



Novel experimental protocol to increase specific plasma nonesterified fatty acids in humans

CARINE BEYSEN,¹ ABIGAIL K. BELCHER,¹ FREDRIK KARPE,¹
BARBARA A. FIELDING,¹ EMILIO HERRERA,² AND KEITH N. FRAYN¹

¹Oxford Centre for Diabetes, Endocrinology and Metabolism, Radcliffe Infirmary, Oxford OX2 6HE, United Kingdom; and ²Faculty of Experimental and Technical Sciences, University San Pablo-CEU, E-28668 Madrid, Spain

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Beysen, Carine, Abigail K. Belcher, Fredrik Karpe, Barbara A. Fielding, Emilio Herrera, and Keith N. Frayn. Novel experimental protocol to increase specific plasma nonesterified fatty acids in humans. *Am J Physiol Endocrinol Metab* 284: E18–E24, 2003. First published September 17, 2002; 10.1152/ajpendo.00113.2002.—This study reports a novel protocol to increase plasma monounsaturated, polyunsaturated, and saturated nonesterified fatty acids (NEFA) in eight healthy volunteers (age 29–54 yr, body mass index 23–26 kg/m²). This was achieved by feeding small boluses of fat at different time points (35 g at 0 min and 8 g at 30, 60, 90, 120, 150, 180, and 210 min) in combination with a continuous low-dose heparin infusion. Olive oil, safflower oil, or palm stearin were used to increase monounsaturated, polyunsaturated, or saturated NEFAs, respectively. Plasma NEFA concentrations were increased for 2 h, when fat and heparin were given (olive oil: 745 ± 35 μmol/l; safflower oil: 609 ± 37 μmol/l, and palm stearin: 773 ± 38 μmol/l) compared with the control test (no fat and no heparin: 445 ± 41 μmol/l). During the heparin infusion, 18:1 *n*-9 was the most abundant fatty acid for the olive oil test compared with 18:2 *n*-6 for the safflower oil test and 16:0 for the palm stearin test (*P* < 0.01). The method described here successfully increases several types of plasma NEFA concentrations and could be used to investigate differential effects of elevated individual NEFAs on metabolic processes.

monounsaturated nonesterified fatty acids; polyunsaturated nonesterified fatty acids; saturated nonesterified fatty acids

INAPPROPRIATELY HIGH CONCENTRATIONS of plasma nonesterified fatty acids (NEFAs) have been linked to an impairment of insulin-stimulated glucose utilization (10) and an increase in hepatic glucose production (9). Adverse consequences of increased plasma NEFAs on very low-density lipoprotein production, myocardial function (23), insulin-mediated vasodilatation, and nitric oxide production (26) have also been recognized. Recently, attention has focused on the short- and long-term effects of elevated plasma NEFAs on insulin secretion (1, 6).

The traditional method to increase plasma NEFA concentrations experimentally in vivo is by intrave-

nous infusion of a triacylglycerol (TAG) emulsion in combination with a continuous low-dose heparin infusion. Heparin releases lipoprotein lipase (LPL) from the endothelium into the circulation. This results in an increase in LPL action on emulsion particles and leads to an increase in plasma NEFA concentrations. In contrast to intravenous protocols, oral fat feeding results in several metabolic changes, such as the secretion of gastrointestinal hormones, including glucagon-like peptide 1 and glucose-dependent insulinotropic peptide, and an increase in splanchnic blood flow, which may also be relevant for the metabolic effect investigated.

Most previous studies of elevated NEFA concentrations are based on the effect of a particular mixture of fatty acids (mostly polyunsaturated fatty acids), since only a limited number of intravenous TAG emulsions with similar fatty acid composition are available (e.g., Intralipid, Soyacal, Lipofundin, Liposyn II). Intravenous fat emulsions high in saturated fatty acids are not commercially available. Therefore, the traditional approach to increasing plasma NEFA concentrations excludes the possibility of comparing effects of different classes of plasma NEFAs on any of the aspects mentioned above. Until now, no method has been available to increase specific plasma NEFAs, partly because of the lack of fat emulsions high in specific fatty acids.

When heparin is infused intravenously, in vitro lipolysis becomes a problem. The LPL released into the circulation by heparin will still be active in the blood sample and needs to be inhibited immediately to reflect true plasma NEFA concentrations. This is not a widely recognized problem, and inadequate handling of the blood samples could explain the extremely high and varying plasma NEFA concentrations reported in several previous studies (7, 15). Methods to inhibit the postsampling in vitro lipolysis in such protocols are needed. Tetrahydrolipstatin (THL), a pancreatic lipase inhibitor, is also an inhibitor of LPL activity (18), and we have tested the use of THL for inhibiting in vitro lipolysis in the protocol developed here.

Address for reprint requests and other correspondence: K. Frayn, Oxford Lipid Metabolism Group, Oxford Centre for Diabetes, Endocrinology and Metabolism, Radcliffe Infirmary, Oxford OX2 6HE, UK (e-mail: keith.frayn@oxlip.ox.ac.uk).

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Table 1. Demographic and baseline (fasting) characteristics of the 3 study groups for all men and women

	Study Group					
	In vitro lipolysis		Protocol development		Final protocol	
	Men	Women	Men	Women	Men	Women
<i>n</i>	2	2	5	2	4	4
Age	50 ± 3	24 ± 3	23 ± 2	20 ± 1	34 ± 7	30 ± 1
BMI	22 ± 1	21 ± 1	23 ± 1	19 ± 1	24 ± 2	22 ± 1
NEFA, μmol/l	711 ± 113	873 ± 114	338 ± 82	600 ± 40	248 ± 18	285 ± 27
TAG, μmol/l	920 ± 116	555 ± 86	954 ± 356	608 ± 141	935 ± 122	827 ± 127
Glucose, mmol/l	5.1 ± 0.1	4.9 ± 0.1	5.0 ± 0.5	4.8 ± 0.1	4.7 ± 0.2	4.7 ± 0.2
Insulin, mU/l	8.1 ± 1.1	7.8 ± 0.7	4.2 ± 1.5	6.2 ± 1.7	10.1 ± 1.1	8.5 ± 0.9

Data are *n* or means ± SE. BMI, body mass index; NEFA, nonesterified fatty acid; TAG, triacylglycerol.

The main aim of this study was to develop a novel protocol to achieve an acute and sustainable steady-state increase in mainly plasma nonesterified monounsaturated (MUFA test), polyunsaturated (PUFA test), or saturated NEFAs (SFA test). Here, small portions of fat were given orally every 30 min for 3.5 h. This is in contrast to the traditional method for increasing plasma NEFAs, where fat is continuously infused intravenously. Three studies were performed. First, the use of THL was tested as mentioned above (*in vitro lipolysis study*). Second, a study was undertaken to obtain an acute steady-state increase in total plasma NEFA concentrations by titration of the oral fat to the amount of heparin administered intravenously (*protocol development study*). Once a satisfactory protocol to increase total plasma NEFA concentrations was established, different sources of fat (olive oil, safflower oil, and palm stearin) were used for elevating specific classes of NEFAs. This was assessed in eight healthy volunteers and compared with a control experiment where no fat or heparin was given (*final protocol study*).

METHODS

Subjects

Healthy subjects were recruited to take part in one of the three studies described. Characteristics of the subjects are given in Table 1. All of the subjects were nonsmokers, and no medication was taken likely to affect lipid metabolism. All subjects maintained a stable weight at least a month before and during the study, and no changes in diet and exercise were reported. The same standard low-fat meal (380 kJ, 6 g fat, 54 g carbohydrate, and 27 g protein) was given the evening before each experiment to all volunteers. The female subjects were studied during the second half of their menstrual cycle on each occasion. All volunteers gave informed consent, and the studies were approved by the Central Oxford Research Ethics Committee.

For the *in vitro lipolysis study*, four healthy subjects volunteered, and in the morning after breakfast, they were given an intravenous heparin bolus (500 IU, Leo Laboratories, Dublin, Ireland) to increase plasma LPL action. A blood sample was taken 15 min later.

For the *protocol development study*, seven healthy subjects volunteered. Two subjects took part in one experiment, three in two experiments, one in three experiments, and one in four experiments. They fasted overnight for 12 h.

The *final protocol study* was assessed in eight healthy volunteers. Each volunteer participated on four occasions with an interval of ≥2 wk. They fasted overnight for 12 h and then, in random order, mainly plasma monounsaturated NEFAs (MUFA test), polyunsaturated NEFAs (PUFA test), saturated NEFAs (SFA test) or no fatty acids (control) were increased.

Fat Administration

The fat was given orally as hot chocolate-flavored drinks (50% fat emulsions). The ingredients are given in Table 2. The fat emulsion was prepared as follows. The oil plus the monoglyceride emulsifier were heated to 65–70°C in a microwave oven (2–3 min, 750 W) until the emulsifier was melted in the oil. When the palm stearin was used, it was particularly important to melt the oil thoroughly to achieve a stable emulsion. At the same time, ~150 g of water were heated to the boiling point (microwave or pan). The beaker with the hot oil and the emulsifier was placed in a container with hot water to prevent the oil from cooling during the rest of the process. The cocoa powder and sweetener (aspartame) were

Table 2. Ingredients for 200 g of chocolate-flavored fat emulsions and %fatty acid composition of different oils used in protocol development and the final protocol studies

Ingredients	Amount, g		
	PUFA test	MUFA test	SFA test
Oils			
Safflower oil ^a	100		
Olive oil ^b		100	
Palm stearin ^c			100
%Fatty acid composition of oil			
16:0	8	14	59
18:0	3	3	5
18:1 <i>n</i> -9	15	69	28
18:2 <i>n</i> -6	74	11	6
Monoglycerides ^d	1	1	1
Sweetener ^e	2	2	2
Cocoa powder ^f	6	6	6
Water	91	91	91

PUFA, MUFA, and SFA, polyunsaturated, monounsaturated, and saturated fatty acids, respectively. ^aRefined safflower oil (Anglia Oils, Hull, UK); ^bOlive oil (Tesco, Oxford, UK); ^cPalm stearin (Anglia Oils); ^dEmulsifier (HYMONO 8903K, Quest International, Zwijndrecht, The Netherlands); ^eSweetener (Canderel, High Wycombe, UK); ^fCocoa (Cadbury, Premier Brands UK, Stafford, UK; contains <30 mg caffeine/6 g).

added to the oil. All ingredients were mixed with a hand mixer (the end of the hand mixer was warmed before use by being placed in hot water) while 91 g of the hot water (no longer boiling) were added very slowly. This mixture was blended for ≥ 2 min to become a smooth drink. The drinks were kept warm (65–70°C) until needed or were reheated 15–20 min before use in a bottle warmer. The subjects consumed the drinks within 2 min.

For the *protocol development study*, olive oil was used.

For the *final protocol study*, different oils were used on each occasion to increase the fatty acid of interest. For the MUFA test, olive oil high in 18:1 *n*-9 was used; for the PUFA test, safflower oil high in 18:2 *n*-6 was used; and for the SFA test, palm stearin high in 16:0 was used (Table 2). For the control study, the oil was replaced with an equal amount of water. The fat (or water for the control study) was given orally in small portions during the first 210 min of the experiment. Thirty-five grams of fat were given at 0 min followed by 8 g of fat at 30, 60, 90, 120, 150, 180, and 210 min.

THL Stock Solution

Because pure THL was not available, Xenical capsules (Orlistat, Hoffman-La Roche) containing 120 mg of THL were used. The contents of one capsule were finely ground with a mortar and pestle. This was dissolved in 40 ml of ethanol. The opaque solution was filtered or centrifuged for 10 min at 3,000 rpm. The stock THL solution (3 g/l in ethanol) was stored in the fridge for up to several months.

Sample Handling to Prevent In Vitro Lipolysis

To prevent ongoing lipolysis in the test tube when heparin was given intravenously, blood samples were collected in precooled heparinized syringes (Monovette; Sarstedt, Leicester, UK), and the plasma was immediately separated by centrifugation at 2°C. THL (30 μ g/ml plasma) was immediately added to the plasma. It was found that addition of ethanolic THL to whole blood caused hemolysis, which interfered with the enzymatic analysis of NEFAs and TAGs. Samples were kept in the fridge for analysis on the same day or frozen at –20°C for later analysis.

Experimental Protocols

For the *in vitro lipolysis study*, the postheparin blood sample was separated immediately and split into four portions. The portions were kept 1) at room temperature, 2) on ice, 3) at room temperature plus THL (30 μ g/ml plasma), and 4) on ice plus THL (30 μ g/ml plasma). Plasma NEFA concentrations were analyzed at 0, 30, 60, 150, and 210 min to test lipolysis of plasma TAGs in the test tube.

For the *protocol development and final protocol studies*, a cannula was inserted retrogradely into a vein draining a hand. This hand was warmed in a hotbox (air temperature of 65°C) throughout the experiment to provide arterialized blood samples. An antecubital vein was also cannulated for infusion of heparin or saline (control). Two fasting samples were taken at –30 min and at 0 min.

For the *protocol development study*, five different protocols were tested and are described in Fig. 2.

Analytical Methods

Total plasma NEFA (Wako NEFA C kit; Alpha Laboratories, Eastleigh, UK), plasma TAG (Randox, Crumlin, UK), and plasma free glycerol concentrations (16) were measured enzymatically with an IL Monarch automated analyzer (Instrumentation Laboratory, Warrington, UK). When heparin

was administered, an aliquot without THL had to be used for TAG measurement (see DISCUSSION). The result gives total glycerol (TAG plus free glycerol). This was corrected for free glycerol by use of an aliquot of plasma that had been deproteinized immediately with perchloric acid to inhibit LPL activity (16). Perchloric acid samples are not suitable for TAG and NEFA measurement due to inhibition of LPL used in the TAG kit and denaturing of the albumin-NEFA fraction. For analysis of specific fatty acids, lipids were extracted from plasma by using chloroform-methanol (2:1, vol/vol). The plasma NEFA fractions were then separated from total plasma lipid extracts by solid-phase extraction using aminopropyl silica columns (Harbor City, CA) (4) followed by methylation of fatty acids with methanolic sulfuric acid. Gas chromatography was used to analyze the fatty acid composition of the plasma NEFA fractions and oils (11). Plasma lipase activities were measured in the absence and presence of 1 M NaCl. LPL activity was determined by subtracting non-LPL-dependent activity (high salt) from the total lipolytic activity (20). LPL activity is expressed as pkatals (pmol fatty acid released per second) per milliliter.

Statistical Analysis

Results are presented as means \pm SE unless stated otherwise. Repeated-measures analysis of variance (ANOVA) was used for comparisons between tests and between fatty acids. A *P* value ≤ 0.05 was considered statistically significant.

RESULTS

In Vitro Lipolysis Study

The effect of temperature and THL on *in vitro* lipolysis is presented in Fig. 1. At 0 min, plasma NEFA concentrations were the same for all four conditions. Plasma NEFA concentrations from samples kept at room temperature increased 25, 56, and 80% after 30, 90, and 210 min, respectively, compared with baseline, and these values were higher compared with samples stored on ice or with THL throughout the experiment.

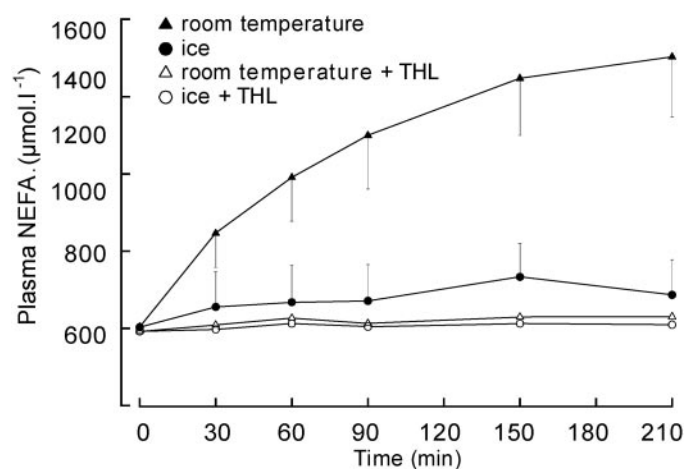


Fig. 1. Effects of temperature and tetrahydrolipstatin (THL) on *in vitro* lipolysis of postheparin plasma samples. Plasma nonesterified fatty acid (NEFA) concentrations increased significantly when samples were kept at room temperature compared with ice ($P < 0.02$), ice with THL, and room temperature with THL ($P < 0.01$). Plasma NEFA concentrations were also significantly different when samples were kept on ice compared with samples stored with THL ($P < 0.02$). Data are means \pm SE of 4 subjects.

Plasma NEFA concentrations from samples kept on ice were significantly higher than samples with THL. No difference was seen between samples stored on ice or at room temperature once THL was added.

Protocol Development Study

Total plasma NEFA and TAG concentrations for the five protocols are shown in Fig. 2. When 0.2 and 0.3 IU·kg⁻¹·min⁻¹ heparin were given in combination with a total of 69 g of fat, a slow rise in total plasma NEFA concentration was seen toward the end of the experiment (*protocols 1 and 2*). This coincided with a slow but substantial increase of total plasma TAG concentration toward the end of the experiment. To achieve an earlier rise in plasma NEFA and TAG concentrations, the heparin infusion and the total and the first fat load were increased (*protocol 4*). Despite these changes, a similar pattern was seen. When saline was given instead of heparin, total plasma NEFA concentrations increased but less than with the previous protocols (*protocol 3*). The heparin bolus given at 120 min resulted in a sharp increase in total plasma NEFA concentrations (*protocol 5*). This was not sustained

until the end of the experiment, probably because of the decline in plasma TAG concentrations.

Final Protocol Study

Total plasma NEFA and TAG concentrations. Final adjustments were made to *protocol 5*. The heparin was given earlier (at 90 min) to obtain a faster increase in plasma NEFA concentrations. Ninety minutes was chosen to allow for the fat to be absorbed by the gut and appear in the circulation. To prevent depletion of the precursor molecules and subsequently a drop in plasma NEFA concentrations, more oral fat was given. Plasma TAG concentrations were comparable during fasting (MUFA test, 852 ± 60 μmol/l; PUFA test, 727 ± 60 μmol/l; SFA test, 836 ± 62 μmol/l; and control, 894 ± 76 μmol/l) and feeding (MUFA test, 943 ± 88 μmol/l; PUFA test, 786 ± 96 μmol/l; SFA test, 887 ± 142 μmol/l; and control, 851 ± 81 μmol/l), and no significant difference was seen between the tests. Although plasma TAG concentrations tended to be greater for the MUFA test toward the end of the experiment, this was not significantly different from the other two fat tests. No significant difference was seen

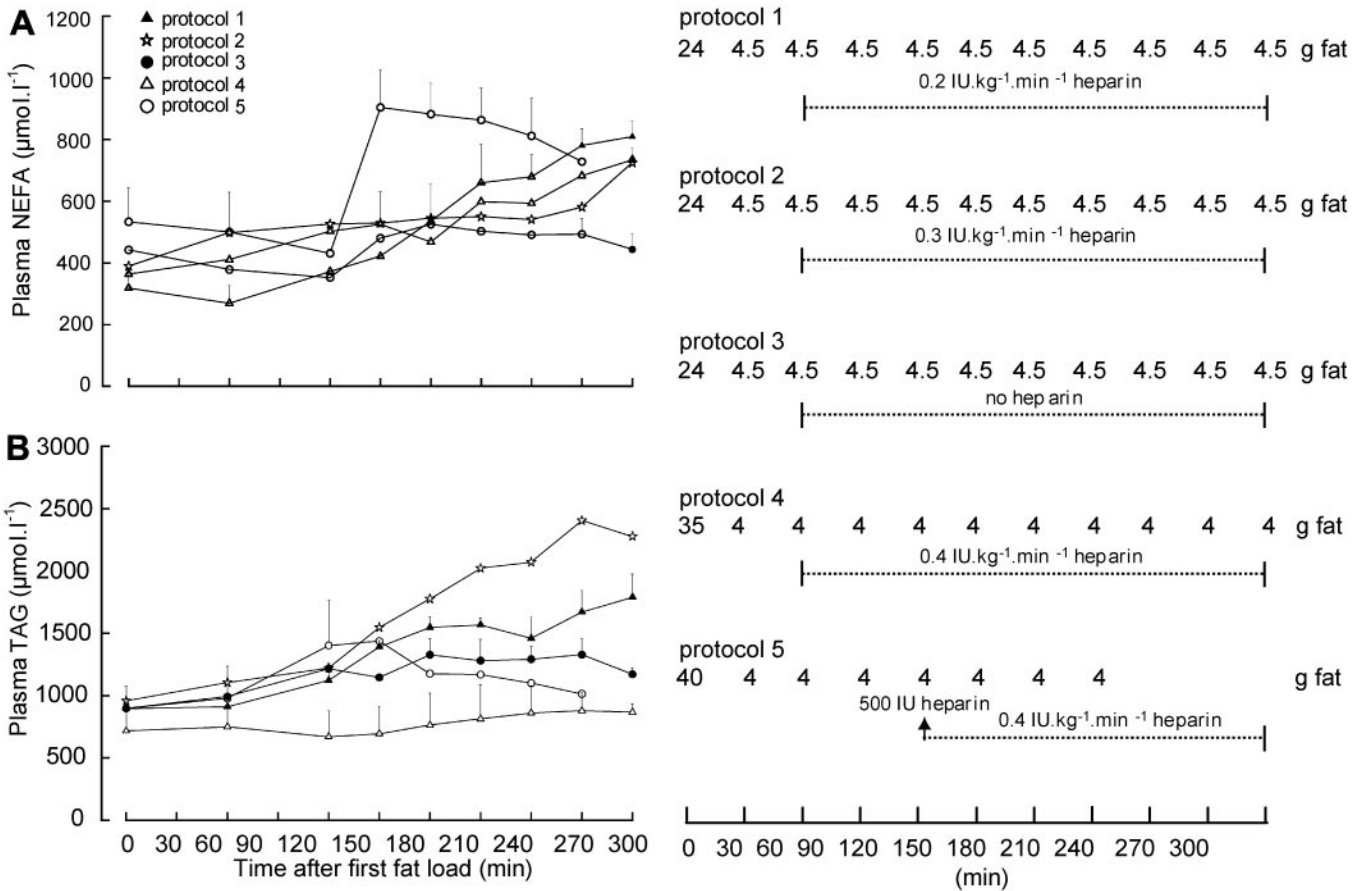


Fig. 2. Total plasma NEFA concentrations (A) and total plasma triacylglycerol (TAG) concentrations (B) for protocols 1–5 of the protocol development study. During these experiments, the amount and/or timing of fat and heparin was changed to produce a 2-h steady-state increase in plasma NEFA concentrations. During *protocols 1, 2, and 3*, different heparin infusion rates (0.2 or 0.3 IU·kg⁻¹·min⁻¹ or no heparin) were tested in combination with 69 g of fat. For *protocols 4 and 5*, the amount of fat was increased (75 g), and more heparin was given (0.4 IU·kg⁻¹·min⁻¹). Data are means ± SE.

for fasting (0 min) and preheparin (0–90 min) total plasma NEFA concentrations between visits (Fig. 3A). From 90 to 210 min, a steady-state rise in total plasma NEFA concentrations was seen for all three fat tests. The mean absolute concentrations were significantly greater for the three fat tests (MUFA test, $745 \pm 35 \mu\text{mol/l}$; PUFA test, $609 \pm 37 \mu\text{mol/l}$; SFA test, $773 \pm 38 \mu\text{mol/l}$) when fat and heparin were given compared with the control visit ($445 \pm 41 \mu\text{mol/l}$). When adjusted for baseline (0–90 min), the degrees of increase com-

pared with control were 3.7-fold for the MUFA test, 2.5-fold for the PUFA test, and 3.5-fold for the SFA test.

Specific plasma NEFA composition. Preheparin absolute concentrations of individual plasma NEFAs did not differ among the MUFA, PUFA, and SFA tests, but a significant difference was seen between fatty acids ($P < 0.001$; Table 3). Before the heparin infusion, 18:1 *n*-9 followed by 16:0 were the predominant fatty acids for all four visits. During the heparin infusion, the fatty acid composition changed between visits ($P < 0.02$). Significant differences were seen for 16:0, 18:1 *n*-9, and 18:2 *n*-6 when compared between visits (Table 3). During the heparin infusion, 18:1 *n*-9 was the most abundant fatty acid for the MUFA test compared with 18:2 *n*-6 for the PUFA test and 16:0 for the SFA test, respectively. No significant differences were seen for 18:0, 16:1 *n*-7, and 18:3 *n*-3 during the heparin infusion among the MUFA, PUFA, and SFA tests (Table 3). When individual plasma NEFA concentrations were compared before and during heparin infusion within each fat test, only 18:1 *n*-9 increased significantly for the MUFA test ($P < 0.002$), 18:2 *n*-6 for the PUFA test ($P < 0.002$), and 16:0 ($P < 0.02$) and 18:1 *n*-9 ($P < 0.002$) for the SFA test. No significant increases were seen for the other fatty acids.

Plasma LPL activity. Plasma LPL activity before heparin infusion and during the control visit was not distinguishable from zero (Fig. 3B). Intravenous heparin infusion resulted in an increased LPL activity for the MUFA, PUFA, and SFA tests. This was significantly higher compared with baseline and the control visit. Although mean LPL activity was lower for the MUFA fat test during the heparin infusion, this was not statistically significantly different from the two other fat tests.

DISCUSSION

A protocol was developed in healthy subjects to increase total plasma NEFA concentrations to values commonly seen in obesity and type 2 diabetes by feeding several small boluses of fat orally and infusing a low dose of heparin. This is different from the traditional approach, where fat is administered intravenously and the process of fat absorption and its gastrointestinal responses are bypassed. This new procedure allows the use of various oils with different fatty acid composition to increase specific plasma NEFAs. In this study, olive oil, safflower oil, and palm stearin were used to increase plasma monounsaturated, polyunsaturated, and saturated NEFAs, but other oils could be used (e.g., fish oils). The fatty acids from the oils are present as TAGs, and although each oil was high in one particular fatty acid, others were present as well. Attempts were made to emulsify pure fatty acids, but the end product was not suitable for consumption; the drinks were very acidic, and pure palmitic acid did not emulsify. We also tried to emulsify pure tripalmitin but without success.

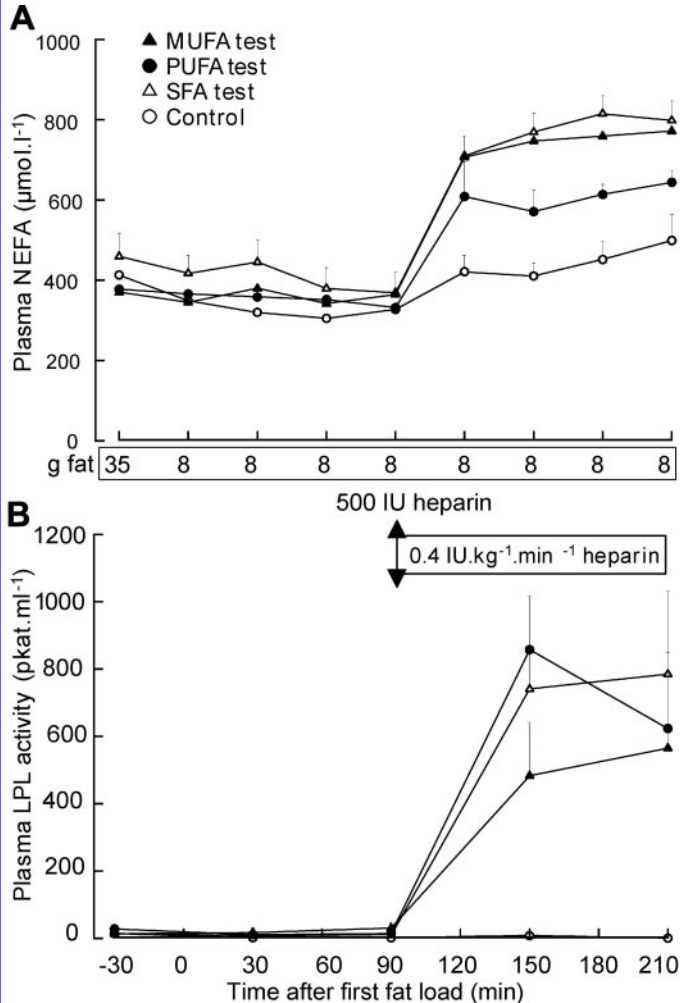


Fig. 3. Total plasma NEFA concentrations (A) and plasma lipoprotein lipase (LPL) activity (B) during the monounsaturated fatty acid (MUFA) test (olive oil), the polyunsaturated fatty acid (PUFA) test (safflower oil), the saturated fatty acid (SFA) test (palm stearin), and control visit (no fat, no heparin) from the final protocol study. For the 3 fat tests, 91 g of fat (35 g at 0 min and 8 g at 30, 60, 90, 120, 150, 180, and 210 min) were given orally, and 500 IU of heparin were given at 90 min followed by a $0.4 \text{ IU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ heparin infusion (from 90 until 300 min). Between 90 and 210 min, the NEFA concentrations were significantly different for the MUFA test ($P < 0.01$), PUFA test ($P < 0.05$), and SFA test ($P < 0.02$) compared with control. A significant difference in NEFA concentrations with repeated-measures ANOVA was seen when the 3 fat tests were compared during the heparin infusion ($P < 0.02$). Baseline LPL activity was significantly lower than during heparin infusion ($P < 0.001$) and this was so for the MUFA, PUFA, and SFA tests ($P < 0.05$). Data are means \pm SE of 8 subjects.

Table 3. Plasma concentrations of individual NEFA before heparin or saline (90 min) and during heparin or saline infusion (mean of 180 and 210 min) for the 4 tests of the final protocol study

	16:0	18:0	16:1 <i>n</i> -7	18:1 <i>n</i> -9	18:2 <i>n</i> -6	18:3 <i>n</i> -3
MUFA test						
Preheparin	124 ± 28	73 ± 23	20 ± 6	137 ± 36	49 ± 12	9 ± 5
Heparin	136 ± 11	49 ± 12	16 ± 3	475 ± 61*	68 ± 9	9 ± 6
PUFA test						
Preheparin	74 ± 11	46 ± 5	11 ± 3	116 ± 28	52 ± 10	7 ± 2
Heparin	109 ± 10	62 ± 6	14 ± 5	163 ± 26	217 ± 17*	7 ± 3
SFA test						
Preheparin	97 ± 8	55 ± 8	10 ± 3	146 ± 19	49 ± 7	7 ± 3
Heparin	291 ± 25*	100 ± 25*	11 ± 2	257 ± 20	74 ± 9	6 ± 3
Control						
Presaline	92 ± 24	51 ± 5	28 ± 14	138 ± 42	72 ± 20	13 ± 10
Saline	111 ± 10	58 ± 4	16 ± 3	168 ± 18*	67 ± 8	5 ± 1

Values are means ± SE ($\mu\text{mol/l}$) of 8 subjects. During the heparin infusion, palmitic acid (16:0) concentrations were significantly higher for the SFA test compared with the MUFA and PUFA tests ($P < 0.001$), but no difference was seen between the MUFA and the PUFA tests. Oleic acid (18:1 *n*-9) concentrations were significantly different for the MUFA test compared with the PUFA ($P < 0.01$) and the SFA ($P < 0.02$) tests and also between the SFA and the PUFA ($P < 0.05$) tests. Linoleic acid (18:2 *n*-6) concentrations were significantly different for the PUFA test compared with the MUFA and the SFA tests ($P < 0.001$) but not between the PUFA and the SFA tests. *Significant differences for fatty acids before and during heparin or saline infusion.

Intravenous heparin infusion was used to enhance lipolysis by dislocating LPL from its endothelial binding sites. Varying amounts of heparin have been reported to increase a wide range of plasma NEFA concentrations (7, 12, 25). In this study, a bolus of 500 IU and a $0.4 \text{ IU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ infusion were needed to increase plasma NEFA concentrations to $\sim 800 \mu\text{mol/l}$. These are rather low NEFA concentrations compared with other studies using a similar or lower amount of heparin for higher plasma NEFA concentrations (5, 25). Butler et al. (5) infused heparin at the same rate as we did in combination with an intravenous infusion of 12 g TAG/h. This resulted in an increase in plasma NEFA concentrations to $\sim 3,000 \mu\text{mol/l}$. Our study shows that immediate inhibition of LPL activity is important to prevent *in vitro* lipolysis. Most likely, the high plasma NEFA concentrations are the result of *in vitro* lipolysis, since LPL is still active in the test tube when no LPL inhibitor is used. Lowering the temperature of the harvested plasma, as is often done to prevent lipolysis of TAGs in the test tube, does reduce LPL activity but does not stop it.

Adding THL to samples may have consequences for some analyses. Measurement of plasma TAG concentrations usually involves hydrolysis of the TAGs to free glycerol by a lipase, which will also be inhibited by the THL. For that reason, plasma total glycerol concentrations (TAG and free glycerol) are better measured from samples without THL and later adjusted for free glycerol analyzed from plasma samples immediately added to perchloric acid, which will inactivate the enzyme.

Although similar amounts of fat and heparin were used for the MUFA, PUFA, and SFA tests, a lower increase in total plasma NEFA concentrations was seen when safflower oil was used, but it was still significantly increased compared with control. Lower total plasma NEFA concentrations when safflower oil was given could be due to a slower gastrointestinal handling of the oil compared with the other oils. Gastric emptying is a limiting factor for the entry of the

oils into the small intestine and, hence, their subsequent digestion and absorption. Possible differences in gastric emptying of the oils used in the present study were investigated by Jones et al. (17) and in our laboratory by Robertson et al. (24). Here, 10 healthy women consumed, on three separate occasions, 40 g of either palm oil, safflower oil, or olive oil. There were, however, no differences in gastric emptying between safflower oil and the other test oils (17, 24). To our knowledge, there is also no evidence that polyunsaturated fatty acids, in particular linoleic acid, are less well or less rapidly absorbed compared with monounsaturated and saturated fatty acids when given in smaller amounts. One study suggests that the absorption of labeled palmitic acid was poor compared with the almost complete absorption of labeled oleic and linoleic acid (22). In that study, the labeled fatty acids were administered by simply mixing the fatty acids with the rest of the test meal (22). On the other hand, when palmitic acid was given as an oil as part of a mixed meal, or as a labeled fatty acid as part of an emulsion, no difference in absorption or gastric emptying was seen with oleic or linoleic fatty acids (17, 24). To improve absorption, in particular of the palm stearin high in palmitic acid, a stable fat emulsion (50%, wt/wt) was developed and made palatable as a chocolate drink. The drink was given hot to prevent solidification of the palm stearin, and mechanical force (blending) was used to improve the emulsification.

A difference in fractional uptake of individual fatty acids may also explain the reduced increase in plasma NEFA concentrations when mainly *n*-6 polyunsaturated fatty acids were present. Nevertheless, Hagenfeldt et al. (14) reported no difference in fractional uptake among palmitic, oleic, and linoleic acid across resting forearm and kidney. On the other hand, splanchnic fractional uptake was higher for linoleic acid than for palmitic or oleic acid. When exercise was performed, the muscle showed a slight preference for linoleic and oleic acid compared with palmitic acid (13).



Tracer studies in rats showed greater oxidation of ^{14}C -labeled linolenic and oleic acid than that of ^{14}C -labeled stearic acid (2). Also, Leyton et al. (19) found that fatty acid oxidation increased with higher degrees of unsaturation but was inversely related to chain length. On balance, therefore, the lower plasma NEFA concentrations found with safflower oil might reflect greater clearance of linoleic acid.

Also, differences in total LPL activity between the visits may result in different plasma NEFA concentrations. However, this can be ruled out, because plasma LPL activity was similar for the three fat tests. Additionally, the fatty acid composition of the TAG molecules could affect susceptibility to lipolysis by LPL on chylomicron TAG molecules. Some studies have shown a faster lipolysis for rat chylomicron TAGs or human serum TAGs derived from polyunsaturated fats than from saturated fatty acid-containing TAG (3, 8, 27), but others have not (21). The preferential lipolysis by LPL on human chylomicron TAG derived from olive oil, safflower oil, and palm stearin has not been investigated as yet. This needs further investigation.

In conclusion, the use of this protocol may provide novel insights into metabolic effects of individual plasma NEFAs and makes direct comparison of the effects between the different classes of acutely elevated plasma NEFAs possible.

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