Mechanisms of Inhibition of Triacylglycerol Hydrolysis by Human Gastric Lipase*

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In the human stomach, gastric lipase hydrolyzes only 10 to 30% of ingested triacylglycerols because of an inhibition process induced by the long chain free fatty acids generated, which are mostly protonated at gastric pH. The aim of this work was to elucidate the mechanisms by which free fatty acids inhibit further hydrolysis. In vitro experiments examined gastric lipolysis of differently sized phospholipid-triolein emulsions by human gastric juice or purified human gastric lipase, under close to physiological conditions. The lipolysis process was further investigated by scanning electron microscopy, and gastric lipase and free fatty acid movement during lipolysis were followed by fluorescence microscopy. The results demonstrate that: 1) free fatty acids generated during lipolysis partition between the surface and core of lipid droplets with a molar phase distribution coefficient of 7.4 at pH 5.40; 2) the long chain free fatty acids have an inhibitory effect only when generated during lipolysis; 3) inhibition of gastric lipolysis can be delayed by the use of lipid emulsions composed of small-size lipid droplets; 4) the release of free fatty acids during lipolysis induces a marked increase in droplet surface area, leading to the formation of novel particles at the lipid droplet surface; and 5) the gastric lipase is trapped in these free fatty acid-rich particles during their formation. In conclusion, we propose a model in which the sequential physicochemical events occurring during gastric lipolysis lead to the inhibition of further triacylglycerol lipolysis.

Dietary fat digestion and absorption is a complex process involving enzyme activities and physicochemical changes (1–5). In humans, hydrolysis of dietary triacylglycerols starts in the stomach where it is catalyzed by an acid-stable gastric lipase, a globular protein of about 50 kDa with a broad pH range (6, 7). Triacylglycerol hydrolysis continues in the duodenum, by the synergistic actions of gastric and colipase-dependent pancreatic lipases and bile secretion (1). A characteristic feature of these lipases is their specificity to act on insoluble emulsified substrates (1, 2). A few in vitro and in vivo experiments have shown that the extent of lipid emulsification, which directly affects the lipid/water interface area, modulates the activity of digestive lipases (8–10). Dietary lipids are organized mainly in the form of droplets in the aqueous digestive system (1, 3, 4). The lipid droplets consist of a hydrophobic core containing the majority of the triacylglycerol molecules, esterified cholesterol, and fat-soluble vitamins, surrounded by an amphipatic surface monolayer of phospholipids, free cholesterol, and a few triacylglycerol molecules (11, 12). Earlier studies on lipoprotein models (11, 13, 14) and a recent investigation using dietary emulsions (12) have shown that 2–5 mol % of the droplet surface lipid is triacylglycerol, thereby enabling lipase action at the surface of the lipid droplet.

In healthy humans, gastric lipolysis leads to the hydrolysis of 10–30% (3, 4, 10, 15) of ingested triacylglycerols, generating mainly free fatty acids and diacylglycerols (1, 16, 17). This facilitates subsequent triacylglycerol hydrolysis by pancreatic lipase by allowing fat emulsification (3, 4) and promoting enzyme activity (8, 17). Furthermore, in physiological (preterm or full-term infants) (18) and pathological (cystic fibrosis, pancreatitis) (19–21) pancreatic insufficiencies, gastric lipolysis plays a key role in the digestion of dietary fat by hydrolyzing 10–40% of fat in the stomach (18–21), as well as acting more effectively in the duodenum because of acid pH conditions (19). The relatively limited extent of lipolysis by the gastric lipase under physiological or pathological conditions suggested that a feedback inhibition by the products of lipolysis probably occurs (1, 22).

It has been hypothesized that the inhibition of gastric lipase activity may be due to the progressive release of protonated free fatty acids (8, 23) that might accumulate at the lipid droplet surface (1, 16, 22). At present, however, the mechanism by which free fatty acids inhibit gastric lipase action in the stomach is unknown. It can be suggested from the literature that long chain free fatty acids prevent further gastric lipase lipolysis by modifying the physicochemical properties of the lipid/water interface, especially the interfacial tension or the surface pressure (2, 13, 16, 24, 25); they could prevent the interfacial binding of gastric lipase or promote its release from the droplet surface (2), or they could limit the number of triacylglycerol molecules located at the droplet surface by steric hindrance (13). However, thus far no study has provided direct evidence for the mechanisms involved. A few previous studies have examined the effect of free fatty acid on gastric lipase activity (8, 23) or the distribution of fatty acids in model systems (26–28), however, they were not performed under physiological conditions. In the present work we have performed several in vitro experiments using conditions close to those occurring physiologically, to understand the mechanism of inhibition of gastric lipolysis in vivo, and to begin to elucidate conditions that will enable modulation of the extent of gastric lipolysis.
**EXPERIMENTAL PROCEDURES**

**Purification of Human Gastric Lipase and Lipase Activity Measurements**

Human gastric juice was collected from healthy adult patients for diagnostic purposes after pentagastrin stimulation (6 mg/kg) (a generous gift from Dr. J. Peyrot and Mr. J. Salducci, Gastroenterology Department, Nord Hospital, Marseille, France). Pure human gastric lipase (HGL) with a specific activity of 910 units/mg on tributyrin was obtained according to Thiruppathi and Balasubramanian (29). Gastric lipase activity of gastric juice (100–200 µl) or purified lipase was determined using a pH-stat titrator (Metrohm, Herisau, Switzerland) at pH 5.40 and 37 °C with tributyrin as substrate (ICN Biomedicals Inc., OH) as previously described (3, 23). One lipase unit corresponds to the release of 1 nmol of fatty acid per min.

**Lipid Mixtures**

The relative proportion of lipids used was chosen in accordance with human daily dietary intake (1). The lipid mixture contained 93.5% triolein (w/w) (ICN), 6% phospholipids (PL) (w/w) (13) (α-phosphatidylcholine, XVI-E from egg yolk), and 0.5% free cholesterol (w/w) (both from Sigma, La Verpillière, France). Lipids were solubilized in chloroform/methanol (2:1, v/v), mixed, dried under nitrogen, and desiccated using a rotavapor under vacuum at 30 °C. The lipid mixture was stored at −20 °C. For lipolysis experiments, the mixture contained [3H]- or [14C]-triolein (3 × 10^9 dpm/µmol of triolein or 1.5 × 10^9 dpm/µmol of triolein when a low HGL/TO ratio was used) and [14C]-1-palmitoyl-2-oleoyl phosphatidylcholine (3.5 × 10^9 dpm/µmol PL) (PerkinElmer Life Sciences, Dreieich, Germany). Lipid mixtures enriched with different concentrations of oleic acid (OA) (2 and 6.9% oleic acid (w/w), i.e. 0.063 and 0.216 µmol of OA/µmol of TO) contained [14C]-triolein (PerkinElmer Life Sciences), 3 × 10^9 dpm/µmol of triolein, and [3H]-oleic acid (1.2 × 10^4 dpm/µmol of OA) (Amersham International plc, UK). These OA/TO ratios were selected to mimic the amount of free fatty acids released after 2 or 25 min of gastric lipolysis under present physiological conditions, respectively. To study the distribution of lipolysis-generated free fatty acids between the core and surface of the lipid droplet, the lipid mixture was radiolabeled with [14C]-triolein (10^9 dpm/µmol of triolein), [3H]-1-palmitoylphosphatidylcholine (10^5 dpm/µmol PL), and [3H]-cholesterol (10^5 dpm/µmol of free cholesterol (PerkinElmer Life Sciences). The lipid mixture for fluorescence studies was labeled with dansyl cholesterol (gift from Drs. A. Misharin and C. Alquier).

**Emulsification Procedures and Determination of Emulsion Droplet Size**

A fine emulsion (about 0.7 µm in median diameter) was prepared by sonication of 100 mg of lipid mixture in 6 ml of distilled water for 10 min at 95% power level and a frequency of 20,178 Hz (Sonoreactor, Unda- tim, Japan), in ice/ethanol. A medium-size emulsion (about 2 µm) was obtained by sonicating 100 mg of lipid mixture in 3 ml of distilled water for 5 min at 25 watts power in an ice/ethanol cooling bath using a microtip probe (Brandson 250 W sonifier, Osi, France). A coarse emulsion (about 15 µm) was obtained by sonicating 100 mg of lipid mixture in 1 ml of distilled water for 1.5 min at room temperature. The emulsions obtained were collected after concentration and removal of excess phospholipids as follow: the coarse emulsion was allowed to stand for 10 min in ice, and the medium-size and fine ones were centrifuged 10 min or 1 h at 4,000 rpm and 10 °C, respectively. The resultant triacylglycerol/phospholipid ratios (w/w) were found to be 50/1, 40/1, and 14/1 for the coarse, medium, and fine emulsions, respectively. The emulsion droplet sizes were determined as previously reported (3) using a particle-size analyzer (Capa-700, Horiba) and are expressed as percentage of total volume occupied by lipid droplets from 0.1 to 40 µm (about 70% total particles by volume). The medium-size emulsion (Fig. 1A), lipid droplets sized from 0.1 to 40 µm and about 80% of total particles ranged from 1 to 4 µm. The fine emulsion (Fig. 1C) was mainly composed of small size droplets from 0.1 to 2 µm, with 75% of total particles sized between 0.1 and 1 µm. Emulsion surface area varied inversely with the emulsion median diameter. Both parameters were significantly different for the three emulsions (ANOVA, p < 0.05).

**Lipolysis Experiments**

**Lipolysis of Differently Sized Emulsions by Pure HGL**—Experiments were carried out at 37 °C and pH 5.40, using polycarbonate test tubes (13 × 51 mm, Beckman Instruments, Palo Alto, CA) to limit the loss of lipid molecules on the inner surface of the test tube during lipolysis. The reaction medium was a 2.5-ml mixture containing 100 mM sodium acetate, 150 mM NaCl, 6 mM CaCl2 (buffer L), 1.6 µM bovine serum albumin, and 25 µmol of triolein emulsified as described above. The HGL/TO ratio was selected to mimic physiological conditions (3, 10, 15), i.e. excess enzyme, and was 2.5 units (53.2 pmol)/µmol of triolein. Samples (200 µl) were collected at intervals from 0 to 100 min, and lipids were extracted immediately after the incubation aimulating the Folch method (30). Lipids were separated by thin-layer chromatography (TLC) on silica gel (Ready plastic sheet F1500, Schleider and Schuell, Germany) according to Bitman and Wood (31). After exposure to iodine vapor, individual lipid spots were scraped and the radioactivity was measured by scintillation counting (1600TR, Packard, Meriden, CT).

**Lipolysis in the Presence of Exogenous OA**—The reaction medium was a 0.8 ml of mixture of buffer L with medium-size [3H]-triolein emulsion, at pH 5.4 and 37 °C. Three HGL/TO ratios were used corre-

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1 The abbreviations used are: HGL, human gastric lipase; PL, phospholipid; OA, oleic acid; FITC, fluorescein isothiocyanate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; TRITC, tetramethyl rhodamine isothiocyanate; TO, triolein.
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Samples of a lipolysed medium-size emulsion collected at intervals from 0 to 80 min were mixed volume/volume with 1% OsO₄ in distilled water at pH 5.4 and room temperature. The mixture was gently shaken, put on a microscope cover glass, and fixed overnight in a moist chamber at room temperature. The emulsion deposit was then gently washed with distilled water, first dried with filter paper followed by drying 1 day in a silica gel desiccator at room temperature. Preparations were gold-palladium coated and then examined at magnification ×4,800 to 6,600 with a JSM-35CF scanning electron microscope (JEOLs, Paris, France) operated at 35 kV accelerating potential.

Fluorescence Microscopy

Immunolocalization of HGL during Lipolysis of Lipid Droplets—An aliquot (50 μl) of a medium-size emulsion previously incubated with HGL for 60 min was mixed in a shaking bath at 37 °C with a medium-size emulsion labeled with dansyl cholesterol to distinguish from the first emulsion, at pH 5.4 for 15 min. Then the mixture was incubated for 30 min on ice with 10 μl of purified specific polyclonal anti-HGL antibodies (6.2 mg/ml) from rabbit (diluted 10 times in buffer L) followed by incubation for 30 min on ice with 10 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA) (diluted 10 times in buffer L). High levels of antibodies were used to obtain sufficient labeling at acid pH and cold temperature. A negative control was performed without the enzyme.

HGL Rhodamine Labeling—HGL was mixed with TRITC (Molecular Probe, Inc.) (25 mg/mg of protein) in 25 mM sodium carbonate buffer, pH 9.0, at room temperature and then quickly neutralized to pH 6.0, dialyzed against sodium acetate 50 mM, 150 mM NaCl, pH 6.0, at cold temperature overnight and concentrated on PEG-6000 to 4°C. Under these conditions HGL retained about 60% of its initial activity.

Visualization of Lipolysis-generated Free Fatty Acids—Based on the method of Holczinger (33), a copper acetate solution (Sigma) (0.15% final concentration) was mixed carefully into the reaction medium after 90 min of lipolysis of a medium-size emulsion. The copper-free fatty acid soaps formed were then visualized with FITC-Gly-Gly-His (Molecular probe) (38 μg/ml final concentration), a marker with high selectivity and sensitivity for Cu²⁺ due to the presence of the tripeptide commonly called copper-binding peptide (34).

Microscopy—All specimens were examined under a Leitz Dialux 20 microscope (Jena, Germany) equipped with a Photomak 3.1 epifluorescence system using filters specific for FITC (filter block Leitz model L3), dansyl cholesterol (filter block Leitz model A2), or TRITC (filter block Leitz model N2), at a final magnification of ×1,250. Photomicrographs were taken using a CCD color Camera (DC 100, Leica, Switzerland).

Isolation and Analysis of the Gastric Lipid Particles

A [¹⁴C]triolein, [¹³C]HPL, free [¹⁴C]cholesterol medium-size emulsion was incubated with HGL for 90 min. The lipid particles generated during lipolysis were isolated by FPLC using a Superose 6 column (6 × 57 cm) at room temperature with a flow rate of 0.3 ml/min with buffer L as eluent. The lipid droplets are retained on the column. The fractions obtained were analyzed for lipid composition by TLC and radiodestroyne counting as described above. The size of the lipid particles was determined with a quasielectric light-scattering detector (SEMAtech, Nice, France). The presence of HGL was ascertained by immunoblotting (35) followed by incubation with 10 μl of the various fractions on a polyvinylidene difluoride membrane; the membrane was shaken for 30 min at room temperature in 5% skim milk in a TBS Tween buffer, washed, and incubated with HGL-polycarboxylic acid antiserum (final dilution 1:5,000); immunodetection was carried out with alkaline phosphatase-labeled goat antirabbit IgG (final dilution 1:5,000) (Sigma).

Statistical Analysis

Statistical significances were analyzed by one-way analysis of variance (ANOVA) and the differences were determined by the Fisher’s test at a probability of 95%. Correlation coefficient was obtained from linear regression (StatView II; Abacus, Berkeley, CA) (36).

RESULTS

Kinetics of Hydrolysis of Differently Sized Emulsions by Gastric Lipase—The amounts of free fatty acid released by pure HGL at pH 5.4 on three differently sized emulsions are shown in Fig. 2. The rate of lipolysis was inversely related to the emulsion size and decreased with time with all the emulsions.
used. The amounts of OA released after 5 min lipolysis were $3.0 \times 10^{-2}$, $2.0 \times 10^{-2}$, and $0.78 \times 10^{-2} \mu$mol of OA/min for the fine, medium, and coarse emulsions, respectively. Thus, a 50% loss of lipase activity was reached after about 10 min incubation at 37 °C. The rate of lipolysis reached a plateau after 40 to 60 min and at this time the amounts of OA released were 0.444 (fine emulsion), 0.325 (medium size emulsion), and 0.124 (coarse emulsion) per μmol of initial triolein. Maximum rates of triacylglycerol lipolysis obtained over time were, expressed as mean ± S.E., 22.6 ± 0.7, 16.3 ± 2.8, and 7.1 ± 0.4% for the fine, medium, and coarse emulsions, respectively. Thus, the extent of lipolysis was 1.4 to 3.2 times higher ($p < 0.05$) with the fine emulsion as compared with the medium and coarse emulsions, respectively, showing that HGL activity is directly related to the available interface area ($r^2 = 0.73, p < 0.05$).

Kinetics of Hydrolysis of Emulsions in the Presence of Exogenous OA—To investigate whether the presence of protonated free fatty acids is specifically responsible for the inhibition of gastric lipolysis, we studied the kinetics of hydrolysis of a medium-size emulsion after adding OA exogenously to reach or exceed the free fatty acid concentrations found during lipolysis, or of preformed OA-enriched emulsions (Fig. 3). The addition of 0.3 μmol of OA/μmol of triolein to the reaction medium 5 min after lipolysis started, as well as the addition of a very high amount of OA (1.4 μmol of OA/μmol of triolein) before and 5 min after the beginning of lipolysis, did not modify significantly the kinetics of hydrolysis (Fig. 3A). The rates of hydrolysis reached a plateau after 60 min and the amounts of OA released were similar to those shown in Fig. 2. We had expected to observe an inhibitory effect of the added free fatty acid on triacylglycerol hydrolysis (8, 23). However, previous in vitro experiments showing such an inhibitory effect were done in excess of substrate (8, 23). Therefore, we have performed two other experiments changing the HGL/TO ratio, one with a moderate physiological ratio (10.6 pmol of HGL/μmol of triolein) or D, comparing the hydrolysis of a medium-size emulsion without exogenous OA to a 2.0% OA-enriched emulsion and a 6.9% OA-enriched emulsion, using a high physiological amount of HGL (53.2 pmol of pure HGL/μmol of triolein). Values are mean ± S.D. of three experiments. The incubations were done at 37 °C, pH 5.40, using 10 mM [3H]triolein emulsions, 100 mM sodium acetate, 150 mM NaCl, 6 mM CaCl₂. Inhibition of Gastric Lipolysis

Fig. 3. Kinetics of hydrolysis of emulsions by pure HGL in the presence of exogenous OA. The effect of adding exogenous free oleic acid on the release of oleic acid from triolein-phospholipid emulsions (medium-size emulsion) by pure HGL was examined by adding 0.3 or 1.4 μmol of OA/μmol of triolein either before or 5 min after lipolysis began with: A, a high physiological amount of HGL (53.2 pmol of HGL/μmol of triolein); B, a moderate physiological amount of HGL (10.6 pmol of HGL/μmol of triolein); C, a large excess of substrate (0.21 pmol of HGL/μmol of triolein); or D, comparing the hydrolysis of a medium-size emulsion without exogenous OA to a 2.0% OA-enriched emulsion and a 6.9% OA-enriched emulsion, using a high physiological amount of HGL (53.2 pmol of pure HGL/μmol of triolein). Values are mean ± S.D. of three experiments.
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FIG. 4. Sequential kinetics of hydrolysis of emulsions by HGL following addition of new enzyme or new emulsion. A, a medium-size emulsion was hydrolyzed by pure HGL for 80 min and then a new amount of pure HGL was added. Incubation was at 37 °C and pH 5.40, using 5 mm [3H]triolein emulsions, 100 mm sodium acetate, 150 mm NaCl, 6 mm CaCl₂, with 53.2 pmol of pure HGL/μmol of triolein. Values are mean ± S.D. of three experiments. B, ability of HGL to hydrolyze a new [14C]emulsion after having hydrolyzed a [3H]emulsion for 90 min. Incubation was at 37 °C and pH 5.40, using 6.25 mm [3H]- and [14C]-triolein emulsions, 100 mm sodium acetate, 150 mm NaCl, 6 mm CaCl₂, with 53.2 pmol of pure HGL/μmol of triolein or with gastric juice having lipase activity equivalent to 53.2 pmol of pure HGL/μmol of triolein. The amounts of [3H]OA released from the first [3H]emulsion and the amounts of [14C]OA released from the second [14C]emulsion were measured.

ated after the first 5 min of lipolysis of 2.0 and 6.9% OA-enriched emulsions were 1.98 × 10⁻² and 2.66 × 10⁻² μmol of OA/min, compared with 1.82 × 10⁻² μmol of OA/min for the control. After 60 min lipolysis 0.521 and 0.534 μmol of OA/μmol of triolein were released, respectively, compared with 0.306 μmol for the control, and higher rates of lipolysis were reached (about 26% for OA-enriched emulsions versus 15% for the control emulsion). Thus, when OA was present in emulsions at 2.0 and 6.9% concentration, the hydrolysis of the emulsion was enhanced (about 1.7 times). This cannot be attributed to differences in emulsion size, as the OA-enriched emulsions exhibited similar size to the control emulsion, i.e. 2.5 and 1.3 μm for the 2 or 6.9% OA-enriched emulsions, respectively (data not shown), versus 2 μm for the 0% OA control emulsion. These results indicate that the reported inhibitory effect of protoneated free fatty acids on gastric lipolysis is more complex than previously envisioned, and that physicochemical events occurring within or at the lipid droplet surface during lipolysis under physiological conditions warrant further consider.

Lipolysis of a Pre-lipolysed Emulsion—Because changes in the lipid/water interfacial tension or in the surface pressure of the emulsion droplet could be involved in the inhibition of lipolysis catalyzed by the gastric lipase (13, 24, 25), we tested the hydrolysis of an already maximally lipolysed [3H]emulsion, i.e. once the rate of lipolysis had plateaued, by a second addi-

tion of gastric lipase (Fig. 4A). In the first step, the lipolysis rate of the emulsion during the first 5 min was 1.06 × 10⁻² OA μmol/min and the amount of OA generated reached 0.275 μmol of OA/μmol of triolein after 60 min of lipolysis. After the second addition of HGL, lipolysis started again, releasing 0.7 × 10⁻² μmol of OA/μmin during the first 5 min, and the total amount of free fatty acids generated over the second 60-min lipolysis period reached 0.149 μmol of OA/μmol of triolein, representing 54% of OA released during the first lipolysis period. These results show that even if the emulsion interface undergoes modification due to lipolysis, the emulsion is still a suitable substrate for newly added lipase. Thus, the enzyme can still bind to and hydrolyze triolein emulsion, even when that substrate has been previously lipolysed for 60 min, implying that changes in the interface alone cannot be solely responsible for HGL inactivation.

Behavior of HGL during Lipolysis of Sequentially Added Emulsions—To examine HGL during lipolysis, a medium-size [3H]emulsion was incubated with pure HGL (53.2 pmol/μmol of triolein) for 90 min, the time necessary to reach a plateau for lipolysis. Subsequently, another medium-size [14C]emulsion was added and the lipolysis was followed for another 90-min period (Fig. 4B). Under these experimental conditions, the HGL/TO ratio was still in the physiological range (3, 10, 15). During the first and the second lipolysis, respectively, 1.72 × 10⁻² μmol of [3H]OA/μmin and 0.38 × 10⁻² μmol of [14C]OA/μmin were released for the first 5 min, and the amounts of free fatty acids generated reached 0.360 μmol of [3H]OA/μmol of triolein and of 0.115 μmol of [14C]OA/μmol of triolein after 90 min. Interestingly, during the second incubation, no further [3H]OA was produced. Thus, the first lipolysis released 6.77 μmol of [3H]OA/μmol of HGL and the second one produced no more [3H]OA and 2.16 μmol of [14C]OA/μmol of HGL. The total amount of OA released during the second lipolysis represented 31.9% of the amount generated during the first lipolysis. To better mimic physiological conditions we performed the same experiment using human gastric juice (Fig. 4B). The amount of free fatty acid released for the first 5 min of incubation was 1.42 × 10⁻² μmol of [3H]OA/μmin, versus 0.16 × 10⁻² μmol of [14C]OA/min during the second lipolysis; at 90 min of lipolysis, 0.346 μmol of [3H]OA/μmol of triolein and 0.173 μmol of [14C]OA/μmol of triolein were produced. In parallel, we observed that after addition of the [14C]emulsion, the lipolysis started again, to a small extent, on the [3H]emulsion, leading to the release of 27.7% of free fatty acids generated during the first lipolysis. On average, the first lipolysis generated 6.50 μmol of [3H]OA/μmol of HGL and the second one, 1.80 μmol of [3H]OA/μmol of HGL and 3.25 μmol of [14C]OA/μmol, i.e. 77.7% of the amount of OA released during the first lipolysis. We noted that HGL in intact juice gave qualitatively similar results than pure HGL but the absolute rate of lipolysis was different. This could mean that additional nonidentified compounds in gastric juice might modify lipolysis. These results show that HGL was not dramatically denaturated by exposure to triolein-PL-emulsion substrate and was still able to hydrolyze a new substrate triolein-PL-emulsion, albeit to a somewhat lesser extent.

Surface-to-core Distribution of Lipolysis-generated Free Fatty Acids—The lipid composition of the surface and the core of the lipid droplets of a medium-size emulsion, before and after lipolysis, was determined using the physicochemical method developed by Miller and Small (11) for artificial lipoproteins. This method is based on separation of the core and the surface of the lipid droplet by ultracentrifugation in a thin space (glass capillary tubes), using the fact that the density of the core and the surface phases are markedly different because of
their lipid compositions (11, 14, 37). Data obtained after 10 min lipolysis by HGL at pH 5.4 are given in Table I. The data obtained after 25 and 60 min lipolysis were deemed not as reliable because the density of the surface became lower as the surface became highly enriched in lipolytic products, making it impossible to effectively separate the surface from the core. Surface-to-core distribution of triolein underwent modification early during lipolysis. The phase distribution coefficient (the weight fraction in the surface phase to the weight fraction in the core phase, Ref. 11) varied greatly for triolein, decreasing from 0.051 to 0.0016, but remained relatively constant for cholesterol (7.77 to 7.53). After 10 min lipolysis, the phase distribution coefficients were 0.89 for diolein, 15.50 for monoolein, and 7.40 for oleic acid. Thus, diolein partitioned almost equally between surface and core, whereas monoolein and oleic acid had a higher affinity for the surface. When the actual weight partition for each lipid was calculated according to Miller and Small (13) taking into account the proportional mass of core and surface, it was found that 8.99, 17.30, and 2.23% of total free oleic acid, monoolein, and diolein generated during 10 min lipolysis, respectively, were located within the surface. In addition, 0.01 and 11.15% of the total triolein and free cholesterol present in the emulsion, respectively, were present in the surface.

**Table I**

| Emulsion         | Control Surface | Control Core | 10-min lipolysed Surface | 10-min lipolysed Core |
|------------------|-----------------|--------------|--------------------------|-----------------------|
| Triolein         | 0.03 ± 1.81     | 99.23 ± 0.03 | 0.15 ± 0.02              | 93.04 ± 0.20          |
| Diolein          | —               | —            | 3.85 ± 0.37              | 4.28 ± 0.18           |
| Monoolein        | —               | —            | 10.65 ± 0.40             | 0.69 ± 0.05           |
| Oleic acid       | —               | —            | 11.04 ± 0.52             | 1.49 ± 0.06           |
| Phosphatidylcholine | 88.99 ± 1.69     | —            | 72.66 ± 1.05             | tr^b                   |
| Free cholesterol | 5.98 ± 0.14     | 0.77 ± 0.07  | 1.64 ± 0.05              | 0.22 ± 0.01           |

^a^, undetectable.
^b^ tr, traces.

**Fig. 5.** Scanning electron micrographs of lipid droplets during lipolysis by HGL. A, before lipolysis, the medium-size emulsion contains lipid droplets that appear as spheres from about 0.5 to 2 μm diameter with smooth surfaces. B, after 5 min incubation with HGL the lipid droplet size increased (range 2.6 to 6.4 μm), and their surface became irregularly covered by small size spherical protrusions (<1 μm, arrow 1, enlargement in E). After 50 (data not shown) and 80 min (C) lipolysis, the droplet size was still greater (range 5.8 to 10 μm), and the droplet surface still appeared irregular containing fewer spherical protrusions with also the appearance of amorphous lipid clusters (C, arrow 2, enlargement in D). Specimens were fixed with OsO₄ at pH 5.4, put on a microscope cover glass, and coated with gold-palladium before viewing. The bar represents 2 μm in all figures. Magnification: ×6,000 for A; ×5,400 for B; ×4,800 for C.

**Fig. 6.** Evolvement of HGL during lipolysis of lipid droplets. An aliquot of a medium-size emulsion incubated with HGL for 60 min was mixed in a shaking bath at 37°C, pH 5.40, for 15 min with a medium-size emulsion labeled with dansyl cholesterol. The mixture was then incubated with polyclonal anti-HGL for 30 min on ice, and then with FITC-conjugated goat anti-rabbit IgG for 30 min on ice. Samples were examined either using filters specific for dansyl (A) (blue, lipid droplets) and for FITC (B) (green, HGL). FITC-labeled HGL was clearly found associated with highly fluorescent lipid droplets from the dansyl-labeled emulsion (arrow 1) and with slightly fluorescent lipid droplets (arrow 2) probably issued from the fusion of a non-labeled droplet with a labeled droplet. Magnification: ×1,200.

Scanning Electron Microscopy of Lipid Droplets during Lipolysis by HGL—Before lipolysis, the lipid droplets of a medium-size emulsion appeared as spheres of about 0.5 to 2 μm diameter with smooth surfaces (Fig. 5A). During lipolysis the size of lipid droplets increased from a range of 2.6 to 6.4 at 5 min to 5.6–10 μm at 80 min. Notably the appearance of the droplets changed (Fig. 5, B and C). The surface of the lipid droplets became irregularly covered with small-sized spherical protrusions (<1 μm) after 5 min (Fig. 5B, arrow 1, enlargement in E) and 25 min (data not shown) of HGL lipolysis. As lipolysis continued, i.e. at 50 (data not shown) to 80 min (Fig. 5C, arrow 2, enlargement in D), the enlarged lipid droplets still appeared irregular, with a surface containing less spherical protrusions and also showing the appearance of amorphous lipid clusters.
Immunolocalization of HGL during Lipolysis of Lipid Droplets—To further examine HGL during lipolysis, a FITC-labeled HGL was incubated for 60 min with a medium-size triolein-PL emulsion and then a medium-size dansyl-labeled triolein-PL emulsion was added (Fig. 6, A and B). FITC-HGL (Fig. 6B) was found associated either with highly fluorescent lipid droplets (Fig. 6A) of the dansyl-labeled emulsion (arrow 1) or with slightly fluorescent lipid droplets (arrow 2). In both cases, the FITC-HGL fluorescence was quite inhomogeneous. The slightly fluorescent droplets probably arise from the fusion of a non-labeled droplet with a labeled droplet, since dansyl-labeled lipid droplets in the absence of nonlabeled lipid droplets show a homogeneous bright fluorescence (data not shown). Thus based on the non-uniform distribution of the FITC-HGL, we suggest that HGL may be associated with lipid clusters formed at the lipid droplet surface during lipolysis. Those clusters trapping HGL might transfer from a droplet to a new one directly (arrow 1) or indirectly by fusion of the two droplets (arrow 2).

Co-localization of HGL and Free Fatty Acids Generated during Lipolysis—To further explore the hypothesis that HGL is associated with free fatty acid clusters formed at the lipid droplet surface during lipolysis, the co-localization of both was investigated by adding copper, that binds specifically to free fatty acids, in a medium-size emulsion hydrolyzed by an active rhodamine-HGL. Visualization of both types of fluorescence showed that the green fluorescence, corresponding to the free fatty acids generated (Fig. 7A), and the red fluorescence, corresponding to HGL (Fig. 7B), were perfectly co-localized. Controls without copper were performed (Fig. 7, C and D), demonstrating that HGL localization was not altered by copper addition per se.

Isolation and Composition of the Lipid Particles Generated during Lipolysis—If the hypothesis that lipid particles form at the interface from lipid clusters during gastric lipolysis were true, it should be possible to isolate these particles. As described under “Experimental Procedures” we have isolated the lipid particles from a medium-size emulsion lipolysed by HGL for 90 min using gel filtration, and analyzed them. These particles were composed of 73.7% free fatty acid, 15.6% phospholipid, 5.0% monoacylglycerol, 3.5% diacylglycerol, 1.2% free cholesterol, and 0.9% triacylglycerol. The composition of these particles was different from the surface composition of the droplets, particularly in that they were highly enriched in free fatty acids. Their apparent size (median diameter) determined after isolation using a quasielastic light-scattering detector.
was 196 ± 16 nm. Moreover, the isolated particles were found to contain HGL as assessed by immunodetection.

**DISCUSSION**

In humans, gastric lipase is the key enzyme achieving the first step of dietary lipid digestion (1, 3, 6). This enzyme has been shown to be markedly inhibited by protonated free fatty acids (8, 23), thereby explaining the limited lipolysis of triacylglycerols under gastric conditions, compared with the complete triacylglycerol hydrolysis by pancreatic lipase in the duodenum (4, 10). Nevertheless, the mechanisms of the inhibition process have not yet been elucidated. To better understand the mechanism of action of gastric lipase, *in vitro* lipolysis experiments were conducted under conditions close to those occurring in the human stomach using three differently sized emulsions, a coarse (about 15 μm), a medium (about 2 μm), and a fine one (about 0.7 μm). These emulsions cover the large range of sizes of dietary emulsions found in human stomach contents (3, 10, 18, 38). As anticipated from prior studies (8, 10), the inhibition of gastric lipolysis is highly dependent on the water/lipid interface area, directly related to the lipid droplet size. In addition, the concentration of free fatty acids generated expressed as micromoles/m² surface area (8, 10), rather than total fatty acid concentration (23), is a key regulatory factor. The lipolysis catalyzed by gastric lipase varied inversely with the droplet size of the emulsion and the maximum extent of lipolysis in our *in vitro* model reached ~7 to 23%, close to values found *in vivo* (10, 18, 20). Lipolysis reached a plateau when the generated free fatty acid concentrations were 122, 107, and 114 μmol/m² lipid surface area, for the fine, medium, and coarse emulsions, respectively. Again, these values are close to those previously found in healthy humans (121–128 μmol/m²) (10) or in children with cystic fibrosis (164–172 μmol/m²).² Taken together, these data indicate that our *in vitro* model is relevant to human gastric lipolysis, and, hence, suitable for the study of the mechanisms involved in the physiological inhibition by free fatty acids.

We found that the presence of long chain free fatty acids per se was not inhibitory of lipolysis, since externally added oleic acid in amounts close to, or even higher than those generated during physiological gastric lipolysis, did not alter the kinetics of triolein hydrolysis by gastric lipase (Fig. 3, A and B). Indeed, free fatty acids somewhat stimulated lipolysis (× 1.7) when added as preformed triolein-PL-OA emulsions (Fig. 3D). An inhibitory effect of the externally added OA was only obtained in the presence of a large excess of substrate (Fig. 3C), i.e. under unphysiological conditions (8, 23). These results indicate that it is the long chain free fatty acids endogenously generated that are potent inhibitors of gastric lipolysis under physiological conditions. This suggests, in turn, an important role of specific lipid-lipid or lipid-protein interactions.

² M. Armand and M. Hamosh, unpublished observations.
To understand how free fatty acids play a role in the inhibition process, we studied the localization of the generated free fatty acids within the emulsion. The present results show for the first time that at pH 5.40 oleic acids generated during lipolysis accumulate in part in the surface monolayer of the lipid droplet, with a surface-to-core molar phase distribution coefficient of 7.4 after 10 min lipolysis. Thus, during gastric lipolysis free fatty acids have higher affinity for the surface than the core of the droplets. This value is close to the surface-to-core distribution coefficients reported for triolein-PL-OA artificial emulsions at neutral pH, i.e. around 7 to 10 (26–28).

Taking into account the relative masses of the surface monolayer and the core in these lipid droplets, the surface being far smaller than the core, we have calculated (13) that about 9% of the total free fatty acids generated are present in the surface of the droplet, while about 91% are within the core. Thus free fatty acids partly accumulate at the droplet surface.

Our results showed that other lipolytic products, such as diolein and monolein, are also present in the droplet surface monolayer, representing 2.2 and 17.3%, respectively, of the total amount of each lipid species generated during lipolysis. It is therefore possible that the presence of diacylglycerols and/or monoacylglycerols (39) at the lipid droplet surface might also be involved in the inhibition of the lipolysis by gastric lipase. We also found that the surface monolayer content of triacylglycerol decreased 33-fold following 10 min lipolysis. This indicates quantitatively that the interfacial availability of triacylglycerols for hydrolysis is a function of the surface composition of the droplet which evolves as lipolysis proceeds, as previously reported for chylomicrons (40). It is noteworthy that the great change in the number of triacylglycerol molecules exposed at the droplet surface occurs very rapidly, within 10 min after lipolysis started, whereas the full inhibition of triacylglycerol hydrolysis by gastric lipase occurs after a longer period of time, ~60 min. This suggests that, at least for gastric lipase, a marked decrease in the number of triacylglycerol molecules present at the surface of the droplet is not a key mechanism involved in the inhibition process. It is possible that a small quantity of triacylglycerol exposed at the surface is enough to permit lipolysis, and that this level is kept more or less constant by a core-to-surface transfer of triacylglycerol molecules (13).

The generation of surface active products can considerably modify the physicochemical properties of the remaining substrate in such a system (2). Indeed, a considerable fusion of the lipid droplets occurs during gastric lipolysis, as previously observed (16), probably due to the presence of free fatty acids, monoacylglycerols, and diacylglycerols that are known to be fusogenic (16). This change in the lipid composition of the droplet surface during lipolysis could modify the interfacial tension or the surface pressure and consequently interfere with gastric lipase binding and activity (2, 24, 25). Alternatively, at a high interfacial energy of the lipid/water interface or at a certain droplet surface pressure, gastric lipase could undergo an irreversible denaturation involving a change from a globular to an unfolded conformation at the interface (2, 25). As HGL contains only one disulfide bridge, it has been postulated that it can be denatured more readily than pancreatic lipase with six disulfide bridges (25). In fact, our data do not support these hypotheses since (i) newly added lipase can further hydrolyze a previously lipolysed emulsion showing that the substrate is still available and (ii) a new emulsion added to a previously lipolysed one can be hydrolyzed by the present gastric lipase, indicating that interfacial denaturation is not a likely mechanism for the inhibition of lipolysis.

It seems likely, therefore, that the accumulation of oleic acid at the droplet surface leads to an inhibition of lipolysis by gastric lipase. The mechanism by which this happens was further investigated using microscopy and the results support this hypothesis. Scanning electron microscopy of hydrolyzed emulsions at pH 5.40 allowed us to observe the formation of clusters at the surface of the lipid droplets. The hypothesis that free fatty acids were present in these clusters was supported by immunofluorescence experiments using copper to localize free fatty acids. The fact that copper readily binds to fatty acids (Fig. 7) suggests that free fatty acids present at the surface of the lipid droplet are not fully organized within the surface monolayer but rather may be exposed in an ionized state, in part, at the surface. Co-localization experiments showed that HGL labeled with rhodamine was present in an identical distribution with the free fatty acids in the clusters formed at the surface of the lipid droplets during lipolysis. Moreover, isolation of these clusters showed that they are particles of about 200 nm mainly composed primarily of free fatty acids (approximately 74%), as well as phospholipids (16%), monoacylglycerols (5%), diacylglycerols (4%), free cholesterol (1%), triacylglycerols (<1%), and HGL. Interestingly, the lipid composition of these particles is close to that of the pelleted material found in the human stomach content during fat digestion (3), suggesting that they could have the same origin. Thus, it is likely that gastric lipase becomes bound to and perhaps trapped in these fatty acid-rich particles that are generated during lipolysis at the droplet interface, thereby preventing further hydrolysis of the substrate. Nevertheless, a fluorescence study using an emulsion labeled with dansyl cholesterol and FITC-labeled HGL clearly demonstrated that HGL can move from a previously hydrolyzed droplet to a new one. Thus, it is possible that the partial lipolysis that was observed after addition of a new substrate is due to a limiting rate of the transfer of HGL, secondary to its being trapped within the fatty acid-rich particles at the droplet surface.

Based on the present findings and previous work of ours and others, we propose the following working model for the mechanism of inhibition of gastric lipolysis (Fig. 8). Step 1, HGL binds at the surface of the lipid droplet. At t = 0, the droplet surface is composed of phospholipids, free cholesterol, and some triacylglycerol molecules. Step 2, lipolysis begins, generating mainly free fatty acids and diacylglycerols, with a small amount of monoacylglycerols. These lipolytic products partition to the surface and core of the lipid droplet according to their physicochemical properties, i.e. a higher proportion of free fatty acids and monoacylglycerols than diacylglycerols partition at the surface. The surface area begins to expand as lipolysis continues. Step 3, at a certain time point, the surface pressure increases such that excess free fatty acid-enriched surface begins to reorganize into peripheral clusters that trap HGL during their formation, leading to the formation of particles budding at the droplet surface as lipolysis proceeds. Step 4, the trapped HGL, although still present at the surface of the lipid droplet, has diminished access to the triacylglycerol that is present in the areas of the droplet surface free of fatty acid-rich particles. The lipolysis inhibition process is completed over 60 min because HGL, even in a trapped form, appears to be able to still bind and act on particle-free lipid droplet surface, by transferring from one droplet to another one.

The role of these newly observed gastric fatty acid-rich particles in lipid digestion needs to be further explored; in particular, it will be important to determine whether the prevention of surface particle formation, through manipulation of emulsion properties, can extend the effectiveness of HGL activity for the benefit of patients suffering from lipid maldigestion and malabsorption due to pancreatic insufficiencies.
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Acknowledgments—We thank Drs. D. M. Small and A. Derksen for the core-to-surface partition technique, Dr. Jacques Peyrot and Profesor J. Salducci for the generous gift of gastric juice, Drs. Alexandre Misharin and Christian Alquier for the generous gift of dansyl cholesterol, Claude Alasia and Jean Luc Ansaldi for technical help with scanning electron microscopy, and Dr. Gérard Piéroni for scientific discussions.

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