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Prevention of in Vitro Lipolysis by Tetrahydrolipstatin

Michael Krebs, Harald Stingl, Peter Nowotny, Daniel Weghuber, Martin Bischof, Werner Waldhäusl, and Michael Roden*

Background: **Metabolic effects of free fatty acids (FFAs) frequently are tested using combined infusion of triglycerides and heparin, which stimulates lipolysis in vivo. Ongoing in vitro lipolysis, however, probably produces falsely high plasma FFA concentrations under these conditions. Therefore, this study aims to assess the efficacy of tetrahydrolipstatin (THL) in inhibiting plasma lipolytic activity and to improve plasma FFA determination.**

Methods: **Plasma concentrations of FFAs and glycerol were measured in five healthy subjects in the presence and absence of THL. Blood was drawn at baseline, during infusion of a triglyceride emulsion (1.5 mL/min), and during infusion of triglycerides plus heparin (0.2 IU · kg^{-1} · min^{-1}). In addition, the effects of storage temperature of the samples were analyzed.**

Results: **In samples frozen immediately after collection, plasma FFAs were 28% lower in the presence of THL than in its absence ($P = 0.008$). When THL-free plasma was incubated for 3 h on ice or at room temperature, plasma FFAs were 22% ($P = 0.02$) and 91% ($P = 0.0004$) higher, respectively, than in samples frozen immediately. The addition of THL blunted temperature-dependent in vitro lipolysis by 88% ($P < 0.01$) and 89% ($P < 0.001$) after incubation on ice and at room temperature, respectively. Changes in plasma glycerol concentrations exhibited similar behavior.**

Conclusions: **THL, which is safe and easy to handle, is a potent inhibitor of in vitro lipolysis and could, therefore, be added to blood samples drawn during triglyceride/heparin infusions to allow more accurate determination of plasma FFA concentrations.**

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Plasma concentrations of free fatty acids (FFAs)[1] are increased in diabetes mellitus type 2 *(1)* and other states of insulin resistance, particularly in obesity *(2)* associated with impaired insulin secretion *(3)*. Because FFAs might play an important role in the development of insulin resistance and regulation of insulin secretion, their effects on glucose metabolism frequently are studied by experimental decreases *(4, 5)* or increases of plasma FFAs. Experimental plasma FFA increases usually are achieved by combined triglyceride/heparin infusions *(6–15)*. Heparin releases lipoprotein lipase (LPL) from the endothelium and thereby stimulates the breakdown of triglycerides to FFAs *(11)*. Because lipolysis initiated intravascularly may continue in plasma samples after collection, efficient inhibition of lipolytic activity is needed to avoid such in vitro lipolysis, which would probably produce artificially high FFA concentrations *(16)*. The broad range of plasma FFA concentrations, from ~0.8 mmol/L *(4)* to ~4.7 mmol/L *(8, 15)*, reported in previous studies despite comparable composition and dosages of triglyceride/heparin infusions indicates that in vitro lipolysis may in fact be a relevant problem for the determination of plasma FFA concentrations under these conditions.

Unfortunately, Paraoxon (diethyl *p*-nitrophenyl phosphate), the only LPL inhibitor tested thus far to reduce in vitro lipolysis *(12)*, is difficult to handle because of its potent neurotoxic properties, which limit its widespread use *(16)*. More recently, tetrahydrolipstatin (THL; Orlistat®) was identified as a nontoxic, active-site inhibitor of mammalian lipases, including LPL *(17)*, which could be used to effectively decrease lipolytic activity in plasma *(18)*. The present study was therefore designed to determine the effects of THL and storage temperature on concentrations of FFA, glycerol, and triglycerides in plasma samples obtained during intravenous infusion of a triglyceride emulsion plus heparin.

Division of Endocrinology and Metabolism, Department of Internal Medicine III, University of Vienna Medical School, Währinger Gürtel 18-20, A-1090 Vienna, Austria.

*Author for correspondence. Fax 43-1-40400-7790; e-mail michael.roden@akh-wien.ac.at.

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[1] Nonstandard abbreviations: FFA, free fatty acid; LPL, lipoprotein lipase; and THL, tetrahydrolipstatin.

Materials and Methods

SUBJECTS

Five healthy male volunteers (age, 26.4 ± 2 years; body mass index, 21.9 ± 3 kg/m^2) without family history of diabetes mellitus, dyslipidemia, or bleeding disorders were included in the study. They did not take any medication on a regular basis. All subjects gave informed consent to the protocol, which was reviewed and approved by the local ethics board.

STUDY PROTOCOL

After subjects fasted for 12 h overnight, studies were begun at 0800 with the insertion of catheters (Vasofix®; Braun) into one antecubital vein of both the left and the right arms for blood sampling and infusions, respectively. Plasma triglyceride concentrations were raised by infusion (0–180 min) of a triglyceride emulsion (1.5 mL/min; Intralipid® 20%, a gift from Pharmacia & Upjohn, Vienna, Austria). To stimulate LPL activity, heparin (bolus, 200 IU; continuous infusion, 0.2 IU · kg^{-1} · min^{-1}; Immuno AG) was infused from 90 to 180 min *(19)*. This heparin dosage is submaximal to release maximum lipolytic activity *(20)* and is commonly applied in metabolic studies in humans *(9, 13)*.

BLOOD COLLECTION AND INHIBITION OF LIPOLYTIC ACTIVITY

A stock solution of 1 g/L THL was prepared from Xenical® capsules, which were a generous gift from Roche (Vienna, Austria), by dissolving the contents of one capsule in 996 mL/L ethanol. The resulting solution was centrifuged for 10 min at 2000*g* to remove insoluble filling materials, such as cellulose, additionally contained in the capsules.

Blood samples with and without THL (final concentration, 1 mg/L) were collected at 0 min (baseline), 90 min (triglyceride infusion), and 180 min (triglyceride/heparin infusion) in two sets of prechilled tubes. Each tube contained 200 μL of EDTA solution (0.33 mol/L in H_2O) as an anticoagulant. In addition, 100 μL of THL (100 mg/L in ethanol) was added to one set of three tubes; the other set contained 100 μL of solvent (996 mL/L ethanol). At the respective time points, 10 mL of blood was added to one tube of each set, which was immediately inverted several times to ensure thorough mixing. Both sets of tubes were placed on ice, and plasma was instantly separated from cells by centrifugation for 15 min at 2500*g* at 4 °C.

To determine the effect of temperature on in vitro lipolysis, plasma from each tube was divided into three equal aliquots. The first aliquot was frozen immediately (−20 °C), the second aliquot was incubated for 3 h on ice, and the third aliquot was incubated for 3 h at room temperature (~20 °C). The second and third aliquots were subsequently also stored at −20 °C until the analyses.

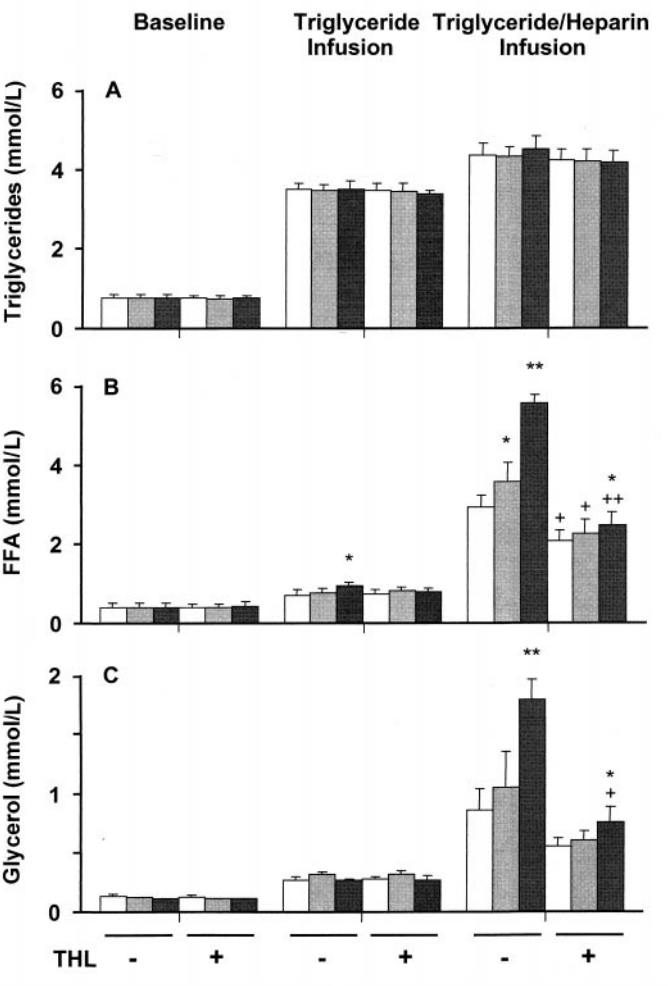

Fig. 1. Plasma concentrations of triglycerides (*A*), FFAs (*B*), and glycerol (*C*) determined in the presence and absence of THL at baseline, during infusion of triglycerides alone, and during infusion of triglycerides plus heparin.

All plasma samples were divided into three identical aliquots, which were frozen immediately (□), incubated on ice for 3 h (▨), or incubated at room temperature for 3 h (■) before analysis. Compared with respective immediately frozen aliquot: *, $P < 0.05$; **, $P < 0.01$. Compared with respective sample without THL: +, $P < 0.01$; ++, $P < 0.001$.

ANALYTICAL METHODS

Plasma FFA concentrations were measured using a microfluorometric method (Wako Chemicals). The intra- and interassay CVs were 4.3% and 5.7%, respectively. Glycerol was determined using a commercially available enzyme assay from Roche with intra- and interassay CVs of 2.0% and 3.4%, respectively. Plasma triglycerides were hydrolyzed by lipase, and the released glycerol was measured by a peroxidase-coupled colorimetric assay (Roche) with intra- and interassay CVs of 5.0% and 6.9%, respectively.

STATISTICAL EVALUATION

All data are given as means ± SE if not stated otherwise. Statistical comparisons were performed using the paired

Student *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

Plasma triglyceride concentrations increased 4.58- and 5.61-fold at 90 and 180 min of lipid infusion, respectively ($P < 0.001$ vs baseline of immediately frozen samples; Fig. 1A and Table 1). Storage temperature and the presence of THL did not affect plasma triglyceride concentrations.

At baseline, FFA concentrations were identical under all conditions of sample storage and treatment (Fig. 1B and Table 1). During triglyceride infusion, plasma FFA concentrations rose moderately ($P < 0.05$ vs baseline). In immediately frozen plasma samples, FFA concentrations were similar regardless of the presence or absence of THL. However, incubation for 3 h at room temperature increased plasma FFA concentrations slightly ($P = 0.05$ compared with immediately frozen samples). This temperature-dependent increase was observed only in plasma collected without added THL.

During combined triglyceride/heparin infusion, plasma FFA concentrations increased by a further 429.4% ($P = 0.001$ vs baseline of immediately frozen plasma containing THL). In immediately frozen samples, FFA concentrations were 28.4% lower in the presence of THL than in its absence ($P = 0.008$). In plasma drawn during triglyceride/heparin infusion, storage temperature markedly affected FFA concentrations. In the absence of THL, plasma FFAs were 22.0% ($P = 0.02$) and 90.6% ($P = 0.0004$) higher compared with immediately frozen plasma after 3 h of incubation on ice and at room temperature, respectively. The addition of THL blunted this temperature-dependent increase of plasma FFAs: FFA concentrations did not increase after incubation of THL-containing plasma on ice (8.0%; *P*, not significant) and rose only slightly after incubation at room temperature (18.3%; $P = 0.025$ vs immediately frozen samples).

The changes in plasma glycerol showed a pattern similar to the corresponding changes in plasma FFAs (Fig. 1C and Table 1). Baseline glycerol concentrations were not affected by storage temperature or THL but increased by 117.6% during infusion of triglyceride emulsion, which already contained 2.5% free glycerol ($P < 0.05$ in immediately frozen samples with THL). The additional infusion of heparin further augmented the rise of plasma glycerol by 100.0% in rapidly frozen samples ($P < 0.05$ vs triglyceride infusion in the presence of THL). Under these conditions, plasma glycerol was 111.5% ($P < 0.01$) higher compared with immediately frozen plasma after 3 h of incubation at room temperature in the absence of THL. The addition of THL substantially diminished this temperature-dependent increase in plasma glycerol concentrations by 77.9% ($P < 0.01$).

Discussion

At baseline, plasma concentrations of triglycerides, FFAs, and glycerol were not affected by storage conditions or by

Table 1. Means ± SD of data presented in Fig. 1 in the absence and presence of THL.

		Baseline			Triglyceride infusion			Triglyceride/Heparin infusion		
	THL[a]	−20 °C	4 °C	20 °C	−20 °C	4 °C	20 °C	−20 °C	4 °C	20 °C
Triglycerides, mmol/L	−	0.775 ± 0.157	0.770 ± 0.181	0.767 ± 0.203	3.500 ± 0.356	3.471 ± 0.360	3.503 ± 0.479	4.355 ± 0.704	4.321 ± 0.561	4.512 ± 0.729
	+	0.752 ± 0.177	0.741 ± 0.170	0.752 ± 0.170	3.460 ± 0.405	3.454 ± 0.423	3.377 ± 0.232	4.221 ± 0.651	4.209 ± 0.644	4.181 ± 0.657
FFAs, mmol/L	−	0.398 ± 0.241	0.405 ± 0.241	0.410 ± 0.246	0.690 ± 0.331	0.769 ± 0.288	0.935 ± 0.206[a,b]	2.914 ± 0.716	3.556 ± 1.076[a,b]	5.554 ± 0.467[c]
	+	0.394 ± 0.241	0.387 ± 0.253	0.440 ± 0.219	0.725 ± 0.253	0.810 ± 0.217	0.779 ± 0.259	2.086 ± 0.557[d]	2.253 ± 0.807[d]	2.467 ± 0.778[b,e]
Glycerol, mmol/L	−	0.130 ± 0.040	0.117 ± 0.018	0.109 ± 0.011	0.266 ± 0.058	0.313 ± 0.040	0.263 ± 0.034	0.847 ± 0.423	1.049 ± 0.671	1.791 ± 0.378[c]
	+	0.125 ± 0.036	0.108 ± 0.016	0.109 ± 0.013	0.272 ± 0.056	0.315 ± 0.069	0.269 ± 0.080	0.544 ± 0.172	0.603 ± 0.174	0.753 ± 0.284[b,d]

[a] −, absence; +, presence.
[b,c] Compared with respective immediately frozen (−20 °C) aliquot: [b]$P < 0.05$; [c]$P < 0.01$.
[d,e] Compared with respective sample without THL: [d]$P < 0.01$; [e]$P < 0.001$.

the presence of THL, indicating that THL itself does not interfere with the assays used for plasma analyses.

In contrast to lipid administration without heparin, combined triglyceride/heparin infusion strongly enhanced in vivo lipolysis, as indicated by a marked rise of both plasma FFA and glycerol concentrations. The plasma FFA concentrations reached in this study are in the mean range of those reported previously in studies using identical triglyceride/heparin infusions (~0.8 to ~4.7 mmol/L) *(6–15)*. In addition to in vitro lipolysis, differences in (*a*) assay methods, (*b*) sample handling, (*c*) duration of lipid infusion, and (*d*) dosage (bolus) of heparin despite identical continuous triglyceride infusion might have contributed to the substantial diversity in plasma FFA concentrations reported previously.

The data presented in this report demonstrate that lipolysis continues in plasma samples obtained after combined infusion of triglycerides and heparin, leading to artificially high plasma FFA and glycerol concentrations. It is of note that the maximum FFA concentrations attained without a lipolysis inhibitor is not possible in vivo because the number of albumin binding sites in plasma is exceeded *(21)*. The rate of in vitro lipolysis is temperature dependent and is substantially blunted by the LPL inhibitor THL. This antilipolytic action of THL applies to the same extent to plasma FFA and glycerol concentrations.

Because FFA concentrations were 28.4% lower in immediately frozen samples with added THL than in those without added THL, even instantaneous freezing of plasma obtained during triglyceride/heparin infusion cannot fully prevent ongoing lipolysis. Despite the presence of THL, slight temperature-dependent increases in FFA and glycerol concentrations were observed. This suggests that a small percentage of plasma lipolytic activity is not inhibited by THL at room temperature, although a >600-fold molar excess of THL/LPL was reached assuming a plasma LPL protein concentration of ~345 μg/L after heparin administration *(18)*. THL is a quick and effective inhibitor of LPL *(17, 22)* that partitions into the surface layer of lipid emulsion particles *(23)*. However, prolonged incubation and the presence of lipid/water interfaces can slowly reverse the interaction between THL and LPL, indicating that THL serves as a pseudosubstrate for the enzyme *(17)*. Although the neurotoxin Paraoxon irreversibly inhibits LPL *(24)*, it is also not able to completely suppress in vitro lipolysis *(16)*. Thus, to avoid residual in vitro lipolysis, plasma samples should be stored on ice before being either assayed immediately or frozen, even when THL is present in the test tubes.

In conclusion, this study demonstrates that (*a*) lipolysis initiated intravascularly by combined infusion of triglycerides and heparin continues in vitro in a temperature-dependent manner and thereby leads to an overestimation of plasma FFA and glycerol concentrations; (*b*) immediate freezing of plasma drawn under these conditions alone is not sufficient to limit in vitro lipolysis; (*c*) THL, which is safe and effectively blocks plasma lipolytic activity without affecting the determination of preexisting FFA and glycerol concentrations, largely prevents the overestimation of plasma FFA and glycerol concentrations.

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