Lingual Lipase Activity in the Orosensory Detection of Fat in Humans

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Running Head: Human Lingual Lipase Analysis
Abstract:

Lingual lipase generates non esterified fatty acids (NEFA) from dietary fats during oral processing by lipolysis. Lingual lipase in rodents has strong lipolytic activity and plays a critical role in oral detection of fats. The functional activity of lingual lipase during oral processing of high fat foods in humans remains poorly characterized. Five commonly consumed high fat foods varying in physical states and fatty acid composition – almond, almond butter, olive oil, walnut and coconut were masticated by 15 healthy human subjects at the rate of one chew/second with and without a lipase inhibitor, Orlistat. Salivary NEFA concentrations were measured. To determine the role of lingual lipase in oral fat detection, sensory ratings were obtained from the same 15 human subjects for almond butter with and without Orlistat. Lingual lipase was active during oral processing of almond and coconut. No activity of lingual lipase was detected during processing of almond butter. There was only weak evidence lingual lipase is a determinant of oral fat detection. Lingual lipase may only contribute to NEFA generation and oral fat detection of fatty foods that require stronger oral processing effort.

Keywords: Human, Lipase, Lingual, Fat, Orlistat
Human Lingual Lipase Analysis

Introduction:

Human lingual lipase is an enzyme secreted into the oral cavity by “Von Ebner” serous glands located in proximity to foliate and circumvallate papillae (4, 5, 17). Lipases are a group of enzymes that hydrolyze ester bonds between the fatty acid and glycerol moieties of dietary triacylglycerol to produce mono and diacylglycerols and free (non-esterified) fatty acids (NEFA). Unlike pancreatic lipase, lingual lipase in human infants can penetrate milk fat globules and initiate lipid digestion. Also, infants have low pancreatic lipase activity as compared to adults. Thus, lingual lipase is important for infant nutrition (5). Lingual lipase also reportedly contributes to the digestion of lipids in adults along with gastric lipase, especially in diseases with pancreatic insufficiency such as cystic fibrosis and alcoholic pancreatic insufficiency (5).

We recently showed that adequate levels of salivary NEFA are achieved to activate potential oral NEFA receptors during oral processing of commonly consumed high fat food items (10). The role of lingual lipase in generation of salivary NEFA is still not clear. While lingual lipase has the capacity to hydrolyze triacylglycerol, questions remain about whether functional concentrations are present in the oral cavity of humans. A number of in-vitro assays for quantification of lingual lipase concentrations and activity have revealed little or no availability and lipolytic activity has varied from absent to weak (2 micromole/min/L) (2, 4, 6, 15, 17, 18). However, the in-vitro experimental conditions used in these early experiments do not completely mimic the natural oral processing of fats. Further, the resting saliva used in these in-vitro experiments may be low in lingual lipase and not representative of concentrations during mastication. The act of chewing stimulates secretion of lingual lipase (13) and the process of chewing facilitates optimum mixing of salivary enzymes with food. A lack of mastication and proper mixing of fat and saliva may contribute to low reported lipase activity. Also, in these laboratory experiments, samples of saliva underwent various preparation steps such as centrifugation, freezing, storage and thawing before assaying the enzyme. Lingual lipase may lose its activity during these steps of sample processing due to denaturing of its protein structure. Further, the antibodies used for quantification of human lingual lipase (17) were against rat lingual lipase and may react poorly with human lingual lipase. Thus, a number of technical issues may have interfered with the precise estimation of the amount and the activity of human lingual lipase. No study has quantified the activity of lingual lipase in the oral cavity (in vivo) during processing of commonly consumed high fat foods in humans.

Orlistat is a lipase inhibitor that suppresses lipolysis and prevents the release of fatty acids from triacylglycerol (1). We tested the first hypothesis that lingual lipase is active during oral fat processing in humans by measuring whether Orlistat suppresses salivary NEFA concentrations. Our in-vivo experimental condition closely approximated the natural oral processing conditions for fats.

Rodent lingual lipase has strong lipolytic activity (50 micromoles of NEFA/mg lipase/min (16)). It hydrolyzes triacylglycerol in the rodent oral cavity to release NEFA (8). Rats express a reduced preference for triacylglycerols in the presence of Orlistat (8). This suggests lipolysis leading to release of fatty acids in the oral cavity contributes to oral detection of fats in rodents. A recent human study demonstrated that Orlistat increases the detection threshold for a pure triglyceride
but not for non-esterified fatty acid (oleic acid) (14). Thus, there is indirect evidence of a functional role of lipase in humans.

It is not known whether suppression of lipolysis interferes with evaluation of the reported fattiness of commonly consumed high fat food items in humans. Our second hypothesis was that the NEFA released by lingual lipase from triacylglycerol in the oral cavity are important contributors to oral fat detection in humans. This was tested by comparing sensory ratings of commonly consumed high fat food in the presence and absence of Orlistat.

As reported in our previous study (10), shredded coconut meat, walnut, whole almond, almond butter and olive oil were used as the test foods (Table 2). These foods varied in physical state (solid-semi solid and liquid) and fatty acid composition (high in saturated fatty acids-SFA, monounsaturated fatty acids-MUFA or polyunsaturated fatty acids-PUFA). This selection enabled the analysis of effects of human lingual lipase on the lipolysis of fats in commonly consumed foods varying in physical state and fatty acid composition.

NEFA inherently present in the food matrix and lipases present in the food matrix that may be activated during oral processing are the two potential sources of salivary NEFA in addition to lingual lipase that may be functional during oral processing of high fat foods (11,12). We also analyzed possible contributions of these two sources to the salivary NEFA pool.

Materials and Methods:

Participants: 15 normal weight (Body Mass Index 18.5-25 kg/m²), self-reported healthy, 18 to 50 year old individuals were recruited by public advertisement. They also met the following eligibility criteria: self-reported non-smoker, not on medications known to affect taste function, normal taste function, self-reported good oral health, no allergies to test foods and palatability scores > 50% on a visual analog scale for each test food. If the participants correctly identified the tastes of 5% sucrose, 5% salt, 2% citric acid and 2% caffeine solutions in deionized water as sweet, salty, sour and bitter respectively, they were considered to have normal taste function.

There were three study sessions including the screening visit held 24 hours apart. Participants were instructed to abstain from eating or drinking anything except water and using any oral hygiene products or procedures such as brushing or flossing of their teeth for two hours before the visits. The study was approved by the University Institutional Review Board.

Screening visit: During the initial session, all participants provided written consent and completed screening taste testing. Eleven females and four males that met the selection criteria were recruited for the study.

Second visit: The aim of this visit was to determine whether Orlistat suppresses salivary NEFA concentrations generated during oral processing of high fat test foods. Each food was offered twice to participants, with placebo or with Orlistat in random order. Thus, ten trials were conducted with each participant.

GlaxoSmithKline provided the Orlistat and placebo capsules that were identical in appearance. The placebo contained a mixture of inert substances that were tasteless, colorless and
odorless. The Orlistat capsule contained the same mixture with 60 mg of Orlistat. Orlistat is also a tasteless, colorless and odorless powder. Thus, the contents of Orlistat and placebo capsules had similar sensory properties.

The contents of an Orlistat capsule were emptied into the participant’s mouth and they were asked to move their tongue to spread the contents in the oral cavity for 5 seconds. The test food was then placed in the participant’s mouth followed immediately by emptying of a second Orlistat capsule in the oral cavity. The participant then chewed the food at a rate of one bite per second for one minute. At the end of the minute, the participant was asked to expectorate the test food into a pre-chilled 15 ml sterile tube. The participants rinsed their mouths with deionized water after expectorating the food. The same procedure was followed with the Orlistat placebo. There was an interval of 10 minutes between each two samples. The expectorated samples were stored on ice during the session.

Processing of samples: The samples were processed using a method described previously (9). In brief, the samples were immediately centrifuged at the rate of 4500 rpm and at 4°C for 30 minutes. Nonadecanoic acid (Nu-Chek Prep, USA) was added to the separated supernatants that were subsequently lyophilized. The lipids were extracted from the lyophilized samples by modified Folch’s extraction (3) using 11 ml of chloroform, 5.5 ml of methanol and 0.5ml of deionized water. The NEFAs in the extracted lipids were selectively derivatized using 1% acetonitrile solutions of diisopropylethylamine and pentafluorobenzyl bromide. The resultant pentafluorobenzyl bromide derivatives of NEFAs were qualitatively and quantitatively analyzed using gas chromatography and mass spectrometry (Agilent Technology, USA).

Third visit: The aim of this visit was to study the effect of Orlistat on the taste of high fat foods. Almond butter (2.5 gms) was used as a high fat test food. A 0.5% Orlistat solution (O) was prepared following the method described by Kawai et al. (8). In brief, 1 gm of Orlistat was mixed with 4 gms of mineral oil, 600 mgs of xanthan gum and 200ml of deionized water with a hand mixer. The same procedure was followed to prepare a control solution (C) without adding Orlistat. The participants completed taste tests under red light wearing nose clips. The participants were asked to rinse their mouth with 10 ml of control solution and then taste and expectorate 2.5 gms of the almond butter sample. After tasting the first sample, participants were asked to rinse their mouth with deionized water and then with 10 ml of control solution. This was followed by tasting the second 2.5 gms of almond butter and again rinsing the mouth with deionized water (C and C). The participants were then asked to answer the question “is the taste of the two samples the same or different?” The taste of two samples in the first pair should have been the same since both were evaluated after rinsing the mouth with the same control solution. Thus, the results of first pair were used as a control. The procedure was repeated for three more pairs of 2.5 gm samples of almond butter while changing the order of solutions used for rinsing the mouth just before testing the almond butter samples as follows: control solution and Orlistat solution for the second pair (C and O), Orlistat and Orlistat solution for third pair (O and O) and Orlistat and control solutions (O and C) respectively for the fourth pair. The effect of Orlistat on sample taste evaluation was tested with the second pair (C and O). The procedure was repeated by reversing the order of solutions in the fourth pairing (O and C) to rule out any residual carry over effect of Orlistat. The objective of the third pair (O and O)
in testing was to determine the effect of Orlistat on salt taste (a taste transduced by another mechanism compared to NEFA). One hundred microliters of 5% sodium chloride solution were added to the second almond butter sample of the third pair to determine whether participants could correctly identify it as more salty than the first sample. Finally the participants were asked to compare the tastes of control and Orlistat solutions. The taste of control and Orlistat solutions had to match to permit comparison of the other test solutions. The presentation order of the five pairs of sensory stimuli was fixed.

All reagents used for the study were from Sigma Aldrich, USA and were of highest purity available.

Analysis of NEFAs inherently present in food: The test food was crushed with a pestle and mortar for a minute and then vortexed with deionized water in a 15 ml disposable tube for one minute. The NEFAs present in the deionized water were analyzed using the same above GC-MS sample analysis. Each test food was analyzed in triplicate.

Analysis of lipase present in the food matrix: Whole almond was crushed with a pestle and mortar for one minute and then mixed with deionized water and vortexed for one minute. The procedure was repeated for another almond, but with 120 mg of Orlistat. NEFA profiles of deionized water from the almond were compared with almond plus Orlistat (both samples were analyzed in triplicate).

Cone probe texture analysis: The amount of force required to break the matrix of solid foods (almond, walnut and coconut) was analyzed with a cone probe (Stable Micro system texture analyzer).

Statistics: Salivary NEFA concentration values were compared using Repeated Measure Analysis of Variance (ANOVA). Treatment differences with a p<0.05 were considered statistically significant.

Results:

**Suppression of salivary NEFA by Orlistat:** As reported previously (9), Palmitic, linoleic, oleic and stearic acids were the salivary NEFAs detected in the saliva obtained from all five foods while lauric and linolenic acids were only present in the saliva after coconut and walnut mastication, respectively. In this analysis, Orlistat significantly suppressed salivary palmitic, oleic, and linoleic acid concentrations as compared to placebo in almond (Fig 1). Orlistat significantly suppressed salivary palmitic and lauric acids as compared to placebo with the coconut samples. Salivary NEFA suppression by Orlistat was not noted in the other test foods.

**Analysis of NEFAs inherently present in the food matrix:** All five test foods released FAs into the deionized water ranging in concentration from 5 to 20 micromolar (Figure 2). The same six NEFA (palmitic, linoleic, oleic, stearic, lauric and linolenic acids) documented in saliva during oral processing of these test foods were noted in these *in-vitro* deionized water extracts.

**Analysis of food lipases:** There was no significant difference between NEFA concentrations obtained from test foods in vitro in the presence and absence of Orlistat (Figure 3).
Effect of Orlistat on the taste of high fat food: Orlistat did not significantly affect the sensory evaluation of almond butter samples in 86% of trials when paired with a control solution (p<0.05).

The second almond butter sample in the third pair (O and O) contained 100 microliters of 5% salt solution. This condition was included to determine whether Orlistat has a non-specific effect as revealed by an altered perception of salty taste that is reportedly transduced by a different mechanism. All 15 participants found the taste of the two samples of the third pair to be different, indicating that the Orlistat did not indiscriminately block all taste sensations.

The taste of the control solution was expected to match the taste of the Orlistat solution as both were reportedly tasteless. In a pilot study with 5 individuals, the taste of the two solutions was similar. In the study, 10 out of 15 participants reported the taste of the two solutions to be the same while 5 reported it to be different. As a result, the sensory ratings by only the 10 participants reporting no differences were considered for further analysis. Seven out of these 10 participants reported the taste of two identical almond butter samples to be the same, after rinsing their mouth with the same control solution before each sample (1st pair, C and C). This step was necessary to assess the veracity of their reports. Thus, sensory ratings by only these 7 individuals were considered for further analysis. The taste of the two almond butter samples after oral rinsing with control and Orlistat solutions (2nd pair, C and O) was the same in 5 out of the 7 participants while all 7 participants reported the taste of the two samples to be the same when the order of solution was reversed (4th pair O and C). Thus, Orlistat did not significantly affect the sensory evaluation of almond butter samples in 12 out of 14 trials (85.71%) when paired with a control solution (p<0.05). This evidence suggests lingual lipase did not play a role in orosensory evaluation of almond butter by these participants.

Cone probe texture analysis: The amount of force required to break the matrix for the solid test foods is given in Table 2. The breaking force was the highest for almond, and lowest for coconut (Table 2).

Discussion:

To-date, analyses of lingual lipase activity in humans have been conducted in vitro, where saliva was mixed with a fat source in the form of chemical reagents (pure diglyceride or monoglyceride or chylomicron triglycerides) and the generation of NEFAs was quantified. These experiments (2, 4, 6, 15, 17, 18) demonstrated absent to weak lingual lipase activity. As the methods followed in these experiments vary (e.g., substrate, compounds used to quantify the lipase activity, methods used to collect saliva) a comparison of results across these reports is problematic. The latest analysis was carried out by Stewart et al. (18) who mixed saliva samples with diglyceride and quantified the release of FAs at 35°C and pH 7.5 with a colormetric test. The lingual lipase activity varied from 0 to 12 micromoles fatty acids /min/L. The median value was 2 micromoles fatty acids/min/L. Thus, in vitro analyses suggest that human lingual lipase has, at best, weak activity as compared to rodent lingual lipase. The aim of our study was to assess lingual lipase activity in the human oral cavity (in vivo) in the presence of commonly consumed high fat foods. The lipase inhibitor, Orlistat was used to isolate the effects of lingual lipase activity. If lingual lipase is active in the oral cavity of humans during oral processing of
high fat food, it will release NEFA from triacylglycerol. Since Orlistat suppresses lingual lipase, the salivary NEFA concentration should be low in the presence of Orlistat as compared to placebo, as reported in rats (8).

Orlistat significantly suppressed salivary palmitic, linoleic, and oleic acids from almond. Orlistat also suppressed lauric and palmitic acids from coconut. Orlistat did not have an effect on salivary NEFA concentrations from walnut, almond butter or olive oil. This evidence suggests that lingual lipase was active, but only in a sub-set of foods.

Almond is high in MUFA while coconut is high in SFA. Lingual lipase was not active during oral processing of almond butter or olive oil even though both of them are high in MUFA. Thus, the action of lingual lipase was not dependent on the fatty acid profile of the high fat food.

We also analyzed the concentration of NEFAs and putative lipases present in the matrix of high fat foods as another source of salivary NEFA liberated or generated during oral processing of high fat foods. Orlistat did not suppress NEFA release from almonds in the in-vitro study. Thus, there was no evidence for functional lipase activity inherently present in the high fat food matrix. Lipases resistant to Orlistat could be present, but there are no reports of such enzymes. These findings suggest the lipase activity documented during oral processing of almond was probably due to the action of lingual lipase.

The NEFA concentrations observed in deionized water were not as high as the salivary NEFA concentrations. During oral processing, salivary NEFA (up to 10 micromolar (9)) are added to the inherently present NEFA released from the food matrix. The NEFA concentrations present inherently in the food matrix that might be released during oral processing were analyzed by grinding the food with a mortar and pestle (to mimic mastication) and further extraction with deionized water (to mimic mixing with saliva). Saliva and deionized water have different viscoelastic properties. This process mimics oral processing, but in vivo oral processing would likely be more efficient at liberating lipids from foods. As a result, the actual inherent concentrations of NEFA in a food matrix are probably higher than reported by the simulation method. Even if this difference is small, it may be sufficient to generate a difference of a few micromolars (the observed NEFA changes). Also, in the case of almond and coconut, additional contributions of lingual lipase might be responsible for the difference. However, we found strong evidence that NEFA present in the food matrix are an additional potential source of salivary NEFA as they were released into the deionized water extract of all five food samples tested in vitro.

The salivary NEFA concentrations were analyzed in our previous study (10) after oral processing of high fat food items. In the current study, the basal salivary NEFA concentrations in response to oral processing of the same five high fat food items are analyzed in the presence of placebo powder. Thus, the basal salivary NEFA concentrations in the two studies were analyzed under different physical conditions. Despite this, the NEFA profiles generated by all 5 food items exhibit the same relationships among NEFA in both the studies. Thus, the results from our current study confirm our previous findings.
Rats lose their preference for triglyceride in the presence of Orlistat (8). This suggests that lipolysis leading to the generation of NEFAs in the oral cavity plays an important role in orosensory detection of fat in rodents. The second aim of our study was to determine the importance of lipolysis for oral detection of fat in humans. If NEFAs generated by lingual lipase are important for oral detection of fat in humans, suppression of lingual lipase by Orlistat should have reduced orosensory fat detection. A recently published study (14) demonstrated that Orlistat increases the threshold (i.e., less sensitive) for pure triglyceride, but not for NEFA suggesting the contribution of lipase in oral detection of fat in humans. We wanted to analyze the effect of Orlistat on fat detection using a common food item, almond butter. The participants were asked to compare the taste of two identical almond butter samples after rinsing their mouth with either the control solution or Orlistat solution before each sample. The concentration of Orlistat solution used previously in rodent studies (8) was 0.5 gm% and was documented as strong enough to significantly suppress lingual lipase and block the oral detection of fat. Rat lingual lipase has been reported to achieve lipolysis at the rate of 50 micromoles of NEFA/ mg of lipase/min (16) leading to lipolysis of 6% of triacylglycerol in 5 seconds (8). Human lingual lipase is present at much lower concentrations (17) and reportedly has much weaker lipolytic action (~2 micromoles of NEFA/min/L) than the rat lingual lipase (7, 18). Although the activity of human and rat lingual lipase has been expressed in different units, physiologically, 2 micromoles per liter of saliva in humans is a very low level of activity compared to rodents. Therefore, the Orlistat solution with a 0.5gm% concentration as used in this study was expected to be strong enough to suppress lingual lipase in humans.

In contrast to the findings by Pepino et al. (14), Orlistat did not suppress salivary NEFA concentrations when masticating almond butter suggesting the lack of lipolysis by lingual lipase during oral processing of this sample. This is consistent with the finding that the presence of Orlistat did not interfere with the sensory evaluation of almond butter in the second experiment. This combination of observations suggests that oral lipolysis is not important for oral detection of almond butter. One explanation for the discrepancy between our finding and those of Pepino et al. (14) is that almond butter might have other naturally present components such as phytochemicals (19) that may interfere with the lingual lipase in the oral cavity. Also, the almond butter used in this study and the pure triglycerides used by Pepino et al. have different matrices. Alternatively, it is possible that the differences in pure triacylglycerol thresholds observed with Orlistat may not necessarily translate into actual differences in sensory evaluation of fat content of high fat foods. Inherent NEFA may obviate the need for lipase activity in fat detection.

Lingual lipase is secreted by von Ebner’s glands into the saliva. Orlistat blocks the active site of the lipase enzyme and does not interfere with the secretion of the enzyme (1, 8). Hence, the lipase activity of the saliva was restored when the oral cavity was rinsed with saline after an experiment with Orlistat solution in rats (8). Similarly, the participants in this study were asked to rinse their mouth with deionized water to prevent any carry-over effect of Orlistat action. In addition, the Orlistat solution was offered after the control solution to prevent any residual action of Orlistat. The order was reversed in another pairing (Orlistat first and then control solution), but the response of five participants did not change and two participants altered their answers.
during sensory evaluation. This observation suggests that the Orlistat solution did not have any residual effect across taste comparisons.

The present finding that the lingual lipase was active only during oral processing of almond and coconut yields new insights on the possible role of lingual lipase on NEFA generation. The physical state of both the almond and coconut samples was solid and required substantial force during mastication to break down the matrix. The inhibitory pattern of Orlistat observed in the study suggests that lingual lipase may be more active during mastication of high fat foods that require strong force to break down their matrix. The dissimilar NEFA concentrations observed with the whole almond and almond butter (just ground almonds) argues strongly for a food form influence. Almond required the highest force, but this was followed by walnut and then coconut. However, the cone probe analysis measures only the force required during the first strike to break the matrix and shredded coconut may require more overall force after the initial break to achieve the optimum size of particles for swallowing and this was not captured by this method. Thus, these findings only partially support a view that lipase activity is directly related to oral processing effort. This is important to verify because if true, it would suggest a lesser role for lipase in detection of lipid from foods requiring little mastication.

In summary, the present evidence suggests that lingual lipase is active during oral processing of some high fat foods, possibly, those that require higher oral processing effort. However, we did not find that lingual lipase contributed to oral fat detection. This is probably because NEFAs inherently present in the matrix of high fat foods may serve as a chemosensory signal for dietary fat in the oral cavity.

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References:


Figure Caption

**Figure 1**
Change in salivary NEFA concentrations in the presence of Orlistat as compared to placebo. Salivary palmitic, linoleic and oleic acid concentrations were significantly suppressed in almond while coconut had significant suppression of salivary palmitic and lauric acid concentrations in the presence of Orlistat as compared to placebo (n=15, statistical analysis: analysis of variance repeated measures).

**Figure 2**
NEFA inherently present in food matrix: Six NEFA palmitic, linoleic, oleic, stearic, lauric and linolenic acids were detected in deionized water extract of the test foods (each food item analyzed in triplicate).

**Figure 3**
Analysis of food lipase: Orlistat had no effect on NEFAs concentrations released from almond in vitro (n=3, statistical analysis: analysis of variance repeated measures).

<table>
<thead>
<tr>
<th><strong>Table 1:</strong> Test foods</th>
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<tbody>
<tr>
<td><strong>Food Item</strong></td>
</tr>
<tr>
<td>Shredded coconut meat (2gm)</td>
</tr>
<tr>
<td>Walnut (2.6gm)</td>
</tr>
<tr>
<td>Almond (raw) (1.2gm)</td>
</tr>
<tr>
<td>Almond butter (2.5gm)</td>
</tr>
<tr>
<td>Olive oil (1gm)</td>
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Table 2: Cone probe analysis of force required to break the matrix

<table>
<thead>
<tr>
<th>Test Food</th>
<th>Force Required to Break Matrix (gms)</th>
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</thead>
<tbody>
<tr>
<td>Almond</td>
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</tr>
<tr>
<td>Walnut</td>
<td>1221.41</td>
</tr>
<tr>
<td>Coconut</td>
<td>203.98</td>
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