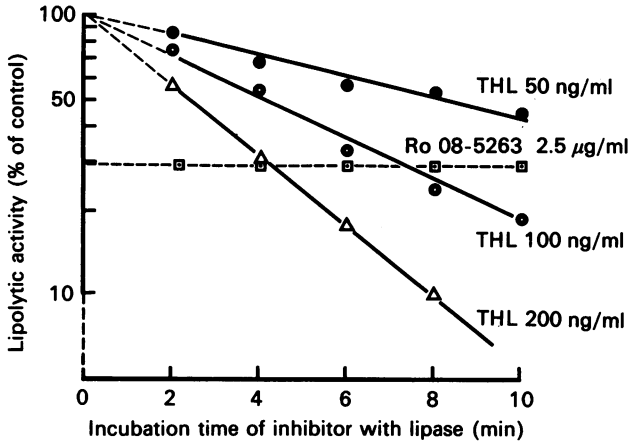


Fig. 2. Time dependence of the inhibition of pancreatic lipase by THL

Percentage inhibition achieved with three different concentrations of THL in the presence of substrate was derived from the slope of the titration curve at the indicated time points and expressed as a percentage of the slope of the control curve after hydrolysis of the same amount of triolein. The inhibition kinetics of a presumed competitive inhibitor, the boron compound Ro 8-5263, are shown for comparison.

THL is irreversible. It can be inferred that the putative covalent intermediate is relatively stable, although it is hydrolysed in buffer over a period of several hours (cf. its stability in acid and instability in alkali noted above). In the usual assay method for irreversible inhibitors [18,19], aliquots of the incubation mixture are diluted into the assay system in order to lower the inhibitor concentration sufficiently to stop the inactivation process. This was not applicable with our test system, because using this method the plot of log(enzyme activity remaining) versus time also gave straight lines but the extrapolated activity at zero time was substantially lower than 100% and dependent on the THL concentration. In contrast to THL, Ro 8-5263, a presumed competitive inhibitor of lipase, provoked a constant inhibition over time (Fig. 2). In analogy to other boronic acid inhibitors of serine esterases [20], the latter compound is expected to be a transition state form inhibitor of lipase, especially as the boron in Ro 8-5263 is tetrahedral as determined by X-ray crystallographic analysis. In the presence of Ro 8-5263 and dependent on its concentration the rate of covalent inhibition of pancreatic lipase by THL is slowed down (measured after 10-fold dilution of the preincubation mixture; not shown).

The substrate triolein strongly retards the rate of inactivation of lipase by THL, as shown in Fig. 3. Since THL is entirely distributed into the lipid phase, a constant concentration of this inhibitor could only be achieved by keeping the total lipid mass constant through substitution of a nonhydrolysable fat, trioleoylglycerol ether, for triolein. Preincubation of lipase with trioleoylglycerol ether in the presence of triolein is without major impact on lipolytic activity, whereas preincubation with the ether without substrate largely inactivates the enzyme. For these reasons preincubation of lipase with THL but without substrate was performed in the absence of a lipid phase but in this case THL was dissolved in the total volume of the sample and not concentrated in the lipid phase. In consequence the effective inhibitor concentrations are different in the two experiments and therefore the results obtained by preincubation without substrate are shown separately (Fig. 3*a*).

Since the inactivation rate of lipase by THL is slowed down by the substrate triolein and by a boronic acid derivative, which is presumed to be a transition-state-form inhibitor, it is probable that THL binds at or near the substrate binding or active site of lipase. Determination of the amino acid residue of pancreatic lipase to which THL is bound is in progress.

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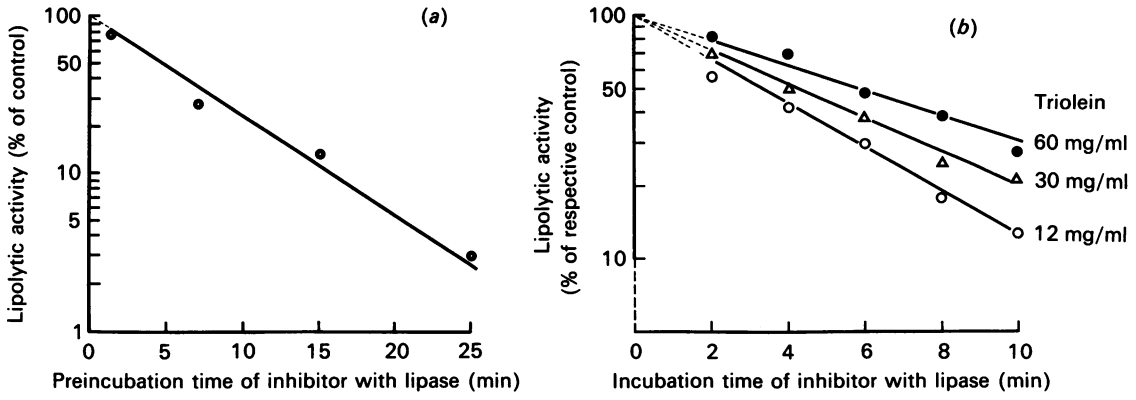

Fig. 3. Influence of substrate concentration on the progressive development of lipase inhibition produced by THL: *(a)* preincubation of lipase with THL without substrate, and *(b)* incubation of lipase with THL in the presence of varying amounts of substrate

(*a*) 0.6 μg of pancreatic lipase was preincubated with THL (100 ng/ml) in a final volume of 230 μl, containing 1% dimethyl sulphoxide. Initial rate of the remaining lipolytic activity (tangent to the titration curve of the liberated fatty acids) was measured after addition of 1.5 ml of triolein emulsion. (*b*) Percentage inhibition achieved with THL (100 ng/ml) was measured as described in Fig. 2. Since THL has a high preference for the lipid phase, the total lipid mass was kept constant (60 mg/ml) by substituting trioleylglycerol ether for triolein. As a result a constant concentration of THL in the lipid phase was achieved.

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