The role of lipolysis in human orosensory fat perception

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Abstract

Taste perception elicited by food constituents and facilitated by sensory cells in the oral cavity is important for the survival of organisms. In addition to the five basic taste modalities, sweet, umami, bitter, sour, and salty, orosensory perception of stimuli such as fat constituents is intensely investigated. Experiments in rodents and humans suggest that free fatty acids represent a major stimulus for the perception of fat-containing food. However, the lipid fraction of foods mainly consists of triglycerides in which fatty acids are esterified with glycerol. Whereas effective lipolysis by secreted lipases (LIPs) liberating fatty acids from triglycerides in the rodent oral cavity is well established, a similar mechanism in humans is disputed. By psychophysical analyses of humans, we demonstrate responses upon stimulation with triglycerides which are attenuated by concomitant LIP inhibitor administration. Moreover, lipolytic activities detected in minor salivary gland secretions directly supplying gustatory papillae were correlated to individual sensitivities for triglycerides, suggesting that differential LIP levels may contribute to variant fat perception. Intriguingly, we found that the LIPF gene coding for lingual/gastric LIP is not expressed in human lingual tissue. Instead, we identified the expression of other LIPs, which may compensate for the absence of LIPF. —Voigt, N., J. Stein, M. M. Galindo, A. Dunkel, J-D. Raguse, W. Meyerhof, T. Hofmann, and M. Behrens. The role of lipolysis in human orosensory fat perception. J. Lipid Res. 2014. 55: 870–882.

Supplementary key words free fatty acid • lipase • taste • triglyceride • von Ebner’s gland • G protein-coupled receptor

The perception of taste elicited by countess chemicals present in food plays an important role for the survival of organisms. The gustatory system monitors not only the caloric content and the appropriate electrolyte supply, but also the presence of putatively harmful substances in food items (1). Detection of food constituents within the oral cavity is achieved by taste receptor molecules expressed by sensory cells specifically devoted to the detection of one of the five taste qualities, sweet, umami, salty, sour, and bitter (2). In the past, the existence of additional taste modalities such as fatty (3), metallic (4), or a taste for water (5) has been speculated. In recent years, particular attention has been paid to the potential existence of fat taste and its putative status compared with the other well-accepted taste qualities. Whereas the textural, olfactory, and postigestive recognition of fat constituents have been considered the dominant cues for fat perception in the past, several recent studies, performed mostly in rodents but also in humans, have pointed to a gustatory component in fat perception [for a recent review see (6)]. The use of anosmic (7) or esophagostomized (8) rat models in combination with texture-masking buffer compositions indicated an orosensory detection mechanism for long-chain fatty acids, in particular. Moreover, a number of candidate receptors for the oral detection of lipophilic molecules were identified by several independent research groups. These putative fat sensors include potassium channels (3) and scavenger receptor/fatty acid transporter CD36/FAT (9, 10), as well as G protein-coupled receptors (GPRs) such as GPR40 (11) and GPR120 (11–13). For some of the candidate receptors, knockout mouse models were analyzed and their contribution to orosensory fat perception confirmed. Mice with a genetic ablation of CD36 exhibit no preference for the polyunsaturated long-chain fatty acid, linoleic acid, in two-bottle preference tests compared with wild-type mice. Moreover, a reduced cephalic phase response upon oral stimulation with oleic, linoleic, and linolenic acid, but not to the saturated long-chain fatty acid, stearic acid, or the medium-chain fatty acid, caprylic acid, suggests pronounced selectivity for particular lipophilic stimuli (10). Similarly, GPR40 as well as GPR120 knockout mice both show a loss of preference for linoleic acid, and

Abbreviations: CV, circumvallate papillae; GPR, G protein-coupled receptor; LIP, lipase; PNLIP, pancreatic lipase; TGF, taste cell-free; TFL, triacylglyceride-free lipid; VEG, von Ebner’s gland; VEGP, von Ebner’s gland protein.

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diminished responses upon acute stimulation with several fatty acids, demonstrated by gustatory nerve recordings (11). However, one of the prerequisites for a bona-fide additional “fat” taste quality would be the identification of a separate dedicated taste cell population expressing these candidate receptors. While all of the above-mentioned fat-taste receptor candidates have been detected within taste buds on the tongue of rodents, evidence on the uniqueness of the corresponding taste cell population is still lacking. Although evidence is also mounting in humans (14–19), much less is known about the orosensory perception of fat constituents relative to rodent models. However, it appears conceivable that the same group of candidate receptors shown in rodents or a subset thereof, such as CD36 (20) and GPR120 (18), may contribute to human fat perception as well.

One of the many open questions concerning human fat perception is what might be the predominant form of the fatty stimulus presented to the oral cavity? Similar to the other two macronutrients, carbohydrate and protein, which are not directly detected by taste receptors, but rather in form of their building blocks, the main dietary lipids are triglycerides and not free fatty acids. In the case of carbohydrates, the contribution of α-amylase, a polysaccharide-degrading enzyme secreted by minor salivary glands directly into the trenches of foliate and vallate papillae, to the slowly developing sweet taste of otherwise tasteless starch is undisputed. Moreover, a recent study reported the presence of this enzyme in a subset of rodent taste receptor cells (21). An almost identical system for the degradation of triglycerides into free fatty acids exists in rodents. In this case, lingual lipase (LIP), a triglyceride degrading enzyme, is produced by minor salivary gland cells and secreted into the trenches of gustatory papillae in the back of the rodent tongue. In fact, it was demonstrated that lingual LIP secreted in saliva of minor salivary glands effectively liberated free fatty acids from trioleate in rats. Moreover, this enzymatic activity was required and sufficient for the development of triglyceride preference in rats, as demonstrated by the blocking activity of the LIP inhibitor, orlistat (22).

For a role of lipolytic activity in human orosensory fat perception, contrasting evidence has been presented. While a recent report demonstrated that human subjects responded to oral stimulation with triolein with thresholds similar to those observed after stimulation with the corresponding free fatty acid, oleic acid, in the absence of orlistat, the thresholds increased, however, indicating an involvement of lipolytic enzymes in stimulus presentation (23). Another recent study performed in human subjects who chewed and expectorated different triglyceride-containing food items for a constant time period before quantifying the amount and nature of free fatty acids concluded that the concentration of free fatty acids in saliva after mastication would reach the required detection threshold. However, the contribution of oral LIP activity was not directly addressed (24). In contrast to these observations, however, other studies demonstrated the absence of lingual LIP by Western blotting, detected only traces of LIP activity in human saliva (25), and questioned the involvement of LIP activity in human orosensory fat perception (19). The latter would argue that only the free fatty acids already present in food may be perceived by human subjects or, perhaps at higher threshold concentrations, triglycerides may be directly detected within the oral cavity [compare (6, 24)].

In order to clarify this important question, we set out to investigate whether triglycerides represent an adequate “fatty” stimulus in vivo and in vitro to determine if lipolytic enzymes are present in the human oral cavity. We performed sensory experiments on human panelists using either triolein or oleic acid as a stimulus in the absence or presence of orlistat. The results of these experiments were compared with data obtained from heterologously expressed fatty acid-sensitive G protein-coupled receptors, GPR40 and GPR120, two main candidates for human and rodent orosensory fat perception. Further, lipolytic activity was measured in human salivary samples. By RT-PCR and in situ hybridization studies, we determined expression of LIPs in minor salivary glands [von Ebner’s gland (VEG)] of human tongue tissue.

**MATERIALS AND METHODS**

**Lipid stimuli**

Oleic acid sodium salt (C18:1), oleic acid (C18:1), glyceryl trioleate, oleyl alcohol 13C3-palmmitoleic acid, and 13C3-glyceryl trioleate were purchased from Sigma-Aldrich (Taufkirchen, Germany).

To minimize potential textural cues induced by the stimuli, human sensory experiments on oleic acid, oleyl alcohol, and glyceryl trioleate were performed in an aqueous triacylglyceride-free lipid (TFL) matrix containing 5.0% (w/v) highly refined mineral oil (white oil, mouse embryo tested; Sigma-Aldrich) as a control for lubricity, 5.0% (w/v) Gum Arabic (Ph Eur, from acacia tree) to minimize viscosity cues, 0.3% (w/v) whey protein (100% Natural Whey Protein Isolate; Olimp) as an emulsifier, and 0.1% (w/v) EDTA (bioUltra) to prevent fatty acid oxidation (18). Prior to sensory analysis, the stimuli were purified by an aerobic low temperature recrystallization from acetone/tributyrin and chromatography, respectively, to afford a purity of each stimulus of >99% (HPLC-evaporative light scattering detector, high resolution gas chromatography-MS).

**Sensory analyses**

Twenty assessors, who gave informed consent to participate in the sensory tests of the present investigation and had no history of known taste disorders, participated for at least 2 years in sensory training sessions and were asked to recognize and quantify the taste of aqueous solutions (5 ml each) of the following purified reference compounds dissolved in bottled water (Evian, low mineralization: 500 mg/l) by means of duo and triangle tests, respectively, using a sip-and-spit procedure (26): sucrose (50 mmol/l) for sweet taste, lactic acid (20 mmol/l) for sour taste, NaCl (12 mmol/l) for salty taste, caffeine (1 mmol/l) for bitter taste, monosodium glutamate (8 mmol/l, pH 5.7) for umami taste, an aqueous emulsion of oleic acid (1 mmol/l, containing 0.02% Emultop) for fatty taste, a water/sunflower oil emulsion (3/6 w/w, containing 0.5% Span 65) for oily mouthfeel, and an emulsion of stearic acid (1 mmol/l, containing 0.02% Emultop) for a grainy powdery mouthfeel.
A group of 12 assessors (4 males, 8 females, 26–40 years of age) was selected from these trained panelists in a preliminary screening test by verifying their oral fatty acid sensitivity using oleic acid (1.4 mmol/l) as the test stimulus (19). The sensory sessions were performed in an air-conditioned room (22°C) with separate booths in three independent sessions. The subjects were instructed to refrain from food, beverages, and oral care products for a minimum of 2 h before the sensory experiments. To prevent cross-modal interactions with odorants, the panelists used nose clips.

For the determination of detection threshold concentrations for the fatty and/or “scratchy” oral sensation of oleic acid, glyceryl trioleate, and oleyl alcohol, respectively, in TFL matrix, an aliquot (2 ml) of sample dilutions differing by 0.25 log units was then presented to the sensory panel in order of ascending concentrations and each dilution was evaluated by means of a two-alternative forced-choice test (ISO 5495:2005) of five sample pairs using a sip-and-spit procedure. During the evaluation, the panelists were asked to rinse their oral cavity with water and wait for 5 min between each sample. The individual threshold concentration of each panelist was calculated as the geometric mean between the last incorrectly identified sample and the first correctly identified sample solution (three repetitions). The threshold concentration of the panel was calculated as the geometric mean of all individual threshold concentrations (P < 0.05).

In an additional set of experiments, the influence of the LIP inhibitor tetrahydrolipstatin (Sigma-Aldrich) on the fatty perception was investigated using glyceryl trioleate (10.0 mg/ml) and free oleic acid (3.0 mg/l), respectively, as stimuli. A solution of the respective stimulus in TFL matrix (2 ml) in the presence or absence of tetrahydrolipstatin (0.25%, w/w) was evaluated by a group of 12 assessors (4 males, 8 females, 26–40 years of age) against the blank TFL matrix (2 ml) by means of a two-alternative forced-choice test (ISO 5495:2005) using a sip-and-spit procedure and asking the question “which sample is more fatty” (three repetitions).

**Lipolytic activity at human foliate papillae**

The experiments on oral lipolytic activity of human volunteers were approved by the ethics committee of the Technische Universität München (application #2311/09) and informed consent of participants was obtained. Twelve trained assessors (4 males, 8 females, 26–40 years of age), instructed to refrain from food, beverages, and oral care products for a minimum of 2 h before the experiment, were asked to rinse their oral cavity for 30 s with bottled water (5 ml). Thereafter, filter paper discs (1 cm², filter paper classic, 94 mm; Melitta, Minden, Germany) loaded with triolein (5 µl, 5.15 µmol/cm²) in the absence and presence of 0.1% (w/w) orlistat (Sigma-Aldrich) were placed on the foliate papillae of the volunteers for 10, 20, 30, 60, 90, and 150 s and, instantaneously after removal, were transferred into glass vials containing diethyl ether (2 ml; Merck, Darmstadt, Germany) for quenching LIP activity. After adding defined amounts of the internal standards 13C₁-palmitoleic acid and 13C₃-triolein, water (5 ml, Milli-Q water advantage A 10 system; Millipore, Schwabach, Germany) was added and, after mixing and pooling the solutions of the individual subjects, lipids were extracted with chloroform/methanol (2/1, v/v; 5 ml, three times) by vortexing the tubes for 30 s, followed by centrifugation for 10 min at 2,000 rpm. The organic phase was separated from solvent under a stream of nitrogen, the residue taken up in acetonitrile, and an aliquot (10 µl) was analyzed by HPLC-MS/MS consisting of a Dionex UHPLC UltiMate® HPLC-system (Dionex, Idstein, Germany) hyphenated by an API 4000 QTRAP mass spectrometer (AB SCIEX Instruments, Darmstadt, Germany). Operating in the negative atmospheric pressure chemical ionization mode, analysis of the fatty acids was performed on a 150 × 2.0 mm, 5 µm, HyperClone ODS C18 column with a flow rate of 250 µl/min starting with 60% acetonitrile and 40% aqueous formic acid (0.1% in water) for 6 min, then increasing the acetonitrile to 100% within 10 min, followed by isocratic elution for an additional 10 min. Analysis of glyceryl trioleate was done on a 150 × 2.0 mm, 4 µm, Hydro-RP column (Phenomenex, Aschaffenburg, Germany) by using a binary gradient with 2-propanol/formic acid (0.1%)/ammonium formate (10 mmol) as solvent A and acetonitrile/formic acid (0.1%)/ammonium formate (10 mmol) as solvent B. Using a flow rate of 250 µl/min, chromatography was done by increasing solvent A from 20 to 30% within 5 min, then to 60% in 5 min, followed by isocratic elution for 8 min. For quantitation of the oleic acid and glyceryl trioleate, a five point calibration curve was recorded using 13C₃-palmitic acid as stable isotope labeled internal standard for the free oleic acid and glyceryl 13C₃-trioleate as the internal standard for glyceryl trioleate, and by plotting the ratios of peak areas of analyte/internal standard versus concentration ratios of analyte/internal standard.

**Correlation of individual lipolytic activity with sensory sensitivity**

For comparison of individual lipolytic activity at foliate papillae with sensory data for fatty perception, two groups of assessors were selected from the 20 subjects, group A (5 subjects) showing high sensitivity for glyceryl trioleate with detection thresholds of less than 0.65 mmol/l, and group B (5 subjects) showing lower triglyceride sensitivity with detection thresholds >1.0 mmol/l. To validate the different triglyceride sensitivity of both groups, a three-alternative forced-choice test was performed with glyceryl trioleate (0.7 mmol/l) in TFL matrix (2 ml) using a sip-and-spit procedure (two replicates). Whereas the sensitive group A was able to correctly pick out the right sample (P < 0.001; ISO 5495:2005), there was no significant outcome for group B, thus demonstrating a clear separation of both subject groups regarding their orosenory triglyceride sensitivity. For determination of the individual lipolytic activity of both subject groups, the assessors rinsed their oral cavity for 30 s with bottled water (5 ml) and were then challenged with filter paper discs (1 cm²) loaded with glyceryl trioleate (5 µl, 5.15 µmol/cm²) and placed on the foliate papillae for 150 s, respectively. After removal, lipolytic activity was terminated by transferring the paper discs collected from each individual subject into glass vials containing diethyl ether (2 ml; Merck, Darmstadt, Germany), followed by quantitation of free oleic acid using the stable isotope dilution assay detailed above (two biological replicates). A Welch’s t-test (27) was applied to demonstrate significant difference in lipolytic activity between group A and B.

**Cell culture**

HEK 293 T stably expressing Ga16-gust44 cells were maintained in DMEM (Gibco) containing 10% fetal bovine serum (Biochrom, Berlin), 4.5 g/l glucose (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (Sigma), 100 mg/ml streptomycin (Sigma), and 400 µg/ml G418 (Calbiochem) at 37°C, 5% CO₂. For calcium-imaging experiments, cells were seeded into 10 µg/ml poly-l-lysine-coated 96-well black clear-bottom plates (µClear, Greiner, Solingen) and after 24 h later, at ~60% confluence, transiently transfected with 150 ng of expression vectors using Lipofectamine 2000 (Invitrogen) (18).

**Functional expression of the GPRs and calcium imaging analyses**

Constructs of human GPR40 or the short variant of human GPR120 in the vector pcDNA5/FRT (18) were transiently transfected into human embryonic kidney cells HEK 293 T-Ga16Gust44. After 22 h, the cells were loaded for 1 h with 1 µM of the Ca²⁺ sensitive dye Fluo-4-AM (Invitrogen) and 2.5 mM probenecid in
serum-free medium. After washing with buffer C1 [130 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 10 mM glucose (pH 7.4)], cells were stimulated with agonists at concentrations referred to in Fig. 3. Agonist stock solutions (C18.1, 10 mM; glycercyl trilolate, 50 mM) were freshly prepared in 100% ethanol before each experiment and then diluted to the working concentrations in buffer C1. The final concentration of the solvent ethanol was kept at or below 0.3% (v/v) to avoid toxic effects on the cells. Receptor responses were measured using a fluorometric imaging plate reader (FLIPR TETRA® system, Molecular Devices) as before (18). Changes in fluorescence after substance application were monitored and, after subtraction of background fluorescence, shown as calcium traces.

RT-PCR

The collection of human biopsy material was approved by the ethics committee of the Charité, Campus Virchow Hospital, Germany (application # 240/2002) and informed consent of participants was obtained. Biopsy materials of human circumvallate papillae (CV), C57BL/6 mouse foliate papillae, and mouse stomach were subjected to total RNA extractions using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. Total RNA of human stomach was purchased from BD Biosciences Clontech. For removal of contaminating genomic DNA, RNAs were subjected to RNase-free DNase-I (Invitrogen) digestion for 30 min at room temperature. Synthesis of cDNA was done using SuperScript III RT and random hexamers (Invitrogen) following the recommendations of the manufacturer. Identities of all cDNA clones were confirmed by sequencing (Eurogentec, Liege, Belgium).

TABLE 1. Oligonucleotides used for cloning and RT-PCR analyses

| Number | Oligonucleotide | Sequence (5’ to 3’) | Annealing (°C) | Amplicon size (bp) |
|--------|----------------|---------------------|----------------|-------------------|
| 1      | hLIPF          | fw GAGGATCCCATGGGCTGCTGTTTTTAAACAT | 58             | 1197             |
| 2      | mLIPF          | fw GACACAGCTTTGAACCAATG | 60             | 1188             |
| 3      | LIPF-RT        | fw CATTGTTACCACTGTTCTTG        | 53             | 303              |
| 4      | Amylase-RT     | fw GCACGAGCTTGCAAGAACAAACGAG  | 55             | 300              |
| 5      | VEGP-RT        | fw GTAAGGAGAGGTGCGTACCCGCA    | 55             | 522              |
| 6      | Pepsinogen-RT  | rv CATTGACAGGAGATGGTATGCAATGCC | 60             | 203              |
| 7      | GAPDH-RT       | rv ACCGAGATGGATGTACCCGCA      | 58             | 454              |
| 8      | hLIPK-RT       | rv CGAGTATGTTAGGTGTTGTTG      | 60             | 410              |
| 9      | hLIPM-RT       | rv GGATTTCATGCCATAGGCAC     | 60             | 393              |
| 10     | hLIPN-RT       | rv CTTTGCGTACCCACGACAGCG   | 61             | 448              |
| 11     | hLIPJ-RT       | rv CAGCGAGCCTTGTGGCAATC     | 59             | 470              |
| 12     | hPNLIP-RT      | rv TCAAGAATGGTGGCAGACATTCA  | 57             | 305              |

fw, forward primer; rv, reverse primer.

Cloning of cDNAs for the generation of riboprobes

For subsequent generation of riboprobes, all cDNAs were subcloned into the transcription vector pBluescript I KS+ (Stratagene). α-Amylase: a 300 bp fragment of human α-amylase cDNA was amplified by PCR from human CV cDNA and subcloned into the EcoRI site of the vector. For mouse α-amylase, a commercially available cDNA provided in the vector pcDNA-Sport6 (Open Biosystems) was digested with EcoRI resulting in a 531 bp long fragment, which was subsequently cloned into the EcoRI site of pBluescript I KS+. LIPF (lingual/gastric LIP): human LIPF cDNA corresponding to the entire coding region was amplified by RT-PCR using cDNA prepared from commercially available stomach total RNA (BD Biosciences Clontech). After the intermediate insertion into the expression vector pcDNA3-FRT (Invitrogen) with BamHI and NotI, a 912 bp long fragment was obtained by BamHI and HindIII digestion and inserted into the transcription vector. The entire coding region of mouse LIPF was amplified by PCR from vallate papillae cDNA of the C57BL/6 strain. Further subcloning of a 902 bp long fragment was performed exactly as described for human LIPF cDNA. Human LIPK, LIPM, and LIPN: ampiclons specific for LIPK, LIPM, and LIPN were generated by PCR from human CV cDNA. After intermediate cloning into pTOPO-TAII or -blunt vectors (Invitrogen), HindIII/XhoI or EcoRI similar sized fragments (LIPK, 434 bp; LIPM, 500 bp; LIPN, 553 bp) were transferred into pBluescript I KS+ vector. The insert orientations and sequence identities of all cDNA clones were confirmed by sequencing (Eurofins MWG Operon, Martinsried). For a list of oligonucleotides,
annealing temperatures, and thermostable DNA-polymerases used for the amplifications, see Table 1.

In situ hybridization of human and mouse gustatory papillae

Linearization of cDNA constructs was done with BamHI (LIPF), HindIII (amylase), Xhol + NotI (LIPK), Xhol + XbaI (LIPM), and Xhol + SpeI (LIPN). Then, in vitro transcription was performed using T3 or T7 RNA polymerase (Roche Applied Science, Mannheim) for sense and antisense probes, respectively. The probes were labeled using Dig-RNA-labeling mix (Roche), treated with RNase-free DNase I (Invitrogen), and then purified by precipitation. In situ hybridizations were performed mainly as before (28). Briefly, cross-sections of human (10 µm) or mouse (14 µm) CV were processed and thaw-mounted onto positively-charged glass slides. Prior to hybridization, the sections were fixed, permeabilized, and acetylated. Prehybridization was done at 50°C for 5 h, followed by hybridization overnight at 50°C. After hybridization the slides were washed several times at low stringency, followed by RNaseA treatment and high stringency washes using 0.4× saline sodium citrate buffer at 50°C. Hybridized riboprobes were detected using an anti-digoxigenin antibody conjugated with alkaline phosphatase and a colorimetric reaction. Images were taken with a microscope (Axioplan; Zeiss, Goettingen, Germany) connected to a CCD camera (RT slider; Diagnostic Instruments, Sterling Heights, MI) or with an automated microscope scanning system (MIRAX Midi; Carl Zeiss MicroImaging GmbH, Jena, Germany).

RESULTS

Human orosensory perception of free and esterified oleic acid

Previously we have shown that human subjects are able to detect free fatty acids of various chain lengths in sensory analyses performed in an aqueous TFL matrix to minimize potential textural cues induced by the stimuli (18). Specifically, long-chain fatty acids, if provided in texture masking fat-like matrix, induce a sensation described as fatty, whereas shorter chain fatty acids and fat alcohols or fatty acids when presented in aqueous solutions were rather described as scratchy (18). In order to test whether esterified long-chain fatty acids were also recognizable by our panelists, and if so, whether identical or similar descriptors would be used, we performed additional sensory tests with the free long-chain fatty acid, oleic acid (C18:1), its esterified form, glyceryl trioleate, and the corresponding fat alcohol, oleyl alcohol. As before, all stimuli were delivered in the aqueous TFL matrix to mask potential texture differences (Fig. 1A). In agreement with our previous observations, oleic acid was recognized and described as fatty at lower concentrations (threshold = 0.4 mM) and as scratchy at higher concentrations (threshold = 2.2 mM). The corresponding fat alcohol, oleyl alcohol, was perceived in a comparable concentration range and attributed exclusively as scratchy (threshold = 0.6 mM), again confirming our previous results. Surprisingly, oral expectoration of triolein resulted in the sensitive recognition of this compound (threshold = 0.5 mM) and this time the only descriptor used was fatty, even at high concentrations. These results suggest that either the esterified form of oleic acid is recognized by human subjects and activates a triacylglyceride-sensitive detection system or an efficient lipolytic enzyme is in place liberating oleic acid from trioleate.

To test whether the long-chain fatty acid-sensitive GPR120, that we suspected to contribute to human orosensory perception of lipids, would equally respond to trioleate, we performed functional calcium imaging experiments. In addition to GPR120, we transiently transfected another long-chain fatty acid receptor, GPR40, implicated in rodent...
fatty acid perception (11), which is, however, not expressed in human tongue tissue, in HEK 293T-Ga16gust44 cells and stimulated the cells with high concentrations of oleic acid (3 μM) and trioleate (3 μM). As a negative control, cells transiently transfected with empty vector were treated accordingly (Fig. 1B). The prominent signals obtained after challenging GRP40 and GPR120 with oleic acid and the absence of calcium traces in receptor transfected cells stimulated with trioleate clearly demonstrate that both receptors respond exclusively to the free fatty acid. Hence, we concluded that humans possess either an effective lipolytic enzyme system within their oral cavity or trioleate is recognized by a yet unknown lipid sensing receptor.

In order to challenge the hypothesis of the involvement of lipolytic enzymes in fatty taste perception, we studied the influence of the LIP inhibitor, tetrahydrolipstatin, on the fatty perception induced by free oleic acid as well, as its ester, glyceryl trioleate (Table 2). By means of a two-alternative forced-choice test (Which sample is more fatty?), randomized TFL solutions containing the respective fatty stimul in the presence or absence of tetrahydrolipstatin were evaluated against the blank TFL matrix. Oleic acid solutions in TFL matrix were significantly judged as the more fatty sample when compared with the TFL matrix alone independent on the absence (α-level 0.02) or the presence of the LIP inhibitor (α-level 0.01). Whereas trioleate could be clearly found to enhance fattiness of the TFL matrix (α-level 0.01), the presence of the LIP inhibitor diminished the sensory impact induced by the triglyceride (α-level >0.2). The data of these human sensory studies showed strong evidence for the active role of lipolytic enzymes in the orosensory perception of triglycerides.

### Determination of lingual LIP gene expression in human taste tissue

To confirm the presence or absence of lingual LIP in human taste tissue, we performed RT-PCR experiments with cDNA obtained from human gustatory tissue. Corresponding mouse tissue was used for control reactions as pronounced lingual LIP activity was reported in mice and rats (29). In fact, the proteins encoded by the orthologous LIPF genes were termed differently in humans ("gastric LIP") and mice ("lingual LIP") because pronounced differences in the peak enzymatic activities in gastric and oral tissues were reported in the two species [compare (29)]. Hence, we also included stomach cDNA of both species in our analyses. In complete agreement with these previous reports, we failed to obtain a PCR product when amplifying cDNA from human CV (Fig. 2A). In contrast, a LIPF-specific amplicon was readily obtained from mouse vallate papillae cDNA. Performing the same analysis using human and mouse stomach cDNA samples gave the opposite result. A LIPF-specific PCR product was evident after amplification of human but not mouse cDNA, corroborating again previous reports about the different expression patterns of this gene in both species. To rule out the possibility that we were unable to detect RNA present in mouse chief cells of the fundus, we amplified mouse stomach cDNA with primers specific for pepsinogen. As pepsinogen is released from the same cell type as LIPF, the pepsinogen-specific product obtained from mouse stomach cDNA demonstrates the presence of this cell type in our sample. As LIPF in rodents is secreted by minor salivary glands into the trenches of foliate and vallate papillae, we performed additional control reactions to confirm the presence of cDNAs originating from gland cell RNA in the investigated tissue samples. Indeed, PCR-products specific for human and mouse α-amylase were obtained from both samples, confirming the presence of minor salivary gland cells in these preparations. Serous minor salivary glands, also called VEGs, produce VEGP (lipocalin 1, tear prealbumin) (25, 30–32), a lipocalin-family member. This gene has been reported to be present in humans but absent in *Mus musculus*. The successful amplification of a VEGP-specific PCR product only from human CV cDNA further confirmed the complete representation of cell types in the

### Table 2. Influence of tetrahydrolipstatin on the recognition of oleic acid and trioleate in a TFL matrix

| Stimuli                          | Positive Answers | α-Level |
|---------------------------------|------------------|---------|
| Oleic acid                      | 24/36            | 0.02    |
| Oleic acid + tetrahydrolipstatin| 26/36            | 0.01    |
| Glyceryl trioleate              | 27/36            | 0.01    |
| Glyceryl trioleate + tetrahydrolipstatin | 29/36 | >0.2    |

*A solution of oleic acid (3.0 mg/l) and glyceryl trioleate (10 mg/l), respectively, in TFL matrix (2 ml) in the presence or absence of tetrahydrolipstatin (0.25%, w/w) was evaluated by a group of 12 assessors against the TFL matrix (blank) by means of a 2-alternative forced-choice test using a sip-and-spit procedure and asking the question (Which sample is more fatty?).

A number of positive answers obtained from a total of 36 responses obtained for 12 assessors in triplicate analysis.
were quantitatively determined in pooled sample by means of HPLC-MS/MS using a stable isotope dilution assay. Compared with a blank vehicle (control), the absolute amounts of free oleic acid on the vehicle steadily increased from about 0.008 ± 0.003 nmol after 10 s to about 0.102 ± 0.012 nmol after 150 s, thus demonstrating a time-dependent release of the free fatty acid (Fig. 4A). Although the presence of the tetrahydrolipstatin could not completely abolish the release of free oleic acid from the triglyceride, the LIP inhibitor induced a significant reduction of the free fatty acid release. Taking the 30 s incubation as the reference, the recovery of triglyceride in the glyceryl trioleate-loaded vehicle after an incubation time of 150 s was about 75% and close to 100% in the presence of tetrahydrolipstatin (Fig. 4B), thus clearly demonstrating the lipolytic activity of foliate papillae secretions resulting in the metabolism of about one-quarter of triglycerides.

Correlation of individual triglyceride sensitivities with lipolytic activities in saliva

For comparison of individual lipolytic activity at foliate papillae with sensory data for fatty perception, subjects were screened for the sensitivity for glyceryl trioleate on the basis of detection thresholds by means of a two-step procedure. First, subjects were evaluated in their detection thresholds for glyceryl trioleate in TFL matrix resulting in a group A of five glyceryl oleate-sensitive subjects showing detection thresholds of <0.65 mmol/l and a group B of five less sensitive subjects with detection thresholds >1.0 mmol/l (Fig. 5A). Second, a three-alternative forced-choice test was performed with glyceryl trioleate in TFL matrix at a level of 0.7 mmol/l. Whereas the sensitive group A was able to correctly pick the right sample (P < 0.001), there was no significant outcome for group B, thus demonstrating a clear separation of both subject groups regarding their orosensory triglyceride sensitivity. For determination of the individual lipolytic activity of both subject groups, glyceryl trioleate loaded vehicles were placed on the foliate papillae of the subjects for 150 s, followed by

Determination of lipolytic activity in human minor salivary gland secretions

To quantitatively monitor the lipolytic activity in human foliate papillae, the release of oleic acid from glyceryl trioleate was measured by placing filter paper discs loaded with glyceryl trioleate and/or without the LIP inhibitor tetrahydrolipstatin onto the foliate papillae of the 12 triglyceride-sensitive volunteers from 10 up to 150 s. Instantaneously after removal and sample work-up, the amounts of free oleic acid as well, as the recovery of glyceryl trioleate, tissue samples subjected to RNA preparation and cDNA synthesis.

In conclusion, the lack of gene expression in minor salivary gland tissue demonstrates that LIPF cannot account for the liberation of free fatty acids from triglycerides in humans and thus does not explain the discrepancy between the detection of triglycerides in our sensory experiments and the observed specificity for long-chain fatty acids of the candidate taste receptors.

Cellular expression of lingual LIPF in human and mouse VEG by in situ hybridization. Ten micron thick cryostat cross-sections of CV containing taste buds (not shown) and VEGs were hybridized with digoxigenin-labeled antisense (A, E) or sense (B, F) riboprobes specific for human (A, B) or mouse (E, F) lingual LIPF. To control the presence of VEGs, sequential sections were hybridized with antisense (C, G) or sense (D, H) riboprobes specific for human (C, D) and mouse (G, H) lingual amylase. Scale bar = 100 µM.
The role of lipolysis in human orosensory fat perception

The discrepancy between the absence of LIPF (lingual/gastric LIP) gene expression in human gustatory tissue and detection of lipolytic activity in saliva of minor salivary glands, forced us to investigate whether other secreted LIPs may account for the observed lipolytic activity. Indeed, several years ago a cluster of LIP genes on human chromosome 10 was identified upon a screening for genes predominantly expressed in human keratinocytes (34). In addition to the well-characterized LIPF [lingual/gastric lipase (35)] and LIPA [lysosomal cholesterol ester hydrolase (36)], four LIP family members, LIPK, LIPM, LIPN, and LIPJ are present in this locus (human chromosome 10q23.31). Of these, LIPK, LIPM, and LIPN, of which the genes are located downstream in close proximity to the LIPF gene and share the identical direction of transcription, possess a putative leader peptide at their amino termini and thus may be secreted enzymes similar to LIPF (Fig. 6A). Hence, we focused on the latter three LIP family members in our expression analyses. By RT-PCR of cDNA from human lingual epithelium using primers specific for putative secretory LIPs and α-amylase, we detected LIPK gene expression in nongustatory lingual tissue [“taste cell-free” (TCF)], as well as in preparations of human CV from two individuals (Fig. 6B). A faint PCR product specific for LIPM was evident in only one of the two CV samples.

LC-MS/MS quantitation of free oleic acid using the stable isotope dilution assay. Comparing the amounts of free oleic acid released upon contact with foliate papillae secretions for the subjects in both groups A and B revealed a clear separation and showed higher amounts of oleic acid, that means higher lipolytic activity, in the samples collected from the fat-sensitive group A (Fig. 5B). On average, concentration of free oleic acid detected for participants from group A (mean = 5.77, SEM = 0.52) was higher compared with samples from group B (mean = 2.27, SEM = 0.49), application of Welch’s t-test revealed a significant difference between both groups (t(17.44) = −4.87, P < 0.001). These data clearly indicate that individual differences in lipolytic activity might play a role in the different release patterns of free fatty acids from triglycerides and in orosensory fat sensitivity.

Identification of LIP family members in human gustatory tissue

Fig. 4. Lipolytic activity in human minor salivary gland secretions. Release of free oleic acid (A) and recovery of glyceryl trioleate (B) from filter paper vehicles (1 cm²) loaded with glyceryl trioleate (5.14 μmol/cm²) in the absence and presence of the LIP inhibitor tetrahydrolipstatin and placed on the human foliate papillae. The recovery of glyceryl trioleate (B) after 150 s is referenced on the levels of glyceryl trioleate found after a short incubation of 30 s.

Fig. 5. Individual trioleate perception and oleic acid release by lipolytic activity. Individuals’ distribution of threshold concentrations (A) and LIP activity (B) expressed as amount of free oleic acid released from glyceryl trioleate (5.14 μmol/cm²) for five glyceryl trioleate-sensitive subjects showing detection thresholds <0.65 mmol/l (group A) and five less sensitive subjects with detection thresholds >1.0 mmol/l (group B).
small deletion, which may have resulted from alternative splicing events. As already shown in Fig. 2, before we could not detect LIPF-specific PCR products, whereas the PCR reactions specific for \( \alpha \)-amylase cDNA were positive in all samples analyzed.

Performing an extensive literature search for other LIPs that may account for the observed oral triglyceride-cleaving activity revealed no further likely candidate enzymes:

Fig. 6. Schematic representation of human LIPs and expression analyses of human LIPK, LIPM, and LIPN in circumvallate tissue. A: Phylogenetic tree and schematic of human acid LIP family members. aa, amino acids. Positions of residues corresponding to the leader peptide and the catalytic triad are labeled. cDNAs originating from the CV of two different human subjects were used as templates to study the expression of LIPK, LIPM, and LIPN (B), as well as LIPJ and PNLIP (C). For comparison and as controls, LIPF, \( \alpha \)-amylase, or VEGP cDNAs were amplified. M, molecular weight marker (Gene Ruler DNA ladder mix; Fermentas); hCV, amplification of cDNA from human CV; TCF, amplification of cDNA from human tissue surrounding the CV without taste cells; +RT, reaction including RT; −RT, control without RT; N, \( \alpha \)-H2O negative control. Pos, cDNA of positive control tissue; LIPJ, testis; PNLIP, pancreas.
The role of lipolysis in human orosensory fat perception

Numerous LIPs do not utilize triglycerides as substrate (e.g., phospholipases such as LIPH, LIPI, phospholipases A–D; mono-/diglyceride LIPs such as DAGLA, DAGLB, MGLL), require coactivators like bile salts (BSSL, CEL) or hormonal stimulation for full enzymatic activity, occur only in intracellular compartments (e.g., LIPA, the lysosomal LIP or ATGL, the adipocyte triglyceride LIP), have been firmly associated with specific tissues or functions (such as the lipoprotein LIPs LIPC, LIPD, LIPG) or share multiple of the above mentioned features disqualifying them as secreted oral triglyceride LIPs. We assessed however, the gustatory expression of two further LIPs, PNLIP (37) and LIPJ (34, 38), because PNLIP is secreted and hydrolyzes triglycerides and LIPJ, even though it lacks a signal peptide required for secretion, because the corresponding gene is located within the same cluster of genes as LIPA, LIPF, LIPK, LIPM, and LIPN (34), and therefore may be subject to similar gene regulation. As shown in Fig. 6C, both cDNAs were not detectable in tongue tissues, thus precluding a role in oral triglyceride breakdown.

Because expression of LIPK, LIPM, and LIPN genes were first reported in human keratinocytes (34), we performed in situ hybridization experiments of human CV sections to determine whether transcripts of these genes are present in VEGs (Fig. 7B). Indeed, in situ hybridizations with all three antisense probes specific for LIPK, LIPM, and LIPN (Fig. 7) resulted in the clear labeling of VEG cells in addition to keratinocytes located in the basal layers of the tongue epithelium (Fig. 7A). The absence of signals when using the corresponding sense probes (Fig. 7) demonstrates the specificity of the hybridization procedure. The identification of putatively secreted lipolytic enzymes expressed in VEG cells indicates that these LIPs can surrogate for LIPF activity and may account for the LIP activity observed in the previous experiment.

**DISCUSSION**

Here, we investigated whether human probands orally perceive triglycerides and whether this may occur directly or via the recognition of long-chain fatty acids liberated from triglycerides by lipolytic activity. We demonstrate that humans perceive triolein similarly to its constituent, oleic acid, as fatty. Moreover, we show that oleic acid is efficiently liberated from trioleyl glycerol upon exposure to saliva secreted from foliate papillae and that blocking of lipolytic activity by tetrahydrolipstatin (orlistat) reduces the generation of oleic acid. Most notably, the subjects’ sensitivity to oleic acid was correlated with their LIP activity. Even though these data argue for an important role of LIP activity in human saliva, we demonstrated that the LIPF gene encoding the human ortholog of lingual LIP (called gastric LIP in humans) is not active in human gustatory tissue. A broader search for enzymes which could compensate for the absence of the LIPF gene product in humans revealed the expression of alternative LIP genes expressed, in particular, in VEGs secreting saliva directly into the trenches of foliate papillae.

Data from rodent studies suggest that the main stimulus for the orosensory perception of fat constituents is free long-chain fatty acids (7, 8, 22, 39). Although long-chain fatty acids are naturally present in fat-containing food, they predominantly occur esterified with a glycerol core in the form of triglycerides (40). In rodent studies, it was demonstrated that triglycerides do not represent an
adequate gustatory stimulus and that the liberation of free fatty acids by the enzyme lingual LIP is required for the orosensory perception of triglycerides (22). Humans detect the free long-chain fatty acid, oleic acid, at lower concentrations than the triglyceride, triolein. Whereas the presence of the LIP inhibitor, tetrahydrolipstatin (orlistat), significantly elevated the orosensory detection threshold for trioleate, detection of oleic acid was not significantly changed (23), suggesting that a similar mechanism for fat detection requiring the liberation of free long-chain fatty acids via lipolytic enzymes may also exist in humans. Our own experiments with human subjects demonstrate that triolein is judged by the panelists as fatty at a concentration range similar to oleic acid (Fig. 1A). However, unlike oleic acid, trioleate is not an appropriate stimulus for GPR40 and GPR120 (Fig. 1B), two prime candidates for orosensory fat perception in humans and rodents (11–13, 18). Because other candidate molecules presumably involved in the perception of fat constituents such as CD36/FAT (9, 10, 23, 41) and delayed-rectifying potassium channels (3) also respond to free fatty acids, another, so far undiscovered, triglyceride-sensitive detection mechanism has to be assumed or, alternatively, lipolytic activity resulting in the generation of appropriate stimuli for fatty acid responsive detection mechanisms should be effective in humans as well.

The question of whether humans possess sufficient lipolytic activity in their oral cavity to release free fatty acids efficiently from triglycerides has resulted in inconclusive answers. Clearly, human saliva exhibits lower LIP activity than rodent saliva (29). However, lipolysis of triglycerides is an important step in the digestive process of this macronutrient. It was shown that the peak production site for LIP activity varies considerably among animal species. Whereas the main production site for LIP in rats and mice is the oral cavity and not the stomach, the opposite is found in humans and other vertebrates (29). It is therefore conceivable that bulk digestion of triglycerides in rats and mice starts at a more proximal point along the alimentary tract compared with humans, however, the question if the minute amount of lipolytic activity found in human saliva is sufficient to liberate sensorially relevant concentrations of free fatty acids remains to be answered.

Previous studies investigating lipolytic activity present in the human oral cavity demonstrated that whole saliva collected from human probands after stimulation with cream contained an average lipolytic activity of 2 μmol fatty acids/min/g wet weight) (19). This is in good agreement with previous data by Spielman et al. (25), who reported identical whole saliva values for lipolytic activity in humans. The same study also collected saliva secreted from VEGs into the trenches of the foliate papillae and reported a 5- to 7.5-fold higher LIP activity, suggesting that minor salivary glands may represent the main source for the secretion of this enzyme. Also, by direct extraction from human VEG tissue, lipolytic activity was obtained (0.3 μmol/min/g wet weight), although at levels several hundredfold below similarly processed gastric tissue, which might in part be due to the fact that lingual gland tissue was obtained post mortem (29). However, the good agreement of these values with those of a previous report (42) substantiates the existence of oral LIP activity. Hence, there is good agreement about the presence of significant lipolytic activity in the salivary secretions of human minor salivary glands paralleling the observations in rodents (7). In order to represent a potent stimulus for the orosensory perception of free fatty acids, the necessary stimulus concentration at the receptor side must be reached. In that respect, the production of LIPS in minor salivary glands which directly secrete their saliva into the trenches of foliate papillae and CV housing the vast majority of oral taste buds, places the generation of the stimulus in the immediate vicinity of the receptive cells. This should allow efficient stimulus generation even with small overall lipolytic activity. Our finding that humans produce LIPS in minor salivary glands (Figs. 5, 6) and that lipolytic activity is detected in minor salivary gland secretions (Fig. 4) attests to a potential role of lipolysis in the processing of fatty stimuli. In light of the rather low overall LIP activity in human saliva, which appears neither sufficient nor necessary for the bulk digestion of triglycerides, a specific function in the generation of orosensory perceived stimuli appears even more likely. Whether this enzymatic activity is indeed required for the orosensory perception of food may depend on the nature and processing grade of food items consumed. In the case of some high fat food items which contain already sufficient amounts of free long-chain fatty acids, the small additional amount of fatty acids liberated by orally secreted LIPS may not be required (24, 43). However, if the liberation of fatty acids from triglycerides is necessary to reach or exceed threshold concentrations, the activity of LIPS may become crucial for orosensory perception of food lipids (23).

Our data demonstrate that not LIPF (lingual/gastric LIP) (Fig. 2), but other LIPS (Figs. 5, 6) likely facilitate triglyceride degradation in human minor salivary gland secretions. Intriguingly, after many speculations about whether the enzymatic activity of human lingual LIP may suffice to effectively degrade triglycerides, it turned out that the LIPF gene is not even expressed in human VEGs, but rather related genes coding for alternative LIPS may replace the function of lingual LIP. Interestingly, DeNigris et al. (29) reported in 1988 that lingual/gastric LIP shows substrate specificity for the medium-chain fatty acid containing triacylglycerin (8:0) over the long-chain fatty acid containing triolein (18:1) by a factor of severalfold. This was also true for human gastric LIP, which exhibited about 4-fold higher enzymatic activity when offered tri[3H]caprylin compared with tri[3H]olein. However, this was not observed for lingual tissue. Instead, lipolytic activity for triacylglycerin was not detectable in contrast to lipolytic activity for triolein. The observed preference of lingual/gastric LIP for medium-chain fatty acid-containing triglycerides over long-chain fatty acid-containing triglycerides is in perfect agreement with data obtained for partially purified rat lingual LIP, which exhibited a 5–10 times higher specific activity for tributyrin compared with triolein (44). It is tempting to speculate that this fact may indeed point to the involvement of different enzymes in humans, such as LIPK, LIPM, and LIPN, as reported here. Moreover, as we have
previously shown that long-chain fatty acids rather than medium-chain fatty acids represent the main orosensory stimulus for fat detection in humans, the preferential liberation of this type of fatty acids would make perfect sense. An important step toward the understanding of human orosensory fat perception would be a careful biochemical characterization of these alternative LIPs to evaluate their individual contribution for stimulus generation.

Intriguingly, we observed that individual variation of triolein detection thresholds correlated with lipolytic activities measured in minor salivary gland secretions of human probands (Fig. 5A, B). While several studies reported considerable interindividual variations of fatty acid sensitivity among humans (15), and some of these studies even correlated the observed variations with nutritional status (19, 45) and/or single nucleotide polymorphisms in candidate “fat taste receptors” (23), none of the previous reports investigated variations in lingual LIP activities. Because the perireceptor milieu constituted through salivary secretions is an important contributor for the perception of ingested substances (46) and salivary gland function is regulated by autonomic innervation (47), lipid sensitivities may even be influenced by the acute or chronic nutritional status of individuals and may involve modifications of the amounts of secreted LIPs.

Our work demonstrates the oral perception of triglycerides by human probands as well as the impact of differential LIP activities on individual threshold concentrations. Moreover, we have shown that the expression of LIPs different from LIPF in human minor salivary gland tissue may account for the release of the stimulating free fatty acids from triglycerides. Together, our results provide evidence for an important role of lingual lipolysis in the generation of appropriate stimuli for the oral perception of fat components in humans. Future work needs to be targeted toward a careful biochemical and functional characterization of the specific enzymes found in this study which seem to underly this mechanism.

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