Dietary lipids and sweeteners regulate glucagon-like peptide-2 secretion

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THE PRIMARY FUNCTIONS of the gastrointestinal (GI) tract are digestion and absorption to meet the metabolic and growth requirements of the body. A recent development in treatment for intestinal failure (8), which means malnutrition and dehydration caused by an intestinal malfunction, has revealed the significance of several growth factors in maintenance for GI tract and stressed their potentials as therapeutic targets. Glucagon-like peptide-2 (GLP-2) is a potent intestinal growth factor derived from enteroendocrine L cells. Although food intake is known to increase GLP-2 secretion, its regulatory mechanisms are largely unknown as a result of its very short half-life in venules. The aims of this study were to compare the effects of luminal nutrients on the stimulation of GLP-2 secretion in vivo using lymph samples and to clarify the involvement of the sweet taste receptor in this process in vitro. Lymph samples were collected from the thoracic duct after bolus administration of dietary lipids or sweetening agents into the duodenum of rats. Human enteroendocrine NCI-H716 cells were also used to compare the effects of various nutrients on GLP-2 secretion. GLP-2 concentrations were measured by ELISA in vivo and in vitro. GLP-2 secretion was enhanced by polysaturated fatty acid- and monounsaturated fatty acid-rich dietary oils, dietary carbohydrates, and some kinds of sweeteners in rats; this effect was reproduced in NCI-H716 cells using α-linolenic acid (αLA), glucose, and sweeteners. GLP-2 secretion induced by sweetening agents was inhibited by lacticose, a sweetness-antagonizing inhibitor of T1R3. In contrast, lactose was unable to inhibit GLP-2 secretion induced by αLA alone. Our results suggested that fatty acid- and sweetener-induced GLP-2 secretion may be mediated by different pathways, with the sweet taste receptor involved in the regulation of the latter.

sweet taste receptor; glucagon-like peptide-2; fatty acids; sweeteners

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Table 1. Fatty acid profile (% of total fat)

<table>
<thead>
<tr>
<th></th>
<th>Perilla</th>
<th>Fish</th>
<th>Olive</th>
<th>Panaceta</th>
<th>Butter</th>
<th>Sunflower</th>
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<tr>
<td>Butyric (4:0)</td>
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<td>NA</td>
<td>NA</td>
<td>ND</td>
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<td>NA</td>
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<tr>
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<td>NA</td>
<td>NA</td>
<td>ND</td>
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<td>NA</td>
</tr>
<tr>
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<td>NA</td>
<td>NA</td>
<td>99.1</td>
<td>1.6</td>
<td>NA</td>
</tr>
<tr>
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<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>3.4</td>
<td>NA</td>
</tr>
<tr>
<td>Lauric (12:0)</td>
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<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>3.4</td>
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</tr>
<tr>
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<td>0.6</td>
<td>ND</td>
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<td>ND</td>
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<tr>
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<tr>
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<td>3.5</td>
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<tr>
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<td>1.2</td>
<td>ND</td>
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<td>74.5</td>
<td>ND</td>
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<td>26.3</td>
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<tr>
<td>Linoleic (18:2)</td>
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<td>3.6</td>
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<td>ND</td>
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<td>ND</td>
</tr>
<tr>
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<td>9.8</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Others</td>
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<td>2.4</td>
<td>0.4</td>
<td>0.9</td>
<td>0.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

NA, not analyzed; ND, not detected.

Nutrient stimulation of GLP-2 secretion

To standard rat chow and water. Animals were allowed to acclimatize for 2 wk before experimentation. The study protocol was approved by the Animal Ethics Committee of the National Defense Medical College (No. 09088). All the following animal experimental procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of National Defense Medical College.

Surgical procedures. Under anesthesia with pentobarbital, the thoracic duct near the cisterna chyli of rats was cannulated according to the method described by Bollman et al. (3). A nylon tube (0.55-mm inner diameter, 1.00-mm outer diameter; EPINEED, Terumo, Tokyo, Japan) was selected for placement. Another plastic tube (1-mm inner diameter, 2-mm outer diameter; Nalgene; Nalge Nunc, Rochester, NY) was placed into the duodenum through an incision in the stomach wall. After the operation, rats were placed in Bollman cages (Natsume Seisakusho, Tokyo, Japan) overnight for recovery, and 0.9% saline was infused at 3 ml/h into the duodenum to compensate for lymph loss.

Lymph collection. The next morning after surgery, the animals were given 3 ml saline, dietary lipids, carbohydrate solutions, artificial sweeteners, or mixed infusions via bolus administration, and lymph was collected at 2 h or continuously for 2, 4, 6, and 8 h. Each sample was accompanied with 10% by volume of an antiproteolytic solution (0.25 mol/l EDTA, 0.80 mg/ml aprotinin, and 80 U/ml heparin). To make up for loss of fluids and electrolytes, rats were maintained on a saline infusion until the end of lymph collection. All samples were centrifuged to remove impurities and stored at −20°C.

Infusion of nutrients and sweeteners. To examine the impact of nutrients and sweeteners on GLP-2 secretion, we administered a 3-ml single bolus of dietary lipids through the duodenal feeding tube to the first group of rats. Dietary lipids included perilla oil (n = 10), fish oil from menhaden (n = 7), olive oil (n = 7), sunflower oil (n = 6), Panaceta (n = 6), and butter (n = 4). The fatty acid profiles of oils are shown in Table 1. Perilla oil was purchased from Ohta Oil Mill (Fukuoka, Japan). Butter was from Megmilk Snow Brand (Tokyo, Japan), and other lipids were from Sigma-Aldrich Japan (Tokyo, Japan). The second group of rats received a 3-ml bolus of dietary lipids and either glucose (n = 5), saccharose (n = 5), or PALSWEET (n = 3) solution.

Blood collection. One group of animals was allotted for the collection of blood samples, which were used for comparison of GLP-2 concentrations between blood and lymph. In these animals, only a feeding tube was inserted into the duodenum. Animals were anesthetized with pentobarbital 2 h after a 3-ml bolus of saline or perilla oil, and blood was collected by cardiac puncture with a 25-gauge needle. Blood samples were centrifuged to separate plasma and stored at −20°C, in the same manner as lymph samples.

Cell culture and GLP-2 secretion. Human NCI-H716 cells were obtained from the American Type Culture Collection (Manassas, VA). For proliferation and maintenance, cells were grown in suspension in RPMI 1640 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin. Two days before each experiment, the cells were seeded into 96-well plates precoated with poly-l-lysine (1 × 105 cells/well). The culture medium was replaced with HEPES buffer before the experiment, and the cells were incubated for 1 h at 37°C with test agents. α-Linolenic acid (αLA), glucose, sacralose, and PALSWEET were tested with or without lactisole, a sweetness-antagonizing inhibitor of T1R3. Each sample contained 50 μM antiproteolytic solution as aforementioned. Viability of the NCI-H716 cells after treatment was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Chemicon International, Temecula, CA), as reported (18).

Measurement of GLP-2. GLP-2 concentrations were determined using a GLP-2 enzyme immunoassay kit (Yanaihara Institute, Fujinomiya, Japan), according to the manufacturer’s protocols. This ELISA kit is able to detect quantities ranging from 0.137 to 100 ng/ml and does not cross-react with glucagon or GLP-1.

Data presentation and statistical analysis. Hourly GLP-2 outputs were calculated by multiplying the hourly lymph flow with the GLP-2 concentration. Data are presented as means ± SE. Differences between mean values for variables were compared by Mann-Whitney U-test or Kruskal-Wallis one-way ANOVA on ranks with Bonferroni’s correction. The Wilcoxon t-test was also used to assess the GLP-2 concentration of the same animal before and after the infusion. P values of <0.05 were considered significant. Correlations were examined by linear regression analysis. Slopes of the best-fit lines were considered significantly different from zero if the P value was <0.05 (yStat 2004, Microsoft Excel 2007; San Diego, CA).
Infusion, 4.76

**P**/H11006

†

P

n

infusion. Blood samples were collected from the hearts of untreated rats (5% and 10% dextrin solution to avoid the effect of osmotic stress induced by glucose solution. In this setting, 5% dextrin solution increased GLP-2 output comparable to 5% glucose solution, and 10% dextrin solution induced a marked increase in GLP-2, suggesting that GLP-2 secretion induced by glucose may not be due to its large degree of osmotic effect (Fig. 3C).

**Effects of lipid composition on GLP-2 secretion.** To compare the effects of different types of lipids on GLP-2 secretion, we tested GLP-2 secretion in response to five types of lipids other than perilla oil (Fig. 4). Among them, increased GLP-2 secretion compared with control (1.51 ± 0.45 ng/h) was found in response to fish oil, which contains high n-3 PUFA (8.81 ± 1.54 ng/h), olive oil, which contains high oleic acid (8.80 ± 2.88 ng/h), Panacet, which consists of caprylic acid (5.14 ± 0.51 ng/h), and sunflower oil, which contains linoleic and oleic acid (4.99 ± 1.09 ng/h). Butter, which mainly contains saturated fatty acids and oleic acid, also increased GLP-2 secretion (3.92 ± 0.76 ng/h) but did not show any significant difference.

**Effects of carbohydrates and sweeteners on GLP-2 secretion.** To examine the effects of different carbohydrates and sweeteners on GLP-2 secretion, we administered sucrose and three different artificial sweeteners to rats (Fig. 5). The administration of sucrose solution significantly augmented GLP-2 output (5.75 ± 1.73 ng/h) compared with control saline. Interestingly, sucralose solution caused a significant increase in GLP-2 output (6.331 ± 0.56 ng/h), similar to the response to sucrose solution. PALSWEET also induced an increase in GLP-2 output (4.01 ± 0.83 ng/h), and only saccharin solution did not cause an increase in GLP-2 output (2.52 ± 0.76 ng/h). These results indicated that some artificial sweeteners stimulate GLP-2 secretion as well as lipids and carbohydrates.

**Additive effects of a mixed infusion of dietary lipids and sweet-tasting agents on GLP-2 secretion.** To examine the effects of a mixture of dietary lipids and carbohydrates or sweeteners on GLP-2 secretion, we prepared 3 ml of a mixture of perilla oil and 5% glucose, sucralose, or PALSWEET. The proportions of each mixture that yielded the data shown in Fig. 6 were as follows: perilla oil:glucose/PALSWEET = 2:1, perilla oil:sucralose = 1:1. Although no difference was seen between perilla oil and the mixture containing glucose solution, the mixture of perilla oil and sucralose solution significantly in-

**RESULTS**

Comparison of GLP-2 concentrations in the lymph and plasma. GLP-2 concentrations in the blood and lymph before and 2 h after perilla oil infusion were shown in Fig. 1. The concentration of GLP-2 in the lymph was significantly higher than that in the blood for both time points (before infusion, 1.34 ± 0.25 ng/ml vs. 0.54 ± 0.08 ng/ml, respectively; after infusion, 4.76 ± 1.09 ng/ml vs. 1.49 ± 0.14 ng/ml, respectively). Increased GLP-2 concentrations after perilla oil infusion were more obvious in lymph samples, indicating that lymphatic sampling may be more sensitive than blood sampling as a means of measuring GLP-2 secretion.

Hourly GLP-2 output and dose-response relationships with intraduodenal infusion of lipid and carbohydrate. Lymph samples were collected every 2 h until 8 h after a 3-ml bolus of saline, perilla oil, 5% glucose solution, or 0.5 g/kg sucralose solution (Fig. 2). GLP-2 output was calculated by multiplying the hourly lymph flow and GLP-2 concentration of each sample. Saline did not induce GLP-2 secretion during study period. Increased GLP-2 output was observed from 2–8 h following perilla oil injection and from 2–4 h after injection for both glucose and sucralose solutions. For all reagents, the mean output peaked at 2 h after the injection. On the basis of these results, subsequent studies used samples collected at 2 h following administration of the indicated reagents.

GLP-2 outputs induced by perilla oil, glucose solution, and dextrin solution were compared before and after infusion (Fig. 3). A 3-ml-dose of perilla oil significantly increased GLP-2 output (9.02 ± 1.27 ng/h) compared with saline injection (1.51 ± 0.45 ng/h), whereas 0.1–1.0-ml doses of perilla oil did not elicit the same effect, suggesting that perilla oil induces dose-dependent GLP-2 secretion (Fig. 3A). Glucose-induced GLP-2 output was increased when concentrations more than 5% of glucose was administered (Fig. 3B), but GLP-2 output apparently declined after 10%. We then examined a glycerol polymer that has an 83-fold greater molecular weight than that of glucose by using 5% and 10% dextrin solution to avoid the effect of osmotic stress induced by glucose solution. In this setting, 5% dextrin solution increased GLP-2 output comparable to 5% glucose solution, and 10% dextrin solution induced a marked increase in GLP-2, suggesting that GLP-2 secretion induced by glucose may not be due to its large degree of osmotic effect (Fig. 3C).

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creased GLP-2 output (13.22 ± 1.35 ng/h) compared with perilla oil (9.02 ± 1.27 ng/h) or sucralose alone (6.33 ± 0.56 ng/h). Furthermore, a similar trend was observed in response to a mixture of perilla oil and PALSWEET. The mixture of perilla oil and PALSWEET augmented GLP-2 output (20.02 ± 5.16 ng/h) to a much greater extent than perilla oil or PALSWEET alone (4.01 ± 0.83 ng/h). These results suggested the possibility that mixtures of dietary lipids and sweeteners or glucose solution were more efficient at inducing GLP-2 outputs while keeping the calorie content of food lower.

**Different pathways mediating GLP-2 secretion and the role of sweet taste receptors in NCI-H716 cells.** The effects of αLA, a major component of perilla oil, and the effects of three different sweeteners (that showed enhanced GLP-2 release in in vivo studies) on GLP-2 secretion in human enteroendocrine NCI-H716 cells are shown in Fig. 7. αLA, glucose, and sucralose elicited dose-dependent increases in GLP-2 secretion (Fig. 7A). Among these agents, glucose- and sucralose-induced GLP-2 secretion was inhibited by 500 μM lactisole, an antagonizing inhibitor of the sweetness receptor T1R3, whereas αLA-induced GLP-2 secretion was not inhibited by lactisole, suggesting that T1R3 signaling may be involved in glucose- and sucralose-mediated effects but not in αLA-mediated effects. Cocktails of αLA and either glucose or sucralose were more effective at inducing GLP-2 than each sweetener alone (Fig. 7B). Intriguingly, GLP-2 secretion induced by combined treatment with this cocktail and lactisole was lower than that induced by each sweetener alone, despite the presence of αLA. The effects of PALSWEET, which is 98.98% erythritol, on GLP-2 secretion from NCI-H716 cells are shown in Fig. 7C. Moreover, 0.04% PALSWEET (estimated 30 μM erythritol) caused increased secretion of GLP-2 and was inhibited by lactisole, similar to glucose and sucralose. A cocktail containing PALSWEET and αLA induced a greater response than PALSWEET alone; similarly, GLP-2 secretion induced by concurrent administration of PALSWEET, αLA, and lactisole was comparable to that induced by PALSWEET alone, despite the presence of αLA (Fig. 7D). Lactisole itself did not affect GLP-2 secretion (Fig. 7E). The viability of the NCI-H716 cells was not significantly affected by administration of either substance (αLA, glucose, sucralose, PALSWEET, or lactisole) under the experimental conditions.

**DISCUSSION**

GLP-2 secretion is stimulated by luminal nutrients, some of which were already known to mediate intestinal growth or...
solution (a glucose polymer with lower osmotic stress, almost taken into consideration, so we examined its impact by dextrin secretion in vivo. Hence, the impact of osmolality was also at concentrations of more than 5% induced significant GLP-2 to lymph flow (data not shown). We demonstrated that glucose GLP-2 concentration tends to decrease inversely proportional dependency on GLP-2 secretion in vivo. This is because

tion and the hourly lymph flow in each sample to assess dose adaptation [e.g., short-chain fatty acids (35) and whey proteins (20)]. However, in contrast to GLP-1, the mechanism of GLP-2 secretion remains to be elucidated. Theoretically, GLP-2 should be cosecreted with GLP-1 in equimolar amounts by proglucagon convertase-1/3 (7, 28). However, some studies depicted the difference between GLP-1 and GLP-2 levels in an in vivo study (20). The difference may be partly due to the different response to protease DPP-IV, which leads to different half-life time (10).

Thus, due to their different circulating profiles (28), their cosecretion in response to luminal nutrients has not been fully demonstrated, and that makes it important to focus on the mechanism of GLP-2 secretion.

To the best of our knowledge, this is the first report showing the impacts of luminal contents, especially long-chain fatty acids and artificial sweeteners, on GLP-2 secretion using rat lymph and human enteroidendocrine NCI-H716 cells. The rationale for using lymph samples to measure GLP-2 was provided by D’Alesso et al. (6), who suggested that sampling intestinal lymph may provide a promising approach to studying gut hormones due to lower levels of DPP-IV in the lymph. Although DPP-IV activities were not compared, we found significantly higher GLP-2 concentrations in the lymph than in the plasma, as predicted. Lymph was collected 2 h after injection, based on the finding that plasma GLP-2 concentrations reached its peaks within 45 min of carbohydrate ingestion in humans (37) and that lymphatic GLP-1 concentrations peaked at 60 min in rats (25).

We adopted hourly output by multiplying GLP-2 concentration and the hourly lymph flow in each sample to assess dose dependency on GLP-2 secretion in vivo. This is because GLP-2 concentration tends to decrease inversely proportional to lymph flow (data not shown). We demonstrated that glucose at concentrations of more than 5% induced significant GLP-2 secretion in vivo. Hence, the impact of osmolality was also taken into consideration, so we examined its impact by dextrin solution (a glucose polymer with lower osmotic stress, almost 3.4 mM per 5% solution). GLP-2 output was also increased by 5 or 10% dextrin solution; therefore, high osmotic pressure of glucose solution itself may not be the main cause of driving GLP-2 secretion from L cells.

GLP-2 secretion induced by dietary lipid was influenced by its composition. We observed a trend in which lipids containing n-3 PUFAs and long-chain monounsaturated fatty acids (MUFA)s strikingly increased GLP-2 output. In contrast, butter, which contained many saturated acids, did not reach a significant increase. Similar findings were observed in the secretion of other proglucagon-derived peptides (PGDPs) and GLP-1 (1, 17, 30). Rocca et al. (30) showed that MUFA:s with chain lengths of greater than 14 carbons stimulated PGDPs, whereas Beglinger (1) reported that olive oil and sodium oleate increased plasma concentrations of GLP-1 in humans. Unexpectedly, we also found that Panacet, which consists of caprylate (C8:0) by 99.1%, also enhanced induced GLP-2 secretion, indicating that middle-chain fatty acid is a potent stimulator for GLP-2 secretion.

Although the mechanism in which these fatty acids stimulate L cells to secrete GLP-2 was not examined in this study, several studies suggested the involvment of fatty acid receptors in GLP-2. Hirasawa (17) discovered that αLA induced GLP-1 secretion through GPR120, a G protein-coupled receptor that binds long-chain free fatty acids (LCFFAs). GLP-1 secretion through GPR120 is mediated by a calcium ion-dependent signaling pathway in both STC-1 cells and NCI-H716 (29) cells, suggesting the involvement of similar fatty acid receptors in GLP-2 secretion. Gpr120 mRNA is abundantly expressed in the rat intestinal tract and in humans (34). In addition, Lauffer et al. (24) demonstrated that NCI-H716 cells also expressed GPR40 and GPR119, as well as GPR120. Both receptors are those for LCFFAs and found in the rat intestine (19, 24). GPR120 and GPR40 are also activated by medium-chain fatty acids (12, 33).

The role of sweeteners in GLP-2 secretion was also demonstrated in this study. Sucralose and PALSWEET increased GLP-2 secretion both in vivo and in vitro. Sucralose has been reported not to induce GLP-1 secretion within 10 min after administration in rats (15). Different responses to sucralose possibly come from the gap between calorie-dependent and -independent pathways on GLP secretion. That is, it may take more time for sweeteners to induce both GLP-1 and GLP-2 than for dietary nutrients to do the same amount. This explanation is also consistent with the results of a previous study that demonstrated increased GLP-1 secretion from GLUTag cells incubated with sucralose for 1 h (26). Some types of sweeteners may be applied as substitutes for glucose to induce GLP-2 secretion if they remain in the intestine for longer times. Inhibition of GLP-2 secretion by lactisole in NCI-H716 cells indicated that sweet-tasting agents induced GLP-2 through sweet taste receptor signaling, i.e., T1R3.

On the other hand, sweetness intensity (32) seems to have little to do with the impact on the extent of GLP-2 secretion. Sucralose is about 600 times as sweet as sucrose (22) and 750 times as sweet as glucose. But, just like data of Jang et al. (21), in which glucose and sucralose stimulated GLP-1 secretion in a dose-dependent manner using NCI-H716 cells, no further increase was seen in GLP-2 secretion for sucralose compared with the same concentration of glucose in vitro. Moreover the
mean GLP-2 output induced by 4% sucralose solution was not greater than that by 5% glucose solution in vivo.

Consistent with previous data, in which more GLP-1 output was induced by mixed infusions composed of dietary lipids and carbohydrates than that by lipids with the same number of calories (25), our results showed that mixed infusions of perilla oil and glucose or sweeteners induced GLP-2 secretion at a similar or greater level than perilla oil alone, even though it contains a higher amount of calories. That is, although the total calories in a mixture of 2 ml perilla oil and 1 ml 5% glucose solution was no more than three-fourths of that in 3 ml of perilla oil, the mixture induced nearly an equal amount of GLP-2 secretion as perilla oil did. In addition, GLP-2 output induced by the mixture, which contained sucralose or PALSWEET, was significantly greater than that by perilla oil alone. These results suggest the existence of receptors responding to sweet-tasting agents may elicit some “additive” or “synergistic” effects on other receptors responding to fat components. NCI-H716 cells also exhibited additive effects of αLA and sweet-tasting agents. It should be noted that GLP-2 induction was completely suppressed by lactisole when αLA was mixed with sweet-tasting agents, whereas GLP-2 secretion in response to αLA alone was not affected by lactisole. The exact mechanisms for this are unknown; however, it can be speculated that the sweet taste receptor signaling may potentiate or modify the signal through which αLA induces GLP-2 secretion.

In conclusion, we demonstrated that 1) the impacts of luminal fat, carbohydrate, and sweeteners on GLP-2 secretion are associated with their components and 2) GLP-2 secretion induced by sweet-tasting agents is mediated by sweet taste receptor, which is a different pathway from that for fatty acids. Although it still remains to be seen how much endogenous GLP-2 is needed to exert its intestinalotropic effects, further studies to understand the regulation of GLP-2 may help the clinical attempt to improve intestinal function by daily dietary components.

Fig. 7. GLP-2 secretion in response to α-linolenic acid (αLA) and sweet-tasting agents in NCI-H716 cells. αLA, glucose, and sucralose caused dose-dependent increases in GLP-2 secretion. These effects were inhibited by lactisole, a sweet taste receptor antagonist, with the exception of αLA (A). Concurrent stimulation with αLA caused a greater induction of GLP-2 than with each sweet-tasting agent, and this effect was negated by treatment with lactisole (B). GLP-2 secretion induced by PALSWEET showed a similar trend to secretion induced by glucose and sucralose (C, D). Lactisole itself did not affect GLP-2 secretion (E). PALSWEET (diluted in HEPES to 0.04%) contains approximately 30 μM erythritol. Values are means ± SE. N.S., not significant. *P < 0.05, **P < 0.01 vs. controls (HEPES buffer alone) using the Kruskal-Wallis H-test. *P < 0.05, **P < 0.01 vs. controls using the Mann-Whitney U-test.
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