Title

Effect of single oral co-ingestion of GABA and Malic acid on postprandial GLP-1, glucose, and insulin responses in healthy volunteers: a randomized, double-blind, placebo-controlled, crossover study

Authorship

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21 **Abbreviations Used**

22	GLP-1	Glucagon-like peptide-1
23	GABA	γ-Aminobutyric acid
24	MA	Malic acid
25	CA	Citric acid
26	DPP-4	dipeptidyl peptidase-4
27	Phe	L-Phenylalanine
28	ZeinS	Aqueous extract from zein
29	OGTT	Oral glucose tolerance test
30	MTT	Meal tolerance test
31	IFG/IGT	Impaired fasting glucose / impaired glucose tolerance
32	NFG/NGT	Normal fasting glucose / normal glucose tolerance
33	RT	Room temperature

34

35 **Keywords**

36 corn; enteroendocrine cell; glucagon-like peptide-1; incretinotropic effect; synergistic effect.

Abstract

Scope:

We examined whether co-ingestion of γ-aminobutyric acid (GABA) and malic acid (MA) before meals enhances glucagon-like peptide-1 (GLP-1) secretion, and which affects subsequent insulin and glycemic responses in humans.

Methods and results:

Initially, a murine enteroendocrine STC-1 cell line was used to verify co-administration of GABA and MA synergistically induces GLP-1 secretion. Next, 22 healthy adults were given water (50 mL) containing 400 mg GABA and 400 mg MA (Test), or only 400 mg citric acid (Placebo) 20 min before meal tolerance test. Interval blood samples were taken postprandially over 180 min to determine GLP-1, insulin, and glucose responses. By comparison to preload of Placebo, preload of Test significantly increased plasma GLP-1 (total/active) levels (incremental area under the curve by 1.2-fold and 1.6-fold), respectively. However, there were no significant differences in postprandial blood glucose and insulin.

Conclusion:

Co-ingestion of GABA and MA before meals enhances postprandial GLP-1

55 secretion. Future studies should explore optimal dosage regimens to find the efficacy of
56 the mixture on insulin and glycemic response.
57
58 **1. Introduction**
59 Glucagon-like peptide-1 (GLP-1) is a hormone primarily released from gut
60 enteroendocrine cells in response to various nutrient stimuli.[1] Once secreted, GLP-1 is
61 rapidly cleaved by dipeptidyl peptidase (DPP)-4. Inhibition of DPP-4 increases the levels
62 of endogenous circulating active GLP-1 hormone, which improves glycemic control by
63 enhancing glucose-dependent insulin secretion thereby helping to attenuate postprandial
64 hyperglycemia. In addition to its insulinotropic effects, GLP-1 is known to contribute to
65 the preservation of metabolic health through involvement in appetite, inflammation, and
66 angiogenesis.[2,3] Consequently, strategies such as administration of GLP-1 analogs
67 and/or DPP-4 inhibitors to augment circulating GLP-1 action are of great interest for the
68 prevention and treatment of metabolic diseases, such as diabetes, obesity-related diseases,
69 and vascular events.[2–4] Unfortunately, however, these approaches often do not work or
70 are associated with risks of adverse events.[4,5] Furthermore, the medical approaches with
71 the analogs and/or the inhibitors are unavailable to subjects with pre-symptomatic status
72 such as pre-diabetes. Therefore, a nutritional-based strategy to enhance GLP-1

availability may offer an alternative or complementary solution alongside current approaches.

A previous human study showed that a high doses of nutrients (20 g of whey peptides for metabolic syndrome,[6] 30 g of glutamine for type 2 diabetes mellitus,[7] and 15 g of glutamine for healthy men [8]) was needed to increase GLP-1 levels. Although these studies have shown no adverse effects at the effective dose, it is desirable to achieve efficacy at lower milligram-level doses.

Our previous studies showed that a hydrolysate prepared from corn protein (zein) attenuated hyperglycemia through enhancement of GLP-1 secretion *in vitro*, and subsequent insulin secretion both *in vitro* and *in vivo* rat models.[9–11] In addition, we found that a water extract (ZeinS) of zein and aqueous components (i.e. GABA and L-phenylalanine (Phe)) in ZeinS activated the secretion of GLP-1. GABA exhibited GLP-1 releasing activity not only alone, but also synergistically with Phe.[12] Therefore, we focused on the potential synergistic effects of combining active ingredients as one of the means for effective dosage reduction.

However, although Phe is safe at levels generally used for flavoring,[13] its safety at higher doses in a functional food has not been established. In our previous study, the active fraction of ZeinS was evaluated with an emphasis on the amino acid component.[12]

LC-MS/MS identification results also confirmed the presence of malic acid (MA) in the active fraction of ZeinS (data not shown). Thus, in the present study, MA, an organic acid newly determined in ZeinS, was evaluated for its effectiveness as an alternative active compound for Phe that displays synergistic action to enhance GLP-1 release. Firstly, we examined the synergistic action of GABA and MA for enhancing GLP-1 secretion using a murine enteroendocrine cell line (STC-1). Subsequently, we examined the effect of single co-ingestion of GABA and MA on postprandial circulating GLP-1 levels and glucose responses in healthy adult volunteers.

2. Experimental Section

In vitro study

GLP-1 secretion assay

Assays for GLP-1 stimulation activity were conducted according to the method described previously [9,14] with some modifications. In brief, STC-1 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) through Summit Pharmaceuticals International Corporation (Tokyo, Japan), and cultured in DMEM (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with 10% fetal bovine serum (ATCC), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified

109 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured by trypsinization after
110 reaching 80% to 90% confluency.

111 STC-1 cells were seeded in 48-well plates at a density of 1.0×10^5 cells/well and
112 then incubated for 48 h. On the day of the experiment, cells were washed twice with
113 HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM
114 $MgCl_2$, 10 mM D-glucose, and 0.1% bovine serum albumin, pH 7.4) and then exposed to
115 various doses of GABA and/or MA in the same buffer at 37°C for 30 min. The supernatant
116 was collected, cleared by centrifugation at 800 × *g* for 5 min, and stored at −80°C until
117 required. GLP-1 (total) concentrations in the supernatant were determined using a GLP-
118 1 ELISA kit (Merck Millipore, Darmstadt, Germany) following the manufacturer's
119 instructions. All data were averaged over three replicates for each treatment.

120 Cytotoxic effects on STC-1 cells were determined by the release of lactate
121 dehydrogenase (LDH) in the supernatant of the cells exposed to the GLP-1 releasing
122 activators in the presence or the absence of the test compounds (by using an LDH
123 cytotoxicity detection kit, Dojindo Laboratories, Kumamoto, Japan).

124 All other chemicals and reagents were of analytical grade and used without
125 further purification. Additional chemicals were purchased from FUJIFILM Wako Pure
126 Chemical Corp. unless otherwise specified.

Intracellular calcium measurements in STC-1 cells

For intracellular calcium $[Ca^{2+}]_i$ measurements in STC-1 cells, cells were cultured at a density of 4.0×10^4 cells/well in a 96-well plate coated with 0.01% collagen solution. Upon reaching confluency 48 h after seeding, cells were loaded with 4.2 μM Fura 2-AM (Dojindo Laboratories) dissolved in a recording medium (140 mM NaCl, 4 mM KCl, 10 mM HEPES, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM D-glucose, pH 7.4) containing 0.05% Pluronic F-127, for 30 min in dark at 37°C. Following incubation, the Fura 2-AM solution was removed and replaced with the recording medium. Fluorescence was measured at an excitation of 340 and 380 nm and emission at 510 nm on FlexStation3 microplate reader (Molecular devices, San Jose, California, US). The recording medium served as control, while various doses of GABA and/or MA were applied as treatment. Ratio of fluorescence intensity at 340 and 380 nm was measured as a function of $[Ca^{2+}]_i$.

Clinical trial

Participants

A total of 192 volunteers were initially screened to verify eligibility (visit 1). Specifically, individuals underwent a physical examination (height, weight, body fat

145 percentage, and blood pressure), determination of health status by self-report, blood tests
146 (HbA1c, fasting lipid status, hematologic profile, liver and renal function) and urine tests
147 (glucose, protein, blood, specific gravity, pH, ketones, urobilinogen, bilirubin) as well as
148 examination of fasting and postprandial glucose, insulin level determination, and GLP-1
149 response. Twenty-two healthy adult men and women, including individuals with pre-
150 diabetes, were recruited for the trial. Participants had to meet the following inclusion
151 criteria: (1) fasting blood glucose less than 126 mg/dL, (2) 120 min blood glucose value
152 less than 200 mg/dL during an oral glucose tolerance test (OGTT) with a 75 g glucose
153 load, (3) body mass index (BMI) of 18.5–29.9 kg/m^2, (4) age 20–49 years. Exclusion
154 criteria included: (1) allergic symptoms in foods and medicines; (2) liver, kidney, heart,
155 respiratory, digestive and/or metabolic disease; (3) blood donation in the past 3 months;
156 (4) stage-2 or higher hypertension (≥ 160/100 mmHg); (5) abnormal vital sign
157 measurements at the screening test (systolic blood pressure < 90 mmHg); (6) pregnancy;
158 (7) lactating; (8) carrier state of syphilis, Hepatitis B, Hepatitis C, or HIV; (9) excessive
159 alcohol consumption (i.e., more than 40 g of pure alcohol per day); (10) smoking more
160 than 20 cigarettes a day; (11) night or shift work; (12) using regular medicines,
161 supplements and/or functional foods; (13) present or past history of drug addiction; (14)
162 participation in other clinical trials during the study period or within the past one month;

(15) classification of unsuitability for the study by the investigator for other reasons. The study protocols were approved by Fukuhara Clinic Clinical Trial Review Committee (IRB number 20000005) and the trial was registered at UMIN-CTR (ref. UMIN000041578). All participants provided written, informed consent prior to participation in the study, which was conducted in accordance with the Declaration of Helsinki.

Study design

A randomized, double-blind, placebo-controlled, crossover study design was used with 2-treatment arms. The daily protocol is shown in Fig. 1. Participants in the test group ingested 50 mL water containing 400 mg GABA (Lactogaban; Pharma Foods International Co., Ltd, Kyoto, Japan) and 400 mg MA (Fuso Chemical Co., Ltd, Osaka, Japan). The participants in the placebo group were given 50 mL water containing 400 mg CA (Fuso Chemical Co., Ltd, Osaka, Japan) as a pH adjuster. The dosage conditions and ingredient selection underwent comprehensive evaluation, focusing on the critical aspects of safety, effectiveness, and commercial viability. GABA (400 mg) and MA (400 mg) were within the dose safely tolerated in a clinical trial [15] or acceptable daily intakes for man.[16] GABA and MA were mixed in equal weight ratio (1:1) based on the results of the present *in vitro* experiments, which demonstrated optimal GLP-1 secretion-inducing activity. Furthermore, we considered the price information of these test

materials in anticipation of their potential future commercialization. Citric acid, used as a placebo, is an acidifier widely used to adjust flavor and appearance, and no GLP-1 secretion activity was observed in the *in vitro* assay (data not shown).

This study consisted of a screening period to determine participants by OGTT (Visit 1), two treatment periods to evaluate the effects of preload test ingredients followed by meal tolerance test (MTT) (Visits 2, 3), and a follow-up period. Participants ate several commercial foods the day before every test day. Randomization, based on the screening data, was conducted using a permuted block method with a Latin square balanced for treatment order and carryover effects. Blinding of treatments was performed by an independent individual unaware of the treatment allocation.

Two 1-day visits were required with a washout period of at least 1 week between visits. The primary outcome was postprandial plasma GLP-1 (total) response during MTT. Secondary outcomes were plasma GLP-1 (active), blood glucose, and insulin response. The participants were assessed during fully supervised study days.

Screening visit

With informed consent each participant received a screening test to confirm body composition, vital signs, and blood measurements. The participants ate several commercial foods and water the day before performing a series of tests. A total of 192

people were screened. The participants arrived at a study facility by 09:00 on the test day in an overnight fasted state, having abstained from exercise and smoking since 21:00 the previous day. After about 20 min at rest in a sitting position, fasting blood for biochemical analyses was collected from a forearm vein. The measurement of body composition was measured (height, weight, Body Mass Index (BMI), and body fat percentage), vital signs (blood pressure, heart rate, and body temperature) and urinalysis were performed. Participants consumed 75 g of glucose (OGTT) at 10:00 and blood sampling was carried out 15, 30, 60, and 120 min after OGTT for determination of GLP-1 (total), glucose, and insulin. During free time between repeated blood collections within OGTT, the participants remained at rest in a sitting position but were free to read, access the Internet on their own devices or perform deskwork activities. The participants were free to leave the facility after completion of blood collection and an examination by a doctor. We identified 22 participants with impaired fasting glucose and/or impaired glucose tolerance (IFG/IGT) and normal fasting glucose and/or normal glucose tolerance (NFG/NGT) based on the trial criteria. They were randomly allocated into two groups of 11 each.

Study visits

Participants arrived at the study facility by 09:00 on the test days in an overnight fasted state, having the other same conditions as the screening visit. After a fasted blood

sample was obtained, each participant was given either a test (50 mL of drinking water containing each 400 mg of GABA and MA) or a placebo (50 mL of drinking water containing 400 mg of CA) 20 min before MTT (at 10:00) comprising 200 g cooked rice (carbohydrate: 67.8 g) with 100 mL water. For placebo treatment, 400 mg of CA was used to make the taste indistinguishable from that of the test treatment. Participants ate the meal within 10 min and blood sampling was carried out 15, 30, 45, 60, 90, 120, and 180 min after MTT for measurement of GLP-1 (total/active), glucose, and insulin.

Blood collection and analytical procedures

Blood for plasma GLP-1 (total) analysis was collected into tubes containing EDTA. Plasma were collected by centrifugation (1500 × *g*, 15 min, 4°C), and stored at -80°C until analysis. Measurement of plasma glucose and serum insulin was outsourced to a commercial laboratory (SRL, Inc. Hokkaido, Japan). Blood samples were collected into tubes containing a serum-separating agent or sodium fluoride, and then stored at room temperature (RT) before being sent to a commercial laboratory (SRL, Inc.). Blood for plasma GLP-1 (active) analysis was collected into K_2EDTA tubes containing a cocktail of protease, esterase, and DPP-4 inhibitors (P800; BD Biosciences, San Jose, CA, USA). Upon collection, samples were centrifuged (1300 × *g*, 10 min, RT) and the plasma was analyzed immediately. Plasma GLP-1 (total/active) concentrations were determined

using a total/active GLP-1 ELISA kit (Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) following the manufacturer's instructions.

Statistical analysis

All data are presented as mean ± standard deviation (SD). For the *in vitro* study, synergistic effect was confirmed by examining the significance of the interaction effect in the two-way ANOVA using both each alone and the combined treatment. Clinical efficacy was analyzed using data from the participants (20) who excluded interfering medication (1) and poor compliance (1) during the study period, aiming to minimize the potential impact of external factors (per-protocol set, PPS). Clinical safety was analyzed using all participants (22) who completed the study, aiming to provide a more comprehensive understanding of how the interventions might perform in practical situations (safety analysis set, SAS). Fluctuations in glucose, insulin, and GLP-1 were analyzed by baseline-adjusted mixed model for repeated measures method (cross-over, treatment and time). The group-by-time interaction at each time point was analyzed by an approximate *t*-test (using Satterthwaite's method for degrees of freedom calculation). Postprandial glucose and hormone responses were converted into incremental area under the curve (iAUC) using the trapezoidal rule. The level of statistical significance was set at 5% for two-tailed. Statistical analyses were performed using the SAS software (R9.4,

253 SAS Institute Japan).

254

255 **3. Results**

256 **Experiment 1： GLP-1 and $[Ca^{2+}]_i$ responses in STC-1 cells to co-administration of**

257 **GABA and MA to investigate their synergistic properties *in vitro***

258 Initially, we assessed the co-administration effect of GABA and MA using STC-

259 1 cells. Although GABA (10 ng/mL) and MA (100 ng/ mL) alone did not significantly

260 increase GLP-1 (total) secretion, synergistic effect was found to be statistically significant

261 (Fig. 2A, $p < 0.01$ using an interaction effect in two-way ANOVA). GABA (2 μg/mL)

262 alone, but not MA (2 μg/mL), had appreciable activity (Fig. 2G, $p < 0.05$). GABA (10

263 ng/mL, 100 ng/mL, and 2 μg/mL) alone significantly increased $[Ca^{2+}]_i$. Although MA

264 (100 ng/mL and 2 μg/mL) alone failed, the combination with GABA (10 ng/mL, 100

265 ng/mL, and 2 μg/mL) synergistically increased $[Ca^{2+}]_i$ (Fig. 2C, F, and I; $p < 0.05$ or $p <$

266 0.1 using an interaction effect in two-way ANOVA).

267

268 **Experiment 2： Effect of single oral co-ingestion of GABA and MA on postprandial**

269 **blood GLP-1, glucose, and insulin response in healthy volunteers**

270 **Participants**

Twenty-two healthy adults randomly assigned into the trial. All participants completed the study periods, with 2 participants excluded for failure to comply with the study protocol (see CONSORT diagram, Fig. 3). Characteristics of the participants are shown in Table 1.

Blood parameters

The effects of the different treatment regimens on blood parameters and iAUC 0–180 min responses of postprandial glucose and regulating hormones (insulin and GLP-1 (total/active)) are shown in Fig. 4, 5. All profiles exhibited a highly significant effect of time ($p < 0.01$) with predictable changes driven primarily by the timing of MTT.

GLP-1

The principal effect of the treatment (Test) was evident in the plasma concentrations of both GLP-1 (total/active) (Fig. 4, both $p < 0.05$). Mean peak concentrations (C_{max}) increased in the Test group (Fig. 4, total; 27.4 ± 10.2 pmol/L; $p < 0.05$, active; 5.56 ± 2.33 pmol/L; $p = 0.0573$) compared with the Placebo group (total; 23.5 ± 7.7 pmol/L, active; 4.54 ± 1.82 pmol/L). The changes from baseline (ΔC_{max}) observed in the Test group increased (total; 16.6 ± 9.7 pmol/L; $p = 0.0715$, active; 3.78 ± 2.13 pmol/L; $p < 0.05$) compared with the Placebo treatment (total; 12.7 ± 6.3 pmol/L, active; 2.59 ± 1.11 pmol/L). A significant effect of treatment was also observed for GLP-

289 1 iAUC 0–180 min responses for both GLP-1 (total/active) (total; 1433 ± 597
290 pmol/L·min; $p < 0.05$, active; 325 ± 169 pmol/L·min; $p < 0.01$) compared with the
291 Placebo treatment (total; 1148 ± 496 pmol/L·min, active; 206 ± 130 pmol/L·min) (Fig.
292 4).

293 ***Glucose***

294 No significant differences were detected in postprandial glucose responses and
295 iAUC 0–180 min between treatment regimens (Fig. 5A).

296 ***Insulin***

297 There were also no significant differences observed in postprandial insulin
298 response and iAUC 0–180 minutes between the treatment regimens (Fig. 5B).

299 ***Adverse events***

300 Some participants reported adverse event symptoms, such as headache, loss of
301 appetite, fatigue, and dizziness during the study period. The analysis of all 22 participants
302 revealed a total of 9 mild to moderate adverse event symptoms reported by 5 participants.
303 Four individuals reported a headache (3) or a loss of appetite (1) in the week following
304 the test treatment during the washout period, and only one individual reported fatigue
305 while on the placebo treatment during the washout period. Given the timing in the onset
306 of these events, they do not appear to be attributable to the treatment.

4. Discussion

The present study demonstrates that co-ingestion of GABA and MA before meals potentiate postprandial GLP-1 secretion at the lowest dose of our knowledge, but has no changes in postprandial plasma glucose levels under this dosage regimen.

GABA is widely used as a food supplement to improve sleep patterns and reduce stress levels in healthy adults.[17] Its biological effects are exerted through the activation of GABA receptors that are expressed in a variety of peripheral tissues and cells, including intestinal enteroendocrine cells and pancreatic islet cells.[18,19] Within the intestine, GABA enhances GLP-1 secretion from L-cells through membrane depolarization.[18] However, to our knowledge, there is a report that evaluated the postprandial GLP-1 responses to oral single and continuous administration of GABA under both fasting and fed conditions in healthy humans, and it suggests that its effectiveness could not be confirmed.[15] MA is also frequently used in foods as an additive ingredient (e.g., acidifier or flavoring agent). Because of its low toxicity, MA is generally recognized as safe (GRAS) for use as a direct food additive.[20] However, the effect of MA on gut hormone enhancement, such as GLP-1, has not previously been investigated. The most abundant organic acid in sweet corn

kernels has been shown to be MA, which followed by α-ketoglutaric acid, citric acid (CA), and succinic acid.[21] Our preliminary tests showed that the amount of MA in ZeinS was approximately 2 mg per 100 g of zein (0.0002% w/w in aqueous extract), measured using a commercial assay kit (J. K. International Inc., Tokyo, Japan) for D/L-malic acid. Thus, we were interested in investigating the potential influence of co-ingestion of GABA and MA on GLP-1 secretory activity.

We first confirmed the synergistic effect of a combination of GABA and MA on GLP-1 responses *in vitro* prior to conducting the present clinical study. We presumed the concentration of GABA at ~2 μg/mL and MA at ~2 μg/mL in ZeinS at 1 mg/mL by quantitative analysis. Based on the experiment employing these concentrations of ZeinS (1 mg/mL), GABA (2 μg/mL), and MA (2 μg/mL), the contribution of GABA and MA to GLP-1-releasing activity of ZeinS (241%, as the mean value of GLP-1 secretion [% Control]) appeared to range between approximately 25.9% and 4.5% for GABA and MA, respectively. However, the contribution of GABA or MA for their synergistic effect (Fig. 2A) is not able to estimate. Synergistic secretion of GLP-1 is thought to result from elevated intracellular Ca^{2+} levels through multiple intracellular signaling pathways, or additionally, elevated intracellular cAMP levels. Hauge et al. demonstrated that the synergistic GLP-1 response is induced by the physiological coaction of two different

343 receptors signaling through Gq and Gs by endogenous ligands.[22] Co-activation of the
344 two pathways leads to marked GLP-1 secretion by increasing the level of intracellular
345 cAMP in addition to increasing levels of IP_3 and Ca^{2+}. In previous study, we also observed
346 that ion channel-type receptors ($GABA_A$) and GPCR (CaSR) act in concert to induce
347 synergistic GLP-1 secretion when GABA and Phe are combined.[12] It was suggested that
348 the synergistic GLP-1 secretion would involve elevation of intracellular Ca^{2+}
349 concentration through the co-activation of $GABA_A$ receptor and CaSR. Therefore, in the
350 present study, our hypothesis was that combined stimulation with GABA and malic acid
351 would induce a synergistic increase in GLP-1 secretion, primarily by elevating
352 intracellular Ca^{2+} levels, or additionally, cAMP levels, compared to individual
353 stimulations.

354 Regarding GABA, our previous study has suggested that the effect on $GABA_A$
355 receptors contributes to the enhancement of GLP-1 secretion,[12] and it also activates the
356 Gq pathway through the $GABA_B$ receptor,[23,24] while the mechanism of action of MA is
357 unclear. MA acts as a ligand for the G protein-coupled receptor GPR81
358 (hydroxycarboxylate receptor 1),[25] which is known to be expressed on the basolateral
359 surface of ghrelin-producing endocrine cells in the stomach and upper gastrointestinal
360 tract.[26,27] Additionally, the agonist L-lactic acid, which exhibits higher affinity for

361 human GPR81 than MA, has been reported to inhibit forskolin-induced cAMP
362 accumulation through GPR81.[25] Therefore in contrast to the above hypothesis of
363 synergistic GLP-1 secretion, the activation of GPR81 by MA may cause cAMP
364 downregulation leading to a decrease in intracellular levels of cAMP. On the other hand,
365 there are reports suggesting that stimulation of alternative signaling pathways by MA
366 may induce an increase in intracellular Ca^{2+} levels. A study reported hydrochloric acid,
367 a principal sour tastant as well as MA, induced GLP-1 release from STC-1 cells, which
368 was diminished in the presence of Ca^{2+} influx blockers.[28] Another study reported that
369 short-chain fatty acids, which are organic acids, increase intracellular Ca^{2+} levels and
370 enhance GLP-1 release from STC-1 cells via transient receptor potential Ankyrin 1.[29]
371 In the present study, the combined with GABA and MA synergistically increased $[Ca^{2+}]_i$.
372 Because intracellular Ca^{2+} acts as a key second messenger in the release of peptide
373 hormones, we reasoned that MA-dependent changes in $[Ca^{2+}]_i$ may play a synergistic role
374 in GLP-1 secretion with GABA. The change in $[Ca^{2+}]_i$ was observed with GABA alone
375 at 10 ng/mL in Figure 2C, but it was not consistent with the change in GLP-1 in Figure
376 2A. If GLP-1 release is initiated by reaching a threshold $[Ca^{2+}]_i$ between 1.2 and 1.5
377 (F340/F380), it might be a reason that GABA alone at 10 ng/mL cannot induce GLP-1
378 secretion, as shown in Figure 2A.

Subsequently, our clinical study demonstrated that GABA and MA (400 mg each), when given together as preloads, lead to significant enhancement of the iAUC 0–180 min and ΔC_{max} of active GLP-1 by 1.6-fold and 1.5-fold, respectively compared to placebo. Previous reports on DPP-4 inhibitors in healthy Japanese adult males have shown that a single dose of anagliptin 100 mg increased the iAUC 0–4h of active GLP-1 by approximately 2-fold compared to placebo.[30] In addition, a single dose of linagliptin 5 mg enhanced the ΔC_{max} of active GLP-1 from baseline (premeal) by approximately 2-fold compared to placebo.[31] Thus, the present findings suggest that co-ingestion of GABA and MA could be an effective nutritional-based strategy for preventing metabolic diseases by enhancing of postprandial GLP-1 availability/action. Besides, there are several advantages to combining the intake of GABA and MA. The reduced requirement for components necessary to achieve effectiveness not only alleviates the burden of ingestion but also reduces production costs for the product.

However, contrary to our expectations, there were no differences in blood glucose and insulin responses between the two groups. Similar to the present study, there are previous studies showing that in clinical trials in healthy subjects, increases in GLP-1 were confirmed, did not elevate insulin, and did not change glucose tolerance. [8,32] These results may be attributed to the lack of a sufficient GLP-1 secretory response to the

397 ingestion of the test sample before MTT (glucose loading). Our previous study showed a
398 kinetic pattern in which a single oral administration of a corn zein peptide before glucose
399 loading induced leading incretin (GLP-1 and GlP) secretion, followed by enhanced
400 insulin secretion that attenuated subsequent hyperglycemia after glucose loading in rat
401 model.[11] These results suggested that it is important to induce GLP-1 secretion prior to
402 increasing glucose levels by glucose loading. A previous report demonstrated that oral
403 administration of glucose or amino acid (glutamine) alone resulted in an increase in
404 circulating levels of glucose or GLP-1, which peaked after 30 min in NGT.[33] Therefore,
405 the ingestion timing of the test ingredients (−20 min) was carefully chosen so that GLP-
406 1 response to the pre-treatment prior to MTT would peak 10 min after MTT. However,
407 under the present protocol, we did not observe the combined GABA and MA-induced
408 GLP-1 secretion before postprandial blood glucose levels increased (e.g., 15 min after
409 MTT). For these reasons, although co-ingestion of GABA and MA potentiated
410 postprandial GLP-1 secretion in healthy adults, this may have been insufficient to affect
411 postprandial insulin and glucose responses. We propose that optimization of dosage
412 regimens for the test ingredients will have the potential to suppress postprandial blood
413 glucose elevation in IFG/IGT and NFG/NGT.

414 Research information on the acute effects of pre-meal intake of GABA and MA

415 in Healthy volunteers is limited. First, a study on GABA reported no difference in GLP-
416 1 response at a meal (lunch) 4 hours after a single intake of 2 g GABA (tablet).[15]
417 Although the blood GLP-1 response was not evaluated, it has also been reported that there
418 was no difference in glucose responses in OGTT (75 g of carbohydrate) after a single
419 intake of 500 mg (capsule) of GABA.[34] On the other hand, when it comes to MA, there
420 is a lack of clinical research information regarding its GLP-1 secretory effects. However,
421 it has been demonstrated that oral administration of 20 g of lactic acid, which is the same
422 organic acid, led to a significant increase in plasma GLP-1 concentrations when compared
423 to intravenous administration.[35] Also, in a study in which the same amount of available
424 carbohydrate was consumed with or without 3 g oral propionic acid, an increase in GLP-
425 1 secretion was observed with the addition of propionic acid.[36] Furthermore, most of the
426 studies supporting a role for acetic acid (vinegar) as a glucose-lowering nutraceutical have
427 typically used the equivalent of 1 g of acetic acid per serving in acute studies of its
428 interaction with carbohydrate-rich diets.[37] In the present study, GABA and MA were
429 consumed at lower doses than in these trials. Therefore, we would not expect to see a
430 difference from the placebo in postprandial GLP-1 responses under each alone condition.
431 However, since these results may change depending on study conditions, future studies
432 should draw conclusions from concurrent testing of each alone and the combined

433 treatment.

434 GLP-1-based agents, including GLP-1 analogs and DPP-4 inhibitors, improve
435 glycemic control by enhancing glucose-dependent insulin secretion. GLP-1, in addition
436 to its glucose-lowering effect, induces other extra-pancreatic activities. Crucially,
437 vascular endothelium is known to play an important role in the maintenance of
438 cardiovascular function.[38] Emerging evidence indicates that GLP-1 and its analogs have
439 direct protective effects on vascular endothelium.[39] DECODE (Diabetes Epidemiology:
440 Collaborative analysis of Diagnostic criteria in Europe) studies have shown that the
441 pathogenesis of diabetic macroangiopathy presents not only in patients with type 2
442 diabetes mellitus but also in individuals with prediabetes (IGT and IFG).[40–42] Thus, the
443 present results suggest that the extra-pancreatic effects of GLP-1 may elicit a protective
444 function in terms of maintaining cardiovascular function in IGT and IFG. The inclusion
445 of healthy participants in this study, who were free of underlying metabolic issues that
446 might confound the overall biological effect, also strengthens these findings. Nonetheless,
447 the corresponding effect of this treatment on NGT requires further investigation.

448 One limitation of this study is that only the short-term glycemic response and
449 side effects could be assessed. Specifically, we only examined the acute effect of co-
450 ingestion of GABA and MA, but the chronic responses to postprandial glucose

metabolism were not investigated. Another limitation of this study is a point of comparative evaluation of the two groups. The GABA alone and MA alone treatment would have allowed us to explore synergistic aspects between GABA and MA on postprandial GLP-1 responses. However, the present study only evaluated the efficacy and side effects of co-ingestion of GABA and MA.

In conclusion, this study provided new insights into the enhancement of postprandial GLP-1 availability through the use of GABA and MA in healthy adults as a part of a nutritional-based strategy. However, the present dosage regimens were found to be ineffective in regulating postprandial blood glucose and insulin responses. These findings are crucial for refining the approach and call for further research in the future.

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Conflict of Interest

The authors declare no competing financial interest.

Data availability

The data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

Author contributions

H. N., T. I., S. U., C. K., N. K., H. H. and T. H. designed the research; H. N., S. U., and C. K. conducted the research and analyzed the data; Y. M. and Y. K. assisted with the statistical analyses; H. N. and T. H. wrote the paper; T. H. had primary responsibility for the final content. All authors read and approved the final manuscript.

References

[1] T. J. Kieffer, J. F. Habener, *Endocr. Rev.* **1999**, *20*, 876.

[2] M. A. Nauck, J. J. Meier, M. A. Cavender, M. Abd El Aziz, D. J. Drucker, *Circulation* **2017**, *136*, 849.

[3] T. D. Müller, B. Finan, S. R. Bloom, D. D'Alessio, D. J. Drucker, P. R. Flatt, A. Fritsche, F. Gribble, H. J. Grill, J. F. Habener, J. J. Holst, W. Langhans, J. J. Meier, M. A. Nauck, D. Perez-Tilve, A. Pocai, F. Reimann, D. A. Sandoval, T. W. Schwartz,

485 R. J. Seeley, K. Stemmer, M. Tang-Christensen, S. C. Woods, R. D. DiMarchi, M. H.
486 Tschöp, *Mol. Metab.* **2019**, *30*, 72.
487 [4] D. J. Drucker, *Cell Metab.* **2018**, *27*, 740.
488 [5] K. Nonaka, T. Kakikawa, A. Sato, K. Okuyama, G. Fujimoto, N. Kato, H. Suzuki, Y.
489 Hirayama, T. Ahmed, M. J. Davies, P. P. Stein, *Diabetes Res. Clin. Pract*. **2008**, *79*,
490 291.
491 [6] A. Bjørnshave, J. J. Holst, K. Hermansen, *Eur. J. Nutr.* **2019**, *58*, 755.
492 [7] D. Samocha-Bonet, O. Wong, E. L. Synnott, N. Piyaratna, A. Douglas, F. M. Gribble,
493 J. J. Holst, D. J. Chisholm, J. R. Greenfield, *J. Nutr.* **2011**, *141*, 1233.
494 [8] J. Chang, T. Wu, J. R. Greenfield, D. Samocha-Bonet, M. Horowitz, C. K. Rayner,
495 *Diabetes Care* **2013**, *36*, 2262.
496 [9] T. Hira, T. Mochida, K. Miyashita, H. Hara, *Am. J. Physiol. Gastrointest. Liver*
497 *Physiol.* **2009**, *297*, G663.
498 [10] T. Mochida, T. Hira, H. Hara, *Endocrinology* **2010**, *151*, 3095.
499 [11] N. Higuchi, T. Hira, N. Yamada, H. Hara, *Endocrinology* **2013**, *154*, 3089.
500 [12] H. Noguchi, N. Kohda, H. Hara, T. Hira, *Biosci Biotechnol Biochem*. **2023**, *87*, 1505.
501 [13] B. L. Oser, R. A. Ford, *Food Technol.* **1978**, *32*, 60.
502 [14] M.K. Sufian, T. Hira, K. Asano, H. Hara, *J. Agric. Food Chem.* **2007**, *55*, 8980.

[15] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J. Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D. L. Greiner, G. J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, *Front. Pharmacol.* **2015**, *6*, 260.

[16] WHO, 1965. NMRS 40 / TRS 339 Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour-treatment agents, acids, and bases; https://apps.who.int/iris/bitstream/handle/10665/39853/WHO_TRS_339.pdf?sequence=1 (accessed Jun 2023).

[17] P. Hepsomali, J. A. Groeger, J. Nishihira. A. Scholey, *Front. Neurosci.* **2020**, *14*, 923.

[18] A. Gameiro, F. Reimann, A. M. Habib, D. O'Malley, L. Williams, A. K. Simpson, F. M. Gribble, *J. Physiol.* **2005**, 569, 761.

[19] N. P. Hyland, J. F. Cryan, *Front. Pharmacol.* **2010**, *1*, 124.

[20] FDA, Code of Federal Regulations Title 21: Food and Drugs, Subchapter B - Food For Human Consumption (continued), Part 184 – Direct food substances affirmed as generally recognized as safe, Subpart B - Listing of specific substances affirmed as GRAS; https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=184.1069, 2023 (accessed Jun 2023).

[21] R. Masuda, I. Yamashita, K. Kaneko, *J. Jpn. Soc. Food Sci. Technol.* **1997**, *44*, 23.

[22] M. Hauge, J. P. Ekberg, M. S. Engelstoft, P. Timshel, A. N. Madsen, T. W. Schwartz, *Mol. Cell Endocrinol*. **2017**, *449*, 64.

[23] L. H. Chen, B. Sun, Y. Zhang, T. J. Xu, Z. X. Xia, J. F. Liu, F. J. Nan, *ACS Med. Chem. Lett*. **2014**, *5*, 742.

[24] A. Karls, M. Mynlieff, *J. Neurochem*. **2015**, *135*, 88.

[25] C. Liu, J. Wu, J. Zhu, C. Kuei, J. Yu, J. Shelton, X. Li, SJ. Yun, T. Mirzadegan, C. Mazur, F. Kamme, TW. Lovenberg, *J Biol Chem*. **2009**, *284*, 2811.

[26] M.S. Engelstoft, W.M. Park, I. Sakata, L.V. Kristensen, A.S. Husted, S. Osborne-Lawrence, PK. Piper, AK. Walker, MH. Pedersen, MK. Nøhr, J. Pan, CJ. Sinz, PE. Carrington, TE. Akiyama, RM. Jones, C. Tang, K. Ahmed, S. Offermanns, KL. Egerod, JM. Zigman, TW. Schwartz, *Mol Metab.* **2013**, *2*, 376.

[27] R.E. Steinert, C. Feinle-Bisset, L. Asarian, M. Horowitz, C. Beglinger, N. Geary, *Physiol Rev.* **2017**, *97*, 411.

[28] M. C. Geraedts, F. J. Troost, W. H. Saris, *Food Chem*. **2011**, *129*, 731.

[29] V. Kumar, P. Khare, K. Devi, J. Kaur, V. Kumar, K. K. Kondepudi, K. Chopra, M. Bishnoi, *Fundam. Clin. Pharmacol*. **2021**, *35*, 1004.

[30] Y. Sunami, S. Sesoko, K. Kaku, *Jpn. Pharmacol. Ther.* **2012**, *40*, 847.

[31] A. Sarashina, S. Sesoko, M. Nakashima, N. Hayashi, A. Taniguchi, Y. Horie, E. U. Graefe-Mody, H. J. Woerle, K. A. Dugi, *Clin. Ther*. **2010**, *32*, 1188.

[32] A. Nilsson, E. Johansson-Boll, J. Sandberg, I. Björck, *Clin. Nutr. ESPEN.* **2016**, *15*, 49.

[33] J. R. Greenfield, I. S. Farooqi, J. M. Keogh, E. Henning, A. M. Habib, A. Blackwood, F. Reimann, J. J. Holst, F. M. Gribble, *Am. J. Clin. Nutr.* **2009**, *89*, 106.

[34] T. H. de Bie, R. F. Witkamp, M. G. Balvers, M. A. Jongsma, *Am. J. Clin. Nutr.* **2023**, *118*, 708.

[35] M. G. B. Pedersen, E. Søndergaard, C. B. Nielsen, M. Johannsen, L. C. Gormsen, N. Møller, N. Jessen, N. Rittig, *Clin. Nutr.* **2022**, *41*, 517.

[36] G. S. Frost, A. E. Brynes, W. S. Dhillo, S. R. Bloom, M. I. McBurney, *Eur. J. Clin. Nutr.* **2003**, *57*, 293.

[37] H. O. Santos, W. M. A. M. de Moraes, G. A. R. da Silva, J. Prestes, B. J. Schoenfeld, *Clin. Nutr. ESPEN.* **2019**, *32*, 1.

[38] J. P. Halcox, W. H. Schenke, G. Zalos, R. Mincemoyer, A. Prasad, M. A. Waclawiw, K. R. Nour, A. A. Quyyumi, *Circulation* **2002**, *106*, 653.

[39] J. Koska, M. Sands, C. Burciu, K. M. D'Souza, K. Raravikar, J. Liu, S. Truran, D. A. Franco, E. A. Schwartz, D. C. Schwenke, D. D'Alessio, R. Q. Migrino, P. D. Reaven,

Diabetes **2015**, *64*, 2624.

[40] DECODE Study Group, the European Diabetes Epidemiology Group. *Arch. Intern. Med.* **2001**, *161*, 397.

[41] M. Hyvärinen, Q. Qiao, J. Tuomilehto, T. Laatikainen, R. J. Heine, C. D. Stehouwer, K. G. Alberti, K. Pyörälä, B. Zethelius, B. Stegmayr; DECODE Study Group, *Diabetes Care* **2009**, *32*, 348.

[42] F. Ning, J. Tuomilehto, K. Pyörälä, A. Onat, S. Söderberg, Q. Qiao; DECODE Study Group, *Diabetes Care* **2010**, *33*, 2211.

Figure 1. Protocol for study visits

[Visit 1] Participants in a fasted state arrived at 09:00. Their body composition and vital signs were measured, and fasting blood samples were collected. The participants consumed 75 g of glucose for OGTT at 10:00, and blood sampling was carried out 15, 30, 60, and 120 min after OGTT.

[Visit 2, 3] Participants in a fasted state arrived at 09:00. Their body composition and vital signs were measured, and fasting blood samples were collected. Each participant was given either a Test (50 mL of drinking water containing each 400

mg of GABA and MA) or Placebo (50 mL of drinking water containing 400 mg of CA) 20 min prior to MTT (at 10:00). The meal consisted of 200 g cooked rice (carbohydrate: 67.8 g) with 100 mL water. Participants ate the meal within 10 min, and blood sampling was carried out 15, 30, 45, 60, 90, 120, and 180 min after the meal.

(OGTT, oral glucose tolerance test; MTT, meal tolerance test)

Figure 2. Experiment to investigate the *in vitro* synergistic effect of co-administration of GABA and MA on GLP-1 (total) secretion and $[Ca^{2+}]_i$ response using STC-1 cells

A, D, G) The relative (%) to control GLP-1 secretion. STC-1 cells were exposed to Control (HEPES buffer), various concentrations of GABA (10 ng/mL, A–C; 100 ng/mL, D–F; and 2 μg/mL, G–I), and MA (100 ng/mL, A–F; and 2 μg/mL, G–I). The gray portion of the bar shows the effects of GABA and MA alone. The black portion of the bar shows the more than additive effect of co-administration of GABA and MA. B, E, H) The ratio of fluorescence intensity at 340 and 380 nm. The Fura 2-loaded cells exposed to Control (recording medium) and the same

593 treatments as the others, and $[Ca^{2+}]_i$ responses were measured ratiometrically
594 using a microplate reader. The conditions represented as follows: Control
595 (quadrangle), MA (triangle), GABA (gray circle), and combined GABA and MA
596 (black circle). C, F, I) Changes in $[Ca^{2+}]_i$ expressed as the ratio of maximum –
597 minimum fluorescence intensity at 340 and 380 nm. The gray and black portion
598 of the bars represent the same as described above. Values are expressed as
599 mean ± SD (n = 3–4). Significance in synergistic effect was analyzed using an
600 interaction effect in two-way ANOVA. Values not sharing the same letter differ
601 significantly ($p < 0.05$ by Tukey's test).
602
603 **Figure 3. CONSORT diagram of study participants**
604 FAS: full analysis set, PPS: per protocol set
605
606 **Figure 4. Changes in plasma GLP-1 (total/active) concentrations during MTT in**
607 **response to oral co-ingestion of GABA and MA**
608 CA (○, 400 mg/serving as Placebo) and a mixture of GABA and MA (●, each
609 400 mg/serving as Test) were preloaded orally 20 min before MTT (equivalent to
610 approximately 70 g carbohydrate). Blood samples were collected from the arm

611 vein before and during MTT. GLP-1 (total/active) concentrations in the plasma
612 were measured. Data were analyzed by baseline-adjusted mixed model for
613 repeated measures method (cross-over, treatment and time). The group-by-time
614 interaction at each time point was analyzed by *t*-test. *P* values were < 0.05
615 (treatment), < 0.01 (time), and 0.13 (treatment × time) for GLP-1 (total) (A); < 0.01
616 (treatment), < 0.01 (time), and 0.11 (treatment × time) for GLP-1 (active) (B).
617 Values are expressed as mean ± SD (n = 20). ** $p < 0.01$ and * $p < 0.05$ differ
618 significantly between treatments at a given time point.
619
620 **Figure 5. Changes in plasma glucose and serum insulin concentrations during MTT**
621 **after oral co-ingestion of GABA and MA**
622 CA (○, 400 mg/serving as Placebo) and the mixture of GABA and MA (●, each
623 400 mg/serving as Test) were preloaded orally 20 min before MTT (equivalent to
624 approximately 70 g carbohydrate). Blood samples were collected from the arm
625 vein before and during MTT. Glucose and insulin concentrations in the plasma
626 and serum were measured. Data were analyzed by baseline-adjusted mixed
627 model for repeated measures method (cross-over, treatment and time). The
628 group-by-time interaction at each time point was analyzed by *t*-test. *P* values were

629 0.67 (treatment), < 0.01 (time), and 0.92 (treatment × time) for plasma glucose

630 (A); 0.49 (treatment), < 0.01 (time), and 0.90 (treatment × time) for serum insulin

631 (B). Values are expressed as mean ± SD (n = 20).

632

633 **Table 1. Characteristics of the participants who completed all two treatment arms**

Characteristic	FAS/SAS			PPS		
Sample size (of which female)	22 (14)			20 (12)		
Age, y	44	±	5	44	±	5
Height, cm	163.3	±	7.2	163.3	±	7.5
Body weight, kg	65.8	±	7.7	66.6	±	7.5
Body fat percentage, %	32.4	±	7.0	32.2	±	7.3
Body mass index, kg m^{-2}	24.7	±	2.5	25.0	±	2.4
Systolic blood pressure, mmHg	125	±	12	125	±	12
Diastolic blood pressure, mmHg	76	±	8	76	±	8
Fasting plasma glucose, mg/dL	88.8	±	7.6	89.4	±	7.6

634 Values are expressed as mean ± SD. All measurements were recorded during

635 the screening visit (Visit 1).

636 FAS, full analysis set; SAS, safety analysis set; PPS, per protocol set