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A comparative kinetic study on human pancreatic and *Thermomyces lanuginosa* lipases: Inhibitory effects of tetrahydrolipstatin in the presence of lipid substrates

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ABSTRACT

The inhibitory effects of tetrahydrolipstatin (THL) on the hydrolytic activity of human pancreatic lipase (HPL) and *T. lanuginosa* lipase (TLL) on various lipidic substrates 'poisoned' with THL as previously described was studied, using either the pH-stat, monomolecular film or oil drop technique.

Prior to adding lipase (method C), an ethanolic solution of THL was injected in a tributyrin (TC4) or a purified soybean oil (PSO) emulsion prepared in a pH-stat vessel. Under these conditions, THL was found to be a potent HPL inhibitor. After being dissolved in the pure triglyceride phase (method D), THL also strongly inhibited HPL. However, with TC4 as substrate TLL was efficiently inhibited by THL only when method C was used and not method D. The very different inhibitory effects on HPL and TLL recorded with method D and PSO as substrate were confirmed using the monomolecular film and oil drop techniques.

With a monomolecular film of dicaprin (di-C10) as substrate, 1 molecule of THL embedded in 400 000 molecules of di-C10 sufficed to reduce the HPL activity to half of its initial value.

HPL was therefore efficiently inhibited by THL with all the methods and substrates tested here. Paradoxically, TLL was inhibited by THL molecules transiently present in the aqueous phase and not by the THL molecules present at the triglyceride/water interface. It should therefore be stressed that the inhibitory effects of THL on each lipase depend strongly on the method and the substrate used.

1. Introduction

Conventional treatments for obesity have focused largely on strategies designed to control energy intake, but the long-term efficacy of these approaches has not yet been proved [1]. Anti-obesity treatments based on the use of digestive lipase inhibitors were therefore developed to reduce fat absorption. Tetrahydrolipstatin (THL, Orlistat), a hydrogenated lipstatin analogue isolated from *Streptomyces toxytricini*, is a potent gastrointestinal lipase inhibitor [2–8]. Several studies on the clinical applications of THL to the treatment of human obesity have been published since 1991 [9–17]. THL was recently found to be a novel inhibitor of the thioesterase domain of fatty acid synthase, an enzyme involved in tumour progression [18,19]. Since THL can block cellular fatty acid synthase, this drug has been tested successfully on various tumour cells [20–23].

It has been suggested that a stoichiometric enzyme–inhibitor covalent complex of the long-lived acyl-enzyme type may be formed between the open β-lactone ring of THL and the catalytic serine of pancreatic lipase [24–26]. However, the physico-chemical aspects of lipase inhibition processes in general are still poorly understood [27,28]. The partitioning of THL between the micellar and oil phases was investigated at our laboratory and THL was found to be mostly associated with the TAG phase, even in the presence of bile salts [29,30]. This interesting physico-chemical property is probably a requirement for prototypic lipase inhibitors to be effective under physiological conditions, i.e., in the presence of bile and dietary lipids [31].

It was established in early *in vitro* studies that the inhibition of porcine pancreatic lipase by THL [2,32] is an irreversible process, whereas that of human carboxyl ester lipase [5] is reversible and non-competitive. It was subsequently reported that bile salts in levels above the CMC are needed for THL to efficiently inhibit HPL

Abbreviations: CMC, critical micellar concentration; π, surface pressure; β-CD, β-cyclodextrin; NaTDC, sodium taurodeoxycholate; 1,2-DO, 1,2-di[*cis*-9-octadecenoyl]-*sn*-glycerol; di-C10, 1,2-didecanoyl-*sn*-glycerol; TC4, tributyroyl-glycerol; PSO, purified soybean oil; TAG, triacylglycerols; THL, tetrahydrolipstatin (Orlistat); TLL, *T. lanuginosa* lipase; HPL, human pancreatic lipase.

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[33] and lipoprotein lipase [34]. The fact that after being inhibited, HPL could be reactivated by reducing the bile salt concentration to below the CMC suggested that bile salts (in concentrations above the CMC) may stabilise the acyl–lipase complex [33]. Potthoff et al. [35] reported that THL had reversible competitive inhibitory effects on *Chromobacterium viscosum* and *Rhizopus oryzae* lipases. In addition, after inhibiting *C. viscosum* lipase, THL remains chemically unchanged, which suggests that a non-covalent THL/lipase complex may be formed. However, Asler et al. [36] more recently provided convincing mass spectrometry evidence supporting the existence of a covalently bound THL molecule at the catalytic serine site in *Streptomyces rimosus* lipase. In a previous study [37], we clearly showed that after pre-incubating HPL with THL in the absence of substrate (method A), the resulting inhibitory effects were reversible.

Although THL blocks the activity of mammalian lipases such as carboxyl ester lipase very effectively, THL was originally not thought to inhibit lipases of microbial origin [5]. In the latter study, in which an emulsified tributyrin substrate was used, THL was found to have no inhibitory effects on extra-cellular *Staphylococcus aureus* or *Rhyzopus arrhizus* lipases; whereas Mosbah et al. [38] reported that THL inhibited a 43-kDa lipase isolated from *Staphylococcus xylosus*. These authors observed enhanced inhibition in the presence of approximately 4 mM sodium deoxycholate and classified *S. xylosus* lipase as a serine enzyme, based on the kinetics of the inhibitory process. Likewise, the lipase produced by a novel strain of *R. oryzae* WPG was isolated, characterized, and also recognized as a serine enzyme, based on the kinetics of inhibition by THL [39]. The same group [40] have also isolated a novel lipase (50 kDa) from the scorpion digestive glands, which was inhibited by THL with a THL/lipase molar ratio of 100.

Homogeneously dissolving THL in the lipid phase of a meal was found to efficiently inhibit the human digestive lipases, since THL mixed directly into butter (the only fat source ingested) inhibited the fat absorption process by more than 90%. When Xenical®, the commercial form of THL, was administered along with a normal diet, the level of inhibition recorded was found to plateau at around 35% [11]. The latter *in vivo* finding prompted us to perform systematic *in vitro* studies on the inhibitory effects of THL dissolved in various lipidic substrates. In this context, it therefore seemed to be of interest to use well defined lipid/water interfaces, which are the main site of action of lipases [41–43]. For this purpose, the inhibitory effects of THL on HPL and TLL activity were therefore investigated using the pH-stat, monomolecular film and oil drop techniques.

2. Experimental

2.1. Lipids

Tributyrin (TC4) of "purum grade", Diolein (1,2-di[*cis*-9-octadecenoyl]-*sn*-glycerol) (1,2-DO) and 1,2-didecanoyl-*sn*-glycerol (di-C10) were purchased from Sigma® (St. Louis, MO). The edible soybean oil was purchased from the company Lesieur (Neuilly-sur-Seine, France) and purified to remove the free fatty acids and partial glycerides on a silicic acid column (Merck) equilibrated with Hexane. The TAG-containing fractions were eluted by increasing the ethyl-ether content stepwise, and dried in a vacuum. The stability with time of the oil–water interfacial tension was checked with the oil drop tensiometer [44], using purified soybean oil (PSO) to ensure that no tensioactive contaminants were present (interfacial tension variations lower than 0.05 mN m^{-1} h^{-1}). The PSO was stored at −20 °C in an argon atmosphere. Sodium taurodeoxycholate (NaTDC) and β-cyclodextrin (β-CD) were purchased from Sigma® (St. Louis, MO). THL (M_w 496) and [^{14}C]THL (specific radioactivity 9.96 μCi mg^{-1}) from Hoffmann-La Roche Ltd. (Basel, Switzerland). A stock solution of 10 mM THL in ethanol was prepared and stored at 4 °C.

2.2. Proteins

Purified HPL [45] and porcine pancreatic colipase [46] were a generous gift from J. De Caro (LLE-Marseille). Purified recombinant TLL was kindly provided by Dr. Allan Svendsen and Dr. Shamkant Patkar from Novo-Nordisk (Bagsvaerd, Denmark) [47].

2.3. Inhibition methods: 'poisoned interface methods'

These methods were developed to test the possible reactions between lipases and inhibitors in the presence of substrate [27]. The residual lipase activity was measured after mixing the inhibitor with substrate prior to adding lipase. In the present study, the premixing of substrate with THL was carried out using the following two methods (see Fig. 1).

2.3.1. Method C

One minute before adding lipase, an ethanolic solution of THL was injected at various final concentrations into the pH-stat lipolysis assay vessel containing the emulsified substrate. Lipase hydrolytic activity was then monitored using the pH-stat technique (Fig. 1, method C).

2.3.2. Method D

With method C, it is not possible to control the 'interfacial quality' and to easily assess the distribution of 'soluble' versus adsorbed THL molecules. More clearly defined THL–substrate systems were therefore obtained by dissolving THL directly in the TAG substrate before carrying out the lipase assay (Fig. 1, method D). This method of inhibition was used with either the pH-stat, monomolecular film or oil drop technique. The process of lipase hydrolysis was then monitored as described below.

2.4. pH-stat experiments

Lipase activity was assayed by measuring the free fatty acids released from mechanically stirred (250 rpm was found in practice to be the optimal stirring rate value) TAG emulsions (either TC4 or PSO), using 0.1 N NaOH with a pH-stat (Metrohm 718 STAT Titrino, Herisau, Switzerland) set at pH 8.0. Each kinetic assay was performed in a thermostated (37 °C) pH-stat vessel containing 0.5 mL TAG and 14.5 mL of 1 mM Tris–HCl buffer, 150 mM NaCl and 5 mM $CaCl_2$. When using HPL, pure colipase was added at a molar excess of 5 with respect to the lipase. When required, 4 mM of NaTDC were added to the assay vessel of the pH-stat prior to HPL injection. The specific lipolytic activities are expressed in international units (IU) per mg of enzyme. One unit corresponds to 1 μmol of fatty acid released per minute.

2.5. Monomolecular film experiments

Measurements were performed with a KSV 5000 Barostat (KSV-Helsinki). The principle of this technique has been described by Verger and De Haas [48]. It involves the use of a "zero-order trough" with two compartments: a reaction compartment (100 cm^2, 100 mL) and a reservoir compartment (367 cm^2) connected to each other by two small surface channels.

Mixed films consisting of substrate (di-C10 or 1,2-DO or PSO) and THL were spread from a chloroform solution (1 mg mL^{-1}), over the surface of the reaction compartment, whereas the reservoir compartment was covered with a film of pure substrate. Enzyme solution was injected into the subphase of the reaction

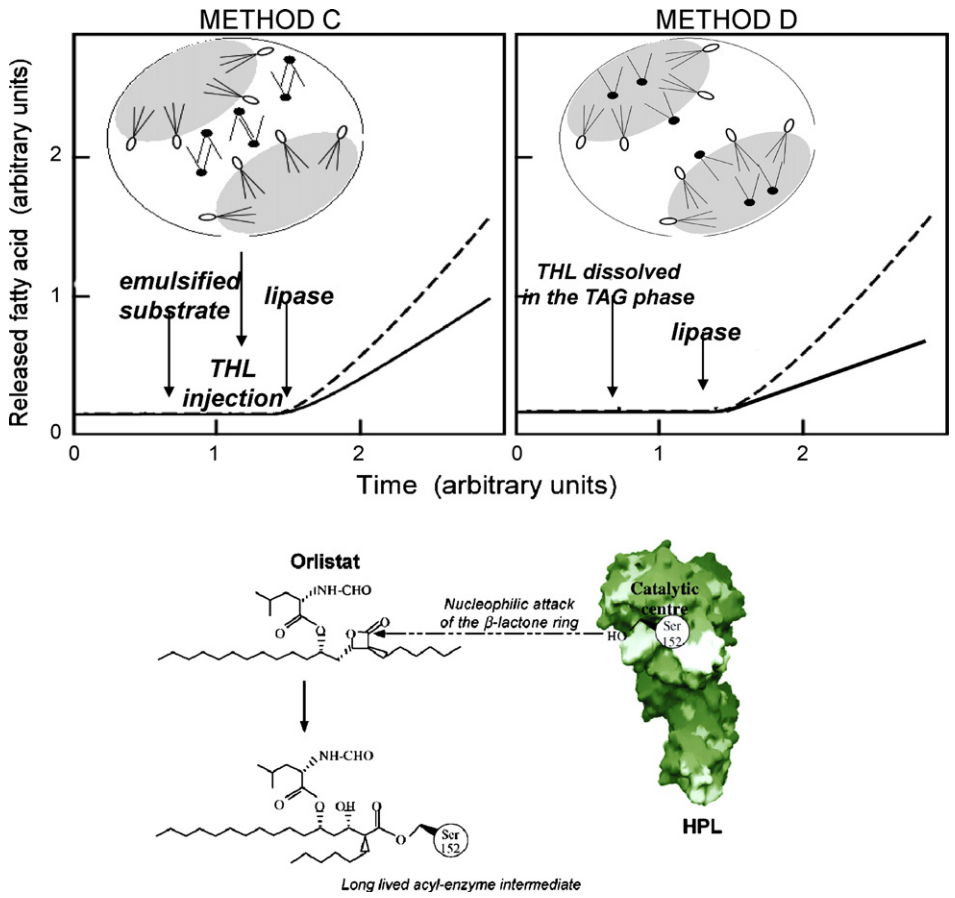

Fig. 1. Upper part: methods C and D ('poisoned interface' methods) used to compare the inhibitory effects of THL on the hydrolytic activity of HPL and TLL. The arrows indicate the order in which the emulsified substrate (TC4 or PSO), the inhibitor (THL from an ethanolic solution), and lipase (HPL + colipase or TLL) were injected. The inserts are diagrams showing the physico-chemical state of THL in either the aqueous phase (method C) or the TAG phase (method D). Empty ovales, with 3 chains, stand for triacylglycerol molecules present at the oil/water interface. Filled circles, with 2 chains, stand for THL molecules. The lines are plots of the kinetic recording in the absence (dotted lines) or the presence (continuous lines) of THL. Further details are given in the text. Lower part: schematic illustration of the covalent adduct between the active site serine (152) of HPL and the β-lactone ring of THL in its opened configuration.

compartment. The surface pressure was measured in the reservoir compartment with a Wilhelmy plate (perimeter 4 cm) attached to an electromicrobalance, which was connected in turn to a microprocessor programmed to regulate the movement of the mobile barrier. The latter, which was automatically driven by the barostat, moved backward and forward over the reservoir to keep the surface pressure constant, thus compensating for the substrate molecules removed from the film by the process of enzyme hydrolysis. The kinetics of the hydrolytic process was recorded for 20–25 min.

In the case of long-chain substrate (PSO), the reaction products which are water insoluble and remain in the surface film, the kinetics of hydrolysis were measured in the presence of 0.8 mg mL^{-1} β-CD in the subphase of the reaction compartment. β-CD is known to trap and dissolve the products of long-chain TAG hydrolysis and the lipase kinetics can therefore be recorded as previously described in the case of medium-chain lipid substrates [49].

Before being used the Teflon trough was systematically cleaned with tap water, and then gently brushed in the presence of distilled ethanol, before being washed again with tap water and finally rinsed with double-distilled water. The aqueous subphase of both compartments was composed of 10 mM Tris–HCl (pH 8.0) containing 150 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA. The aqueous subphase of the reaction compartment was continuously stirred at 250 rpm with two 2-cm magnetic bars at room temperature (23 ± 1 °C).

2.6. Oil drop experiments

The kinetics of PSO hydrolysis by the lipase were monitored by recording the decrease in the interfacial tension with time, using the oil drop tensiometer technique (ITConcept-Teclis, France), as described by Nury et al. [50]. The interfacial tension was measured by automatically analysing the drop profile on-line, using the Laplace/Young equation [51,52]. The tensioactive components, i.e., the lipolytic products accumulating with time at the surface of an oil drop result in a decrease in the interfacial tension, which in turn is correlated with changes in the oil drop profile [44,50,53]. The main advantage of the oil drop tensiometer technique over other interfacial methods is that it can be used to monitor the lipase activity on natural long-chain TAG while controlling the interfacial parameters.

A drop of PSO (5 μL) was delivered using a micro-syringe (Exmire) through a U-shaped stainless steel needle (internal diameter 0.56 mm), into an optical glass cuvette measuring 1 cm × 2 cm × 4.3 cm (Hellma, France) thermostated at 37 °C, containing 5 mL of an aqueous phase which was continuously stirred with a small magnetic bar at 280 rpm. The aqueous phase was composed of a 10-mM Tris–HCl buffer (pH 8.0), 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA. THL samples were previously dissolved at various concentrations in the PSO. HPL (with a fivefold molar excess of colipase) or TLL samples (final concentration: 4 nM) were injected into the aqueous phase (using a Hamilton syringe) 5 min after the formation of the oil drop. Lipase activity is measured by the

Table 1
THL concentration reducing the lipase activity to 50% of its initial value. THL concentrations are expressed as either volumic concentrations (I_{50}, μM) when the pH-stat technique was used, or as molar fractions at the interface (α_{50}) when the monomolecular film or oil drop techniques were used. Lipases were inhibited by THL when method C or D was used, in the absence of NaTDC and the presence of 5 mM $CaCl_2$ (see Section 2 for details).

Technique used	Substrate used	HPL	TLL	I_{50} or α_{50} ratio (TLL/HPL)
Method C				
pH-stat (I_{50}, μM)	TC4	2.2	0.2	0.1
	PSO	0.4	27	90
Method D				
pH-stat (I_{50}, μM)	TC4	2.8	132	47
	PSO	0.2	20	100
Monomolecular films (α_{50})	di-C10	2.5×10^{-6}	10×10^{-3}	4000
	1,2-DO	4.5×10^{-3}	0.1	22
	PSO	2×10^{-3}	0.5	250
Oil drop (α_{50})	PSO	20×10^{-3}	0.8	40

initial rate of decrease of interfacial tension over time, e.g., mN/m per minute.

3. Results

3.1. Method C

The inhibitory effects of THL on HPL and TLL activity were studied using the pH-stat technique. THL at various final concentrations was injected 1 min before the lipase was added to the pH-stat vessel containing emulsified TAG and the rate of hydrolysis of TC4 or PSO was then monitored. The residual lipase activity versus the final THL concentration is shown in Fig. 2. Control assays without THL, corresponding to the full activity (100%) of the lipase, were carried out and the specific activities (IU/mg) obtained were 7310 ± 258 and 5211 ± 367 using TC4, and 1848 ± 97 and 1728 ± 50 using PSO as the substrate with HPL and TLL, respectively.

As shown in Fig. 2, in the absence of bile salts, the rate of TC4 hydrolysis by HPL or TLL decreased sharply with increasing THL concentrations, showing a similar pattern in both cases. The THL concentration (I_{50}) which reduced the activity of HPL and TLL to 50% of the initial value was found to be around 2.2 and 0.2 μM, respectively (Table 1). When PSO was used as the substrate, THL was still found to be a potent HPL inhibitor (I_{50} = 0.4 μM), whereas TLL was found to be much less sensitive to THL inhibition (I_{50} = 27 μM). THL was therefore found to inhibit the hydrolytic activity of HPL on a PSO substrate more efficiently (5.5-fold) than on TC4. In sharp contrast, the inhibitory effects of THL on TLL activity were found to be roughly two orders of magnitude higher when TC4 rather than PSO was used as the substrate (Table 1). It is worth noting that upon comparing the relative efficacy (I_{50} ratio) of the THL inhibition (method C) recorded with 2 different lipases (HPL and TLL), this I_{50} ratio was found to vary by a factor of 900 depending on the type of TG (TC4 or PSO) used (see Table 1). This variability may explain some of the discrepancies observed between studies in which THL was used, with the pH-stat technique, to inhibit lipases of various origins (see Section 1).

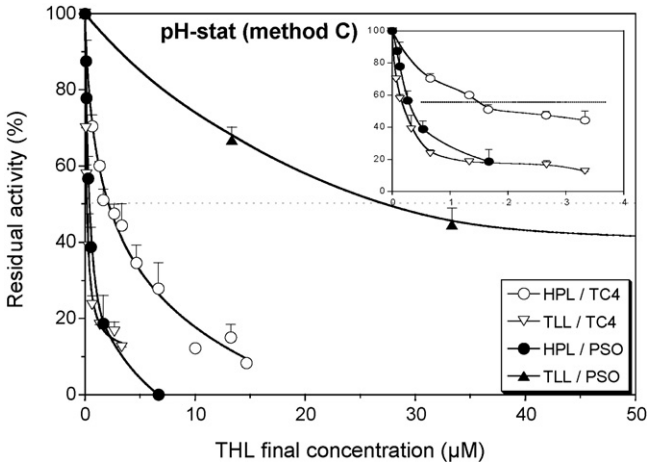

Fig. 2. Effects of increasing THL concentrations on HPL (○, ●) or TLL activity (▽ ▲): residual activity measured using the pH-stat technique on tributyrin, TC4 (○, ▽) or purified soybean oil, PSO (●, ▲) with method C (see Section 2 for details). Kinetic assays were performed in a thermostated (37 °C) pH-stat vessel containing 0.5 mL of TAG previously emulsified mechanically in 14.5 mL of 1 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl and 5 mM $CaCl_2$ in the absence of NaTDC. The final lipase concentration was 2 nM. Colipase was added to the pH-stat assay vessel in a 5-fold molar excess prior to the HPL injection. The dotted line indicates 50% inhibition, which was used to calculate the I_{50} values. The insert is a blow-up showing the residual activity (%) with the THL final concentration (expanded scale from 0 to 4 μM).

The inhibitory effects of THL on HPL were also studied in the presence of 4 mM NaTDC in the lipase assay vessel of the pH-stat and the results obtained are summarised in Table 2. With both substrates tested (TC4 and PSO), the presence of bile salts was found to enhance the inhibitory effects of THL on HPL (I_{50} = 0.3 and 0.1 μM) in comparison with those observed in the absence of NaTDC (I_{50} = 2.2 and 0.4 μM, using TC4 and PSO substrates, respectively), in agreement with previous studies [33,37]. In addition, the absence of calcium in the lipase assay was found to decrease the inhibitory effects of THL on HPL 1.3- to 9-fold (see Table 2).

In order to investigate why TLL was paradoxically so much more strongly inhibited by THL when a TC4 emulsion rather than a PSO emulsion was used as the substrate (see Fig. 2), the following experiments were then performed. The residual catalytic activity of TLL on TC4 emulsion was measured using the pH-stat technique and method C in the presence of THL (at a final concentration of 5 μM, at which TLL activity is inhibited by more than 90%, see Fig. 2). TLL sample was injected into the pH-stat assay vessel at various times ranging from 0 to 60 min after the THL injection and the residual activity of TLL was calculated (Fig. 3). As shown in this figure, THL was found to inhibit the hydrolytic activity of TLL on TC4 decreasingly efficiently as the time elapsing between the injection of THL and TLL increased. This behaviour probably reflects the gradual partitioning of THL between the aqueous and tributyrin phases [29]. It

Table 2
Effects of bile salts (4 mM) and calcium ions (5 mM) on the THL concentration (I_{50}, μM) reducing the lipase activity to 50% of its initial value. HPL was inhibited by THL using method C (see Section 2 for details).

Substrate	0 mM NaTDC		4 mM NaTDC	
	0 mM Ca	5 mM Ca	0 mM Ca	5 mM Ca
TC4	2.8	2.2	2.7	0.3
PSO	1.0	0.4	0.2	0.1

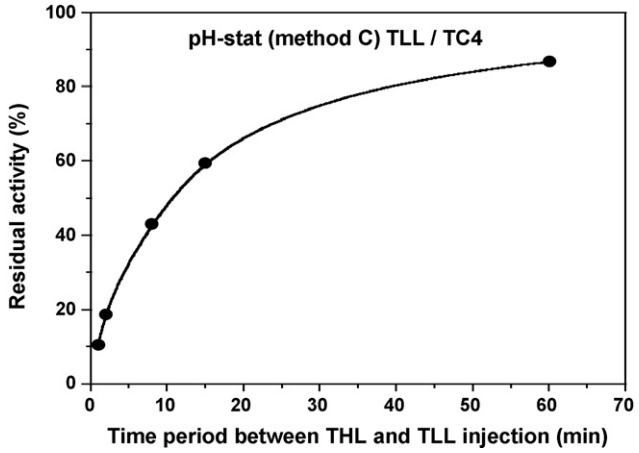

Fig. 3. Relationship between the time elapsing between the successive injections of THL and TLL into the pH-stat vessel and the residual activity of TLL measured using the pH-stat technique on TC4 with method C (see Section 2 for details). Kinetic assays were performed in a thermostated (37 °C) pH-stat vessel containing 0.5 mL of TAG substrate which was mechanically emulsified in 14.5 mL of 1 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl and 5 mM $CaCl_2$ in the absence of NaTDC. The final lipase concentration was 2 nM. As a control experiment, we measured the same inhibition levels after incubating THL, during either 1 min or 60 min, before injecting HPL in the pH-stat vessel containing 0.5 mL of TAG substrate (method C). The stability of THL was thus established under our experimental conditions.

was therefore decided to test another method (method D), whereby THL was initially dissolved in the lipid phase before the lipase was injected.

3.2. Method D

3.2.1. pH-stat experiments

Variable amounts of THL were dissolved directly in pure TC4 or PSO prior to the lipase assay and the rate of TAG hydrolysis by HPL or TLL was determined in the absence of bile salts as a function of the final THL concentration, as shown in Fig. 4.

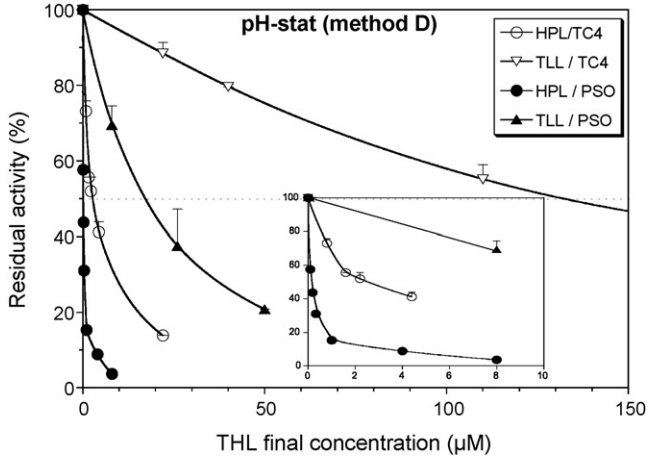

Fig. 4. Effects of increasing THL concentration on the residual activity of HPL (○, ●) or TLL (▽ ▲) measured using the pH-stat technique on tributyrin, TC4 (○, ▽) or purified soybean oil, PSO (●, ▲) with method D (see Section 2 for details). Kinetic assays were performed in a thermostated (37 °C) pH-stat vessel containing 0.5 mL of TAG substrate which was mechanically emulsified in 14.5 mL of 1 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl and 5 mM $CaCl_2$ in the absence of NaTDC. The final lipase concentration was 2 nM. Colipase was added to the pH-stat assay vessel in a 5-fold molar excess prior to HPL injection. The dotted line indicates 50% inhibition, which was used to calculate the I_{50} values. The insert is a blow-up showing the residual activity (%) with the THL final concentration (expanded scale from 0 to 10 μM).

The rates of hydrolysis of both TC4 and PSO by HPL decreased sharply with increasing THL concentrations, contrary to what occurred with TLL. With HPL, I_{50} values of around 2.8 and 0.2 μM were obtained when TC4 and PSO were used as substrates, respectively (see Table 1). In the case of TLL, these values were 132 and 20 μM with TC4 and PSO as substrates, respectively (see Table 1). With *method D*, THL was found to inhibit the activity of both HPL and TLL more efficiently when PSO rather than TC4 was used as the substrate. With both *methods C and D*, the inhibitory effects of THL on HPL were found to be similar (in terms of the I_{50} values) on a given substrate (TC4 or PSO). However, with TC4 as substrate TLL was efficiently inhibited by THL only when method C was used (Table 1) and not method D. It is worth noting that the relative inhibitory efficiency (the I_{50} ratio) of 2 different lipases (TLL and HPL) by THL (pH-stat technique and method D) can therefore vary by 2 orders of magnitude depending on the type of TG (TC4 or PSO) used (see Table 1). Accordingly, method D should be preferred to method C when it is proposed to compare the relative efficiency of a given inhibitor on various lipases. Despite the similarities between the architecture of their respective active sites, which include a nearly superimposable catalytic triad (Ser, His, and Asp), the inhibitory responses of HPL and TLL to THL are very different.

3.2.2. Monomolecular film experiments

The present kinetic studies were carried out at a surface pressure of 12 mN m^{-1} with the two mixed films THL/di-C10 and THL/PSO. At this surface pressure, which is below the collapse pressure of all the components (35, 25 and 13 mN m^{-1} in the case of di-C10, THL and PSO, respectively) [54], all the above pure monomolecular films were stable with time, i.e., no significant decrease in the surface pressure was observed within 20 min. In addition, HPL and TLL were active and linear kinetics were obtained when pure substrates were used.

The residual lipase activities were measured as a function of the THL molar fraction at the interface (α), and the results obtained are shown in Fig. 5. With both lipases tested, the hydrolysis of di-C10

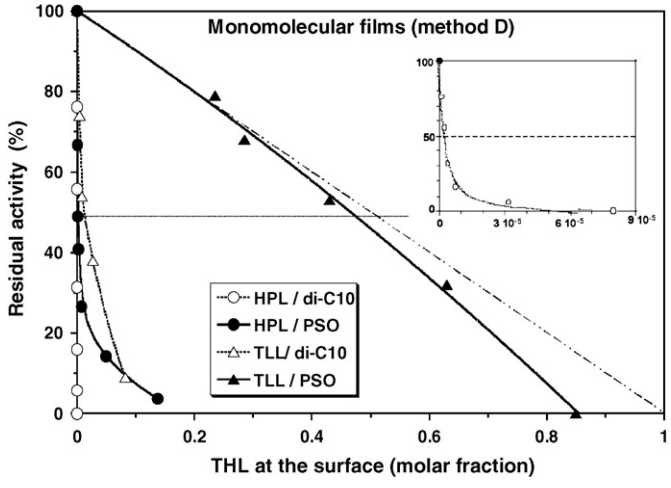

Fig. 5. HPL (○, ●) and TLL (△, ▲) residual activity on 1,2-dicaprin-rac-glycerol, di-C10 (○, △) or purified soybean oil, PSO (●, ▲) monomolecular films spread at the air–water interface at 12 mN m^{-1}, depending on the surface THL molar fraction. The subphase was composed of 10 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA in the absence of NaTDC. The final lipase concentration was 0.16 nM. When a PSO substrate was used, 0.8 mg mL^{-1} β-CD were added to the subphase in the reaction compartment. In all the experiments, the subphase of the reaction compartment was continuously stirred with two magnetic rods at a rate of 250 rpm and the temperature was 23 ± 1 °C (see Section 2 for details). The dotted line corresponds to ideal surface dilution [43]. The insert is a blow-up showing the residual activity of HPL on 1,2-dicaprin-rac-glycerol (di-C10) with the surface molar fraction (expanded scale from 0 to 9×10^{-5}) of THL.

Table 3
Radiolabeled [C^{14}] THL partitioning observed between the TG phase (TC4 or PSO) in the presence (4 mM) or absence of NaTDC in the aqueous phase, using either method C or D. Total radioactivity applied: 610 nCi (see [30] for experimental details).

Substrate	NaTDC (4 mM)	Radioactivity (nCi)		Radioactivity partition aqueous/lipid phase (%)
		Aqueous phase	Lipid phase	
Method C				
TC4	–	1.8 ± 0.1	598.8	0.30
	+	7.3 ± 0.3	600.6	1.22
PSO	–	0.4 ± 0.1	606.7	0.07
	+	3.8 ± 0.2	603.1	0.63
Method D				
TC4	–	1.2 ± 0.2	608.1	0.20
	+	1.7 ± 0.1	609.2	0.28
PSO	–	0.3 ± 0.1	608.2	0.05
	+	1.6 ± 0.1	608.7	0.27

decreased sharply as the surface molar fraction of THL increased. The surface molar fractions of THL (α_{50}) giving half inhibition were found to be 2.5×10^{-6} and 10×10^{-3} with HPL and TLL, respectively (Fig. 5 and Table 1). Comparable results were obtained using the same experimental conditions at $\pi = 20$ mN m^{-1} (data not shown).

When diolein (1,2-DO) was used as the substrate, THL α_{50} values of 4.5×10^{-3} and 0.1 were recorded with HPL and TLL, respectively (Table 1). When PSO was used as the substrate, a surface THL molar fraction of 2×10^{-3} suffice to reduce HPL activity to 50% (Table 1). However, THL had no effect on the lipolytic activity of TLL (Fig. 5), since the plot of TLL residual activity on the mixed THL/PSO film versus the surface THL molar fraction was near the line describing the decrease in the lipase activity only because of the ideal surface dilution [8,43].

With all the substrates tested here, the hydrolytic activity of HPL was therefore found to be much more strongly inhibited than that of TLL by THL: 1 molecule of THL embedded among 400 000 di-C10 molecules at the interface was found to suffice to inhibit the rate of hydrolysis of HPL to half its initial value (see Table 1). When PSO was used as the substrate, an interfacial stoichiometry of one THL molecule per 500 PSO molecules was required to achieve 50% HPL inhibition (see Table 1). These results can be explained qualitatively by the fact that THL is a poor lipase substrate (with a high affinity for lipase and a very low turnover number). The α_{50} or I_{50} values actually reflect the ratio between the relative interfacial lipase affinities for the substrate and THL, respectively (see Ransac et al. [8] for a more quantitative treatment of interfacial inhibition models).

3.2.3. Oil drop experiments

We previously studied the partitioning of THL between the core and the surface of an oil drop as a function of the THL concentration in the oil. The molar fraction of THL at the oil drop surface was estimated using Gibbs' surface excess rule [54]. From these data, it is possible to express THL concentrations in term of surface concentrations (or molar fractions) instead of volumic concentrations. Table 3 summarises the partitioning of THL between the aqueous and oil phases, using TC4 and PSO emulsions (with and without 4 mM NaTDC) with methods C and D.

The residual activities of HPL and TLL on PSO drops 'poisoned' with THL are given as a function of the THL molar fraction at the surface of the oil drop in Fig. 6. The rate of PSO hydrolysis by HPL decreased sharply as the molar fraction of THL increased, reflecting the potent inhibitory effects of THL on HPL activity. However, THL showed no inhibitory effects on TLL lipolytic activity. On the contrary, the curve of the residual TLL activity on the 'poisoned' PSO drops versus the surface THL molar fraction was located above the diagonal line describing the decrease in the lipase activity due to ideal surface dilution [8,43] This behaviour shows that under these conditions, THL paradoxically had moderately activatory effects on TLL. The surface molar fractions of THL (α_{50}) giving half inhibition were found to be 20×10^{-3} and 0.8 with HPL and TLL, respectively (Fig. 6 and Table 1), in agreement with the results obtained using the monomolecular film technique, which were presented above.

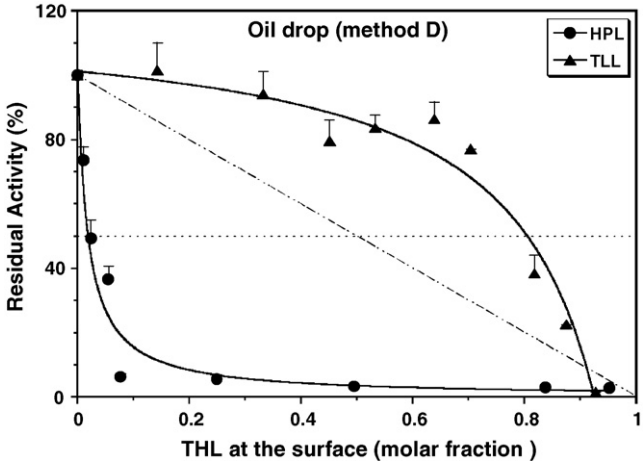

Fig. 6. Residual HPL (●) and TLL (▲) activity on purified soybean oil (PSO) as a function of the THL molar fraction calculated at the oil drop surface [54]. The aqueous solution (5 mL) consisted of 10 mM Tris–HCl buffer (pH 8.0), 150 mM NaCl, 21 mM $CaCl_2$ and 1 mM EDTA in the absence of NaTDC. The final HPL and TLL concentration was 4 and 1 nM, respectively. In all the experiments, the aqueous phase was continuously stirred with a magnetic rod at a rate of 280 rpm and the temperature was kept at 37 °C. The dotted line corresponds to ideal surface dilution [43]. See Section 2 for details.

Using method D, HPL was found to be strongly inhibited by THL on PSO with the pH-stat, monomolecular film and oil drop techniques, whereas TLL was found to be generally insensitive to the presence of THL at the interface (see Table 1).

4. Discussion

4.1. Inhibition of HPL by THL

The results of the present study clearly show that the activity of HPL was efficiently inhibited by THL with both methods of inhibition applied (C and D), all the TAG substrates tested (TC4, PSO, 1,2-DO and di-C10), and all the techniques used (the pH-stat, monomolecular film and oil drop techniques) (see Table 1). This inhibitory effect was enhanced in the presence of bile salts (see Table 2), although some inhibition of HPL activity also occurred in the absence of bile salts, when the activity of the lipase was tested on TC4 and PSO emulsions. Similar enhancing effects of bile salts on the inhibition of HPL activity by THL were observed previously

[37]. This increase in HPL inhibition by THL induced in the presence of bile salts may be attributable to the formation of mixed micelles, which may increase the availability of THL in the micellar solution and thus improve its inhibitory efficiency. This hypothesis is consistent with the solubilisation of THL in the aqueous micellar phase which has been reported to occur in the presence of bile salts [29].

As shown in Table 2, calcium was found to improve the efficiency of the inhibitory effects of THL on HPL activity. Calcium ions are known to increase the turnover of pancreatic lipase on TAG emulsions, which probably form a calcium soap with the hydrolytic products [55].

During the lipase assays performed using methods C or D, no increase with time in the activity of the inhibited HPL was observed (data not shown), contrary to what was observed previously [37]. This was probably due to the large interfacial molar ratio THL/substrate present during the lipase assay when methods C and D were used.

The ability of THL to inhibit the hydrolytic activity of HPL on PSO was found to be 10-fold higher when monomolecular films were used than with the single oil drop technique. This difference was probably due to the difference in the surface behaviour of THL at these two interfaces. We previously reported [54] that the THL molecules are packed very differently at air/water interfaces (70 $Å^2$/molecule) and oil/water interfaces (160 $Å^2$/molecule). This difference in the interfacial packing/orientation/conformation of THL may contribute decisively to the efficiency of the inhibitory effects.

4.2. Inhibition of TLL by THL

Unlike HPL, the activity of which showed a similar pattern of inhibition in response to THL with both methods C and D, TLL was efficiently inhibited only when method C was used with the pH-stat technique with a TC4 substrate (see Table 1). In the latter case, the inhibitory effects of THL on TLL were found to decrease depending on the time elapsing between THL and TLL injection into the pH-stat vessel (see Fig. 3). This difference was probably due to the occurrence of a decrease in the availability of THL in the aqueous phase due to its partitioning into the TC4 phase. In view of this finding, it can be concluded that TLL is inhibited by THL molecules which are transiently available in the aqueous phase. This conclusion is further supported by the results obtained using Method D with all the techniques and substrates tested (see Table 1), whereby THL was previously solubilised in the oil phase and a sufficiently large number of THL molecules were no longer available in the aqueous phase for TLL to be inhibited. In addition, a strong inhibitory effect was observed when TLL was pre-incubated with THL in the absence of TAG substrate (method A) [37].

THL is an amphiphilic compound which forms stable films at the air/water interface [8,54]. In the presence of emulsified TAG substrate, THL is distributed mainly (up to more than 98.8%) towards the oil phase in the presence or absence of NaTDC in the aqueous phase ([30] and Table 3). In addition, the partitioning of THL between the core of the oil droplets and an oil/water interface is in favour of the latter [30,31]. THL is therefore readily available at the oil/water interface, the main site of lipase activity. However, the partitioning and the packing/orientation/conformation of THL at an oil/water interface may depend on the type of TAG used. It seems reasonable to assume that the partitioning of THL between the oil droplet surface and its core is more favourable for the interface in the case of PSO than in that of TC4 due to the difference between their respective hydrophilic/lipophilic balance. This assumption is supported by the fact that when method D was used, THL showed higher levels of inhibitory efficiency on both HPL and TLL with a PSO substrate than with TC4 (see Table 1). In conclusion, the present data show that THL is a potent HPL inhibitor at lipid/water interfaces, in the presence of various lipidic substrates, whereas TLL is less sensitive to the THL molecules present at lipid/water interfaces, which seems to be a paradoxical situation in the case of a true lipase. TLL is mostly inhibited by the THL molecules transiently present into the aqueous phase. It should therefore be stressed that the inhibitory effects of THL on each lipase depends strongly on the method and the substrate used.

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