



Title	Novel Mechanism of Fatty Acid Sensing in Enteroendocrine Cells : Specific Structures in Oxo-Fatty Acids Produced by Gut Bacteria Are Responsible for CCK Secretion in STC-1 Cells via GPR40
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1 **TITLE**

2 Novel mechanism of fatty acid sensing in enteroendocrine cells: Specific structures in  
3 oxo-fatty acids produced by gut bacteria are responsible for CCK secretion in STC-1 cells  
4 via GPR40

5

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26

27 **Key words**

28 Fatty acids; Gut lactic acid bacteria metabolites; Enteroendocrine cells; Cholecystokinin;  
29 Gastric emptying;

30

31 **Abbreviations**

32 CCK, cholecystokinin; C18, octadecanoic fatty acid; GPR40, G-protein coupled receptor  
33 40; GPR120, G-protein coupled receptor 120; LDH, lactate dehydrogenase;

34

35 **ABSTRACT**

36 **Scope**

37       The secretion of gut hormones, such as cholecystinin (CCK) is stimulated by fatty  
38 acids. Although a chain length-dependent mechanism has been proposed, other structural  
39 relationships to releasing activity remain unclear. We aimed to elucidate specific structures  
40 in fatty acids that are responsible for their CCK-releasing activity, and related sensing  
41 mechanisms in enteroendocrine cells.

42 **Methods and results**

43       We examined CCK secretory activities in a murine CCK-producing cell line STC-1 by  
44 exposing the cells to various modified fatty acids produced by gut lactic acid bacteria. The  
45 effects of fatty acids on gastric emptying rate as a CCK-mediated function were examined  
46 using acetaminophen- and phenol red-methods in rats. Out of more than thirty  
47 octadecanoic (C18)-derived fatty acids tested, five oxo-fatty acids potently stimulated CCK  
48 secretion without cytotoxic effects in STC-1 cells. Three fatty acids had a distinct specific  
49 structure containing one double-bond, whereas the other two had two double-bonds,  
50 nearby an oxo residue. CCK secretion induced by representative fatty acids  
51 (10-oxo-*trans*-11-18:1 and 13-oxo-*cis*-9,*cis*-15-18:2) was attenuated by a fatty  
52 acid-receptor GPR40 antagonist. Oral administration of 13-oxo-*cis*-9,*cis*-15-18:2 lowered  
53 the gastric emptying rate in rats in a dose- and structure-dependent manner.

54 **Conclusion**

55       These results revealed a novel fatty acid-sensing mechanism in enteroendocrine  
56 cells.

57

## 58 INTRODUCTION

59 Enteroendocrine systems have a critical role in maintaining whole body homeostasis,  
60 including gastrointestinal digestive and absorptive functions, nutrient metabolism, and  
61 feeding behavior in both pre- and postprandial states, through regulation of gut hormone  
62 secretions [1, 2]. Enteroendocrine cells sense luminal nutrients and then release a specific  
63 gut hormone. Macronutrients are potent stimulants for secretion of various gut hormones.

64 Cholecystokinin (CCK) is a gut hormone produced in enteroendocrine I cells, which  
65 are mainly located in the proximal small intestine [3, 4]. CCK has multiple physiological  
66 actions related to postprandial responses, such as induction of pancreatic enzyme  
67 secretion, and suppression of gastric emptying rate and appetite. Secretion of CCK is  
68 potently stimulated by luminal fatty acids and peptides. Dietary lipids (triglycerides) mainly  
69 consist of long chain fatty acids of more than 16- carbon chain length. Dietary fatty acids  
70 have a variety of structures based on their chain length, degree of unsaturation, position of  
71 double bond, and so on. The relationship between fatty acid structure and fatty  
72 acid-induced CCK secretion activity has only been partly elucidated. Previous in vitro and  
73 human studies revealed chain length-dependent mechanisms, in which fatty acids having  
74 12 carbon chain length or more trigger CCK secretion [5-8]. However, further structural  
75 features that determine the CCK-releasing activity of fatty acids have not been clarified.

76 Understanding the nutrient sensing mechanisms of enteroendocrine cells is  
77 fundamentally important in physiology, but the translational relevance of these mechanisms  
78 is also significant. If the oral administration of specific compounds has potent and selective  
79 stimulatory effects on target enteroendocrine cells, it will be possible to control  
80 physiological reactions, such as postprandial glycemia, lipidemia, and appetite by  
81 enhancing specific gut hormone secretion.

82 Recently, fatty acid derivatives (hydroxy- or oxo-fatty acids) produced by gut lactic  
83 acid bacteria (*Lactobacillus plantarum*) [9] have attracted a lot of attention for their  
84 favorable biological functions in both cell and animal models. Specific fatty acid derivatives  
85 can exert anti-inflammatory effects, cytoprotective effects, modify lipid/energy metabolism,  
86 and protect gut barrier function [9-15]. However, no current research is focused on the  
87 effect of those fatty acids on enteroendocrine function.

88 Fatty acid derivatives produced by gut lactic acid bacteria are a valuable tool to  
89 explore the relationship between the structure and activity of various fatty acids. In the  
90 present study, we aimed to reveal the structural features of fatty acids that directly and  
91 potently stimulate gut hormone secretion from enteroendocrine cells. CCK secretion was  
92 examined in response to more than 30 fatty acids in a murine CCK-producing  
93 enteroendocrine cell line, STC-1. We identified two specific structures responsible for  
94 potent CCK releasing activity in these fatty acids, and further investigated the cellular  
95 mechanism and in vivo effects in rats.

96

## 97 **MATERIAL AND METHODS**

### 98 **Materials**

99 Fatty acid metabolites produced by gut lactic acid bacteria [9] (Supporting Information  
100 Table S1) were provided by NITTO Pharmaceutical Industries, Ltd (Kyoto, Japan). All of  
101 fatty acids had more than 90% (mostly, more than 95%) purity (Table S1). Cell culture  
102 consumables (DMEM, fetal bovine serum, and penicillin/streptomycin) were purchased  
103 from Invitrogen (Carlsbad, CA, USA). Trypsin-EDTA solution, HEPES, and acetaminophen  
104 were purchased from Sigma (St. Louis, MO, USA). A GPR40 antagonist, GW1100, was  
105 purchased from Cayman Chemical (Ann Arbor, MI, USA), and a GPR120 antagonist,

106 AH7614, was purchased from Focus Biomolecules (Plymouth Meeting, PA, USA). Unless  
107 specified, all other reagents were purchased from Wako Pure Chemical Industries (Osaka,  
108 Japan).

109

110 CCK secretion study in STC-1 cells [16, 17]

111 STC-1 cells (a gift from Dr. Hanahan, University of California, San Francisco, CA)  
112 were grown in DMEM (Invitrogen, Cat. No. 12100-038) supplemented with 10% fetal  
113 bovine serum, 50 IU/mL penicillin, and 500 µg/mL of streptomycin. The cells were  
114 incubated in a humidified, 5% CO<sub>2</sub> atmosphere at 37°C. Cells were routinely subcultured by  
115 trypsinization upon reaching 80–90% confluency. Cells at passage numbers 20–40 were  
116 used for experiments.

117 STC-1 cells were seeded in 48-well culture plates at a density of  $1.25 \times 10^5$  cells/well  
118 and grown for 2–3 days until reaching 80–90% confluency. Cells were washed twice with  
119 HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>,  
120 10 mM D-glucose, and 0.1% BSA, pH 7.4) to remove the culture medium and then exposed  
121 to the test agents dissolved in HEPES buffer for 60 min at 37°C. Since the fatty acids were  
122 initially dissolved in ethanol, all test agents contained ethanol at a final concentration of  
123 0.1% (v/v). Following incubation, the supernatants were collected and centrifuged at  $800 \times$   
124 *g* for 5 min at 4°C to remove any cells. The supernatants were then stored at -50°C until the  
125 CCK concentration was measured using a commercially available enzyme immunoassay  
126 (EIA) kit (EK-069-04, Phoenix Pharmaceuticals Inc., Belmont, CA). The primary antiserum  
127 provided in this kit cross-reacts (100%) with sulfated and nonsulfated CCK (26–33),  
128 CCK-33 (porcine), caerulein, gastrin-1 (human), and big gastrin-1 (human). The antiserum  
129 also cross-reacts (12.6%) with CCK (30–33); however, there is 0% cross-reaction with

130 pancreatic polypeptide (human) and vasoactive intestinal peptide (including human,  
131 porcine, and rat). Because STC-1 cells do not express detectable levels of gastrin [5], we  
132 used the EIA kit to measure CCK. The coefficients of the intra- and interassay variation  
133 were <10% and <15%, respectively.

134

#### 135 Measurement of cytotoxicity in STC-1 cells

136 The cytotoxic effects of the fatty acids on STC-1 cells were determined by measuring  
137 the release of lactate dehydrogenase (LDH) from the cell into the supernatant. STC-1 cells  
138 were exposed to test agents, as described above. LDH was measured using a cytotoxicity  
139 detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.  
140 Cytotoxicity was calculated as the relative release (%) of LDH after exposure to the test  
141 agents compared to the total LDH (100%) released upon treatment with lysis reagent.

142

#### 143 Animals experiments

144 Male Sprague–Dawley rats (7-weeks-old) were purchased from Japan SLC  
145 (Hamamatsu, Japan). The experiments were performed in a temperature-controlled room  
146 maintained at  $23 \pm 2^{\circ}\text{C}$  with a 12 h light-dark cycle (8:00–20:00 light period). All animals  
147 had free access to water and were fed a semi-purified diet containing 25% casein, based  
148 on an AIN-93G diet [18], for 4–6 days as an acclimation period, and then divided into test  
149 groups based on body weight. Rats were fasted overnight the day before the experiment.  
150 The study was approved by the Hokkaido University Animal Ethics Committee, and the  
151 animals were maintained in accordance with the guidelines for care and use of laboratory  
152 animals at Hokkaido University (permission no. 14-0013).

153

154 Acetaminophen Test

155 Test agents were suspended in vehicle (saline containing 1.5% (w/v) carboxymethyl  
156 cellulose (CMC) and 1% (w/v) acetaminophen). Acetaminophen (100 mg/kg body weight)  
157 was added as an absorbable marker to assess the gastric emptying rate [19, 20]. A  
158 diunsaturated aldehyde, *trans,trans*-2,4-decadienal (2t,4t-decadienal, Tokyo Chemical  
159 Industry Co., Ltd., Tokyo, Japan) was employed as a positive control, as it has an inhibitory  
160 effect on gastric emptying in rats [21].

161 Test suspensions containing fatty acids or vehicle were orally administered at a dose  
162 of 10 mL/kg body weight through a feeding tube (5 Fr, Atom Medical Co., Tokyo, Japan).  
163 Tail vein blood samples (60  $\mu$ L) were collected prior to (0 min) and 15, 30, 45, 60, 90, and  
164 120 min after oral administration. Blood samples were immediately mixed with heparin  
165 (final concentration at 50 IU/mL, Nacalai Tesque, Inc., Kyoto, Japan) on ice. Plasma was  
166 separated from the blood by centrifugation at  $2300 \times g$  for 10 min at 4°C, and then frozen at  
167 -80°C until analysis. Plasma acetaminophen concentrations were measured using an  
168 acetaminophen detection kit (Kanto Chemical Co., Inc., Tokyo, Japan).

169

170 Phenol Red Test

171 The effects of fatty acids on gastric emptying rate were further assessed using an  
172 unabsorbable marker, phenol red [22, 23]. Phenol red (5 mg/kg body weight) was added to  
173 both test suspensions and vehicle controls. Fifteen minutes after oral administration, rats  
174 were euthanized by exsanguination under isoflurane anesthesia (MSD K.K., Tokyo, Japan).  
175 The stomach was removed after cramping both proximal and distal sites of the tissue. The  
176 stomach content was flushed twice with cold saline, and the washout solution was collected.  
177 The debris was removed by centrifugation at  $8400 \times g$  for 10 min at 4°C. After adding 1 N



178 NaOH to the supernatant (1/10 volume of the supernatant), the concentration of phenol red  
179 was measured spectrophotometrically at 560 nm. The gastric emptying rate was calculated  
180 as follows:

181

182 Gastric emptying rate (%)

183 =  $\frac{\{\text{the amount of phenol red administered (mg)} - \text{the amount of phenol red remaining in}$   
184  $\text{the stomach (mg)}\}}{\text{the amount of phenol red administered (mg)}} \times 100$

185

186 Statistical analysis

187 Results are expressed as mean  $\pm$  SEM. Significant differences among the control  
188 (vehicle) treatment and test groups were determined using Dunnett's post-hoc test ( $P <$   
189  $0.05$ ) as described in the figure legends.

190

## 191 RESULTS

192 The CCK releasing activity of 33 fatty acids was examined in STC-1 cells (Fig. 1A-D).  
193 Out of these, 7 had significant stimulatory effects on CCK release, especially  
194 10-oxo-trans-11-octadecenoic acid (10-oxo-11t-18:1) (Fig. 1A and C),  
195 13-oxo-cis-9,cis-15-octadecadienoic acid (13-oxo-9c,15c-18:2) (Fig. 1B and D),  
196 10-oxo-cis-6,cis-12-octadecadienoic acid (10-oxo-6c,12c-18:2) (Fig. 1D),  
197 10-oxo-cis-6,trans-11-octadecadienoic acid (10-oxo-6c,11t-18:2) (Fig. 1D), and  
198 10-oxo-trans-11,cis-15-octadecadienoic acid (10-oxo-11t,15c-18:2) (Fig. 1D). Out of these  
199 5 fatty acids, 13-oxo-9c,15c-18:2 and 10-oxo-6c,12c-18:2 had a common single oxo  
200 residue located between two *cis*-double bonds, while the remaining 3 fatty acids  
201 (10-oxo-11t-18:1, 10-oxo-6c,11t-18:2, 10-oxo-11t,15c-18:2) had a common

202 “ $\alpha,\beta$ ,-unsaturated carbonyl” or “enone” structure (Fig. 1E).

203 Both 10-oxo-11t-18:1 and 13-oxo-9c,15c-18:2 stimulated CCK secretion in a  
204 dose-dependent manner (Fig. 2A and B). A significant increment in CCK secretion was  
205 observed with  $\geq 100$   $\mu\text{M}$  of 10-oxo-11t-18:1, and  $\geq 50$   $\mu\text{M}$  of 13-oxo-9c,15c-18:2. Five fatty  
206 acids with potent CCK-releasing activity (Fig. 1) did not exert cytotoxic effects in STC-1  
207 cells, as evaluated by LDH release assay (Fig. 2C).

208 We examined the involvement of the major fatty acid receptors GPR40 [24, 25] and  
209 GPR120 [26] as potential sensors of 10-oxo-11t-18:1 and 13-oxo-9c,15c-18:2.  
210 Pretreatment with a GPR40 antagonist (GW1100) abolished CCK secretion induced by  
211 both fatty acids (Fig. 3A and B). In contrast, treatment with increasing concentrations  
212 (50-200  $\mu\text{M}$ ) of a GPR120 antagonist (AH7614) did not affect CCK secretory responses to  
213 both fatty acids (Fig. 3C).

214 The effect of oral administration of the oxo-fatty acids on gastric emptying rate was  
215 examined in vivo by oral co-administration of a test agent and an absorbable marker,  
216 acetaminophen (Fig. 4A, C, 5B), or a non-absorbable marker, phenol red (Fig. 4B). An  
217 unsaturated aldehyde 2t,4t-decadienal, used as a positive control [21], significantly  
218 reduced the appearance of acetaminophen in the peripheral vein (Fig. 4A) and the gastric  
219 emptying rate (Fig. 4B). At the same dose (25 mg/kg), 13-oxo-9c,15c-18:2 administration  
220 resulted in significantly lower acetaminophen levels after 15 min (Fig 4A), and lower gastric  
221 emptying rates (Fig. 4B), while 10-oxo-11t-18:1 administration had no effect compared to  
222 the control (vehicle) treatment. A dose-response study (Fig. 4C) confirmed the suppressive  
223 effect of oral 13-oxo-9c,15c-18:2 administration on gastric emptying rate, for doses of more  
224 than 23.6 mg/kg (80  $\mu\text{mol/kg}$ ).

225 The effect of 13-oxo-9c,15c-18:2 on CCK secretion in STC-1 cells and on gastric

226 emptying rate in rats were further compared with those of other fatty acids sharing partially  
227 common structures (12-oxo-9c-18:1 and 13-hydroxy-9c,15c-18:2). As shown in Fig. 5A,  
228 13-oxo-9c,15c-18:2 significantly increased CCK secretion in STC-1 cells, while other fatty  
229 acids had no effect. No stimulatory effects of 13-hydroxy-9c,15c-18:2 were reproduced (Fig.  
230 1B). Plasma acetaminophen concentrations were significantly lower in rats administered  
231 13-oxo-9c,15c-18:2 (160  $\mu\text{mol/kg}$  = 47 mg/kg), until 60 min after oral administration,  
232 compared to that of rats administered the vehicle only. Oral administrations of the same  
233 dose (160  $\mu\text{mol/kg}$  = 47 mg/kg) of 12-oxo-9c-18:1 or 13-hydroxy-9c,15c-18:2 did not affect  
234 the acetaminophen response observed in vehicle-treated rats.

235

## 236 **DISCUSSION**

237 Dietary fatty acids are known to be potent stimulants for CCK secretion. Chain  
238 length-dependent CCK secretion has been demonstrated previously both in in vivo and in  
239 vitro studies [5-8]. A previous study demonstrated differences in the CCK-releasing potency  
240 of various fatty acids [27], however, the structural features of fatty acids with potent  
241 CCK-releasing activity remain unclear. In the present study, using hydroxy- and oxo-fatty  
242 acids produced by gut lactic acid bacteria as metabolites, we identified two specific  
243 structures responsible for the potent CCK-releasing activity. The stimulatory effects of these  
244 fatty acids are mediated by a fatty acid receptor, GPR40. Further in vivo studies  
245 demonstrated that a single oral administration of a fatty acid possessing the specific  
246 structure exerted inhibitory effect on gastric emptying in rats. These findings revealed novel  
247 specific structure(s) in fatty acids that are responsible for stimulating CCK secretion in an  
248 enteroendocrine cell model, and for reducing gastric emptying rate.

249 In the present study, we tested more than 30 octadecanoic (C18) fatty acids for

250 CCK-releasing activity to elucidate any specific features involved in the activity. Interestingly,  
251 most of the fatty acids tested had almost no effect on stimulating CCK secretion. The  
252 degree of unsaturation, positions of double bonds, cis/trans orientation, conjugation, and  
253 positions of the hydroxy- or oxo-group are not apparently linked to CCK-releasing activity.  
254 Overall, the oxo-fatty acids had more potent activity than the hydroxy-fatty acids, and 5  
255 oxo-fatty acids had an apparent stimulatory effect at the test concentration of 100  $\mu$ M  
256 without cytotoxic effects (Fig. 2C). These results indicate that fatty acids possessing these  
257 specific structures (Fig. 1E) have potent CCK releasing activity in STC-1 cells. Apart from  
258 these potently stimulatory fatty acids, 10-oxo-6c-18:1 and 10-hydroxy,13-hydroxy-6c-18:1  
259 had mild stimulatory effects (Fig. 1C), suggesting that there are other factors involved in  
260 their CCK-releasing activity. There are several limitations in STC-1 cells. The cell line has  
261 different properties from native mouse intestinal CCK cells [28], and co-produces other gut  
262 hormones such as GLP-1 and GIP [29]. Thus, the CCK-releasing activity of certain material  
263 found in STC-1 cells is not necessarily observed in animal/human study after oral  
264 administration. However, the cell line is still useful model for studying nutrient sensing  
265 mechanism in enteroendocrine cells.

266 The fatty acids tested in the present study are not generally recognized as dietary  
267 fatty acids because they are produced as metabolites by gut lactic acid bacteria [9]. Some  
268 of fatty acid metabolites were detected in the intestinal tissue and plasma of mice [9], and  
269 *Lactobacillus plantarum* could survive in the intestine, but the concentrations of fatty acid  
270 metabolites (10-5000 pg/100 mg or pg/100  $\mu$ L range) were apparently lower than the doses  
271 used in the present study. The structures identified in the present study would therefore not  
272 be directly related to CCK secretion in response to dietary fat ingestion. However, our  
273 findings provide a novel insight into the chemical sensing mechanisms in enteroendocrine

274 cells. These structures could be utilized as key molecular features for stimulating gut  
275 hormone secretion.

276 The stimulatory effect of 10-oxo-11t-18:1 and 13-oxo-9c,15c-18:2 were abolished by a  
277 GPR40 antagonist (Fig. 3A and B), but not by a GPR120 antagonist (Fig. 3C), suggesting  
278 that the CCK release is preferably mediated by GPR40 (Fig. 1E). GPR40 and GPR120 are  
279 well known as receptors for medium-long chain fatty acids [30, 31]. Except carbon chain  
280 length dependency, the structure-activity relationships of fatty acid properties such as  
281 degree of unsaturation, position or cis-trans isomerism of double bond(s) have not been  
282 established. Although various GPR40 agonists has been developed [32, 33], none of them  
283 possess the structures found in the present study. The present study revealed two distinct  
284 structures related to potent activation of GPR40-mediated CCK secretion in  
285 enteroendocrine cells. A recent study has found that fatty acids with an  $\alpha,\beta$ -unsaturated  
286 carbonyl (enone) structure have an anti-inflammatory effect in adipocytes [15]. The  
287 possible involvement of peroxisome proliferator-activated receptors and GPCRs were  
288 discussed, but the involvement of GPR40 was not demonstrated.

289 A previous paper [11] demonstrated that 10-hydroxy-12c-18:1 protected the tight  
290 junction barrier via GPR40 in Caco2 cells. In the present study, we found that CCK  
291 secretion was not affected by the fatty acid (Fig. 1A), suggesting that the fatty acid requires  
292 other factors (time, intracellular signal transduction pathway, etc.) in a specific cellular  
293 response for the observed activity through GPR40.

294 We expected that the fatty acids would exert an inhibitory effect on gastric emptying in  
295 vivo, as delaying gastric emptying is a distinct physiological function of several gut  
296 hormones including CCK [3, 4, 34]. Although the effect was relatively smaller than that of  
297 2t,4t-dacadienal [21] at the same weight dose (25 mg/kg), the oral administration of

298 13-oxo-9c,15c-18:2 decreased the gastric emptying rate as evaluated by two independent  
299 methods, while 10-oxo-11t-18:1 did not (Fig. 4A and B). Because the fatty acids were orally  
300 administered, the failure of 10-oxo-11t-18:1 to inhibit gastric emptying could be due to  
301 insufficient delivery of active form fatty acids to the site of action (possibly the small  
302 intestinal lumen). It is possible that the fatty acid was rapidly degraded or absorbed in the  
303 stomach or intestinal lumen, or adhered to the stomach mucosa. However, based on our  
304 results it appears that 13-oxo-9c,15c-18:2 successfully reach the small intestinal lumen  
305 following oral administration, resulting in a reduced gastric emptying rate possibly through  
306 stimulation of CCK-producing enteroendocrine cells. Further studies are needed in the  
307 future to investigate which factors (CCK and/or other gut hormones, such as GLP-1, PYY,  
308 serotonin, etc.) are involved in the effect of 13-oxo-9c,15c-18:2 in vivo. In addition, the  
309 nutritional properties of these modified fatty acids, including their digestion, absorption, and  
310 metabolism, have not yet been characterized. Such information would help to explain the  
311 differences in the observed results between in vitro and in vivo experiments.

312 In the case of 13-oxo-9c,15c-18:2, the oxo-group is essential for the CCK releasing  
313 and gastric emptying inhibitory effect (Fig. 5), since 13-hydroxy-9c,15c-18:2 had no effect.  
314 This result suggests that fatty acids without in vitro activity consistently have no effect on  
315 gastric emptying rate in vivo. It is interesting that the in vitro CCK-releasing activity  
316 correlates well with the observed in vivo gastric inhibiting effect.

317 Recent studies have revealed the physiological functions of fatty acid metabolites  
318 produced by gut lactic acid bacteria [9-15]. Some of these metabolites were found in the  
319 mouse colon [9], as gut microbiota is located primarily in the distal intestine. Continuous  
320 ingestion of substrate fatty acids for gut lactic acid bacteria may provide beneficial effects  
321 through increased gut hormone secretion induced by specific luminal fatty acids, such as

322 13-oxo-9c,15c-18:2 and 10-oxo-11t-18:1. However, the efficacy of digestion or amount of  
323 metabolic products produced in the gut is not yet predicable. Therefore, oral administration  
324 of fatty acids synthesized in vitro rather than a dietary co-supplementation of lactic acid  
325 bacteria and substrate fatty acids could be applicable for reducing glycemia through  
326 suppression of gastric emptying [35], and for reducing appetite through increased secretion  
327 of gut hormones including CCK, peptide-YY, and glucagon-like peptide-1.

328 In conclusion, by examining various fatty acids produced by gut lactic acid bacteria,  
329 we have identified two specific structures responsible for potent CCK secretion in  
330 enteroendocrine cells. The stimulation of CCK secretion was probably mediated by a fatty  
331 acid receptor, GPR40. A fatty acid with the specific structure exerted an inhibitory effect on  
332 gastric emptying after an oral administration in rats. These results revealed a novel fatty  
333 acid-sensing mechanism in enteroendocrine cells.

334

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442

443 **Author contributions**

444 SO, AY, MK, XL and TH performed the experiments;

445 TH, SN, MS, TY, and HH designed and conceived the experiments;

446 SN, MS, TY, SK and JO provided the materials.

447

448 **Conflict of interest**

449 Saki Nishimura, Masayoshi Sakaino, and Takatoshi Yamashita are employee of J-Oil

450 Mills, Inc.

451

452 **Figure legends**

453 **Fig. 1.**

454 CCK releasing activities of various fatty acids derived from lactic acid bacteria (A-D), and  
455 common structures identified in oxo-fatty acids with potent CCK-releasing activity (E).  
456 STC-1 cells were exposed to fatty acids (100  $\mu$ M) for 60 min at 37°C. The concentration of  
457 CCK in the supernatant was measured using a commercial CCK-ELISA kit. Values are  
458 expressed as means and SEM (n=3-4). Plus (+) signs indicate significant differences  
459 compared to the vehicle treatment ( $P < 0.05$  by Dunnett's test).

460

461 **Fig. 2.**

462 Dose-response effects of 10-oxo-11t-18:1 and 13-oxo-9c,15c-18:2 on CCK secretion (A, B),  
463 and cytotoxic effects of fatty acids with potent CCK-releasing activity (C).

464 STC-1 cells were exposed to various concentrations of 10-oxo-11t-18:1 or  
465 13-oxo-9c,15c-18:2 for 60 min at 37°C, and CCK concentrations in the supernatant were  
466 measured (A, B). Values are expressed as means and SEM (n=3-4). Plus (+) signs indicate  
467 significant differences compared to the vehicle treatment ( $P < 0.05$  by Dunnett's test). LDH  
468 activity in the supernatant was measure after 60 min exposure to fatty acids (100  $\mu$ M). For  
469 the total LDH activity control, a lysis reagent was used to release all intracellular LDH. The  
470 values represent LDH activity (%) relative to the total LDH control and are expressed as  
471 means with SEM of cells in three wells.

472

473

474

**Fig. 3.**

475 Effects of GPR40 or GPR120 antagonists on CCK secretion induced by 10-oxo-11t-18:1  
476 and 13-oxo-9c,15c-18:2.

477 STC-1 cells were pre-treated with a GPR40 antagonist (GW1100) (A and B) or a GPR120  
478 antagonist (AH7614) (C) or its vehicle (0.1% DMSO) for 30 min, then exposed to  
479 10-oxo-11t-18:1 (100  $\mu$ M) or 13-oxo-9c,15c-18:2 (100  $\mu$ M) for 60 min. Values are  
480 expressed as means and SEM (n=3-4). Plus (+) signs indicate a significant difference  
481 compared to the vehicle without antagonist ( $P < 0.05$  by Dunnett's test). 'NS' represents no  
482 significant differences within 10-oxo-11t-18:1- or 13-oxo-9c,15c-18:2-treated cells,  
483 respectively.

484

485

**Fig. 4.**

486 The effects of oral administration of 10-oxo-11t-18:1 and 13-oxo-9c,15c-18:2 on gastric  
487 emptying rate in rats.

488 After overnight fasting, 25 mg/kg of 10-oxo-11t-18:1 (84.9  $\mu$ mol/kg), 13-oxo-9c,15c-18:2  
489 (84.3  $\mu$ mol/kg), or 2t,4t-decadienal (164.2  $\mu$ mol/kg) was orally administered with  
490 acetaminophen (A) or with phenol red (B). Blood samples were collected from the tail vein  
491 up to 120 min later, and plasma acetaminophen concentrations were measured (A).  
492 Different doses (40, 80 and 160  $\mu$ mol/kg) of 13-oxo-9c,15c-18:2 were examined using the  
493 same experimental method (C). In a separate experiment, gastric contents were collected  
494 15 min after oral co-administration of test agents and phenol red (B). The gastric emptying  
495 rate was calculated based on the amount of phenol red remaining in the stomach lumen.  
496 Values are expressed as means and SEM (n=4-6). Asterisks (\*) indicate a significant  
497 difference compared to the vehicle treatment (B) at each time point (A, C) ( $P < 0.05$  by

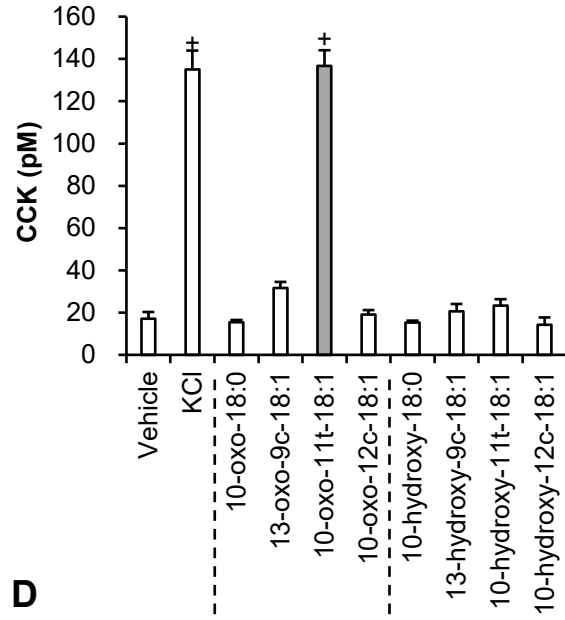
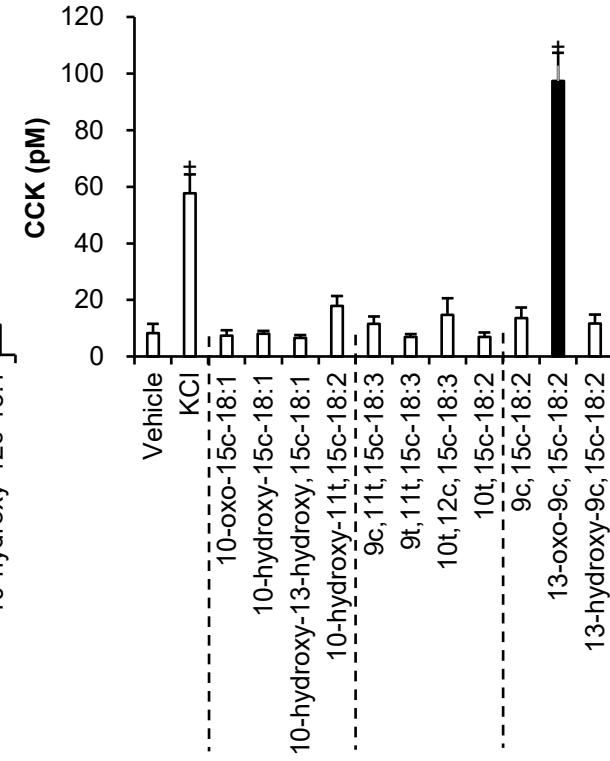
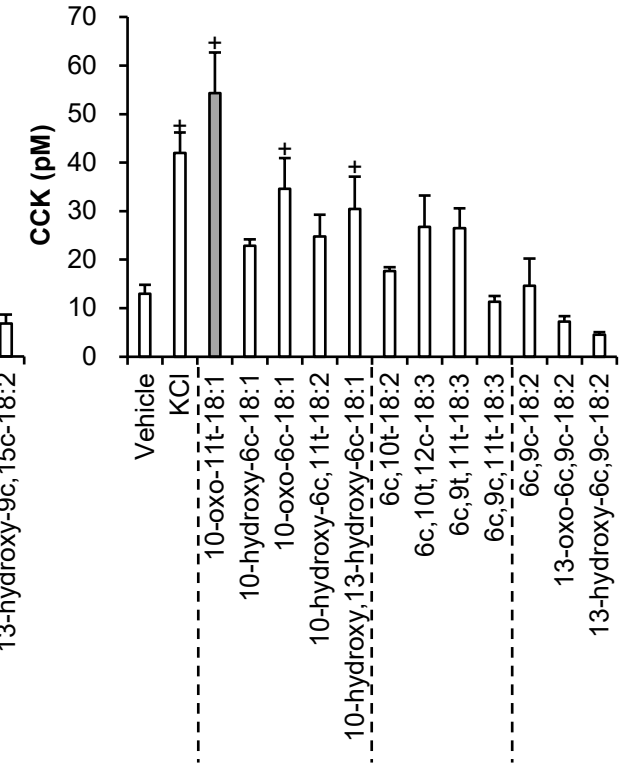
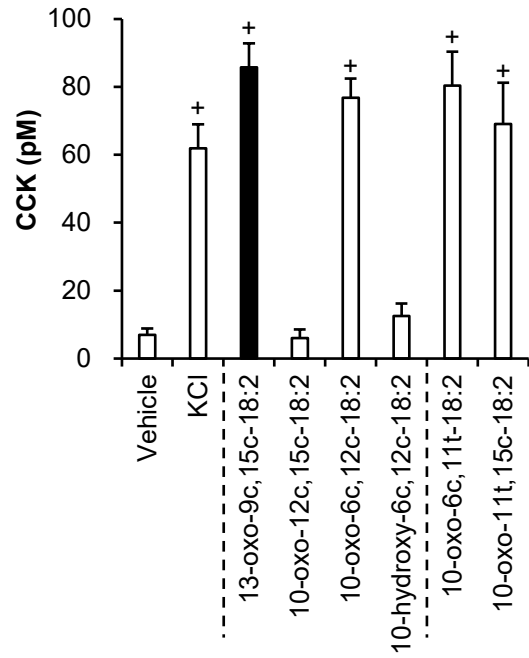
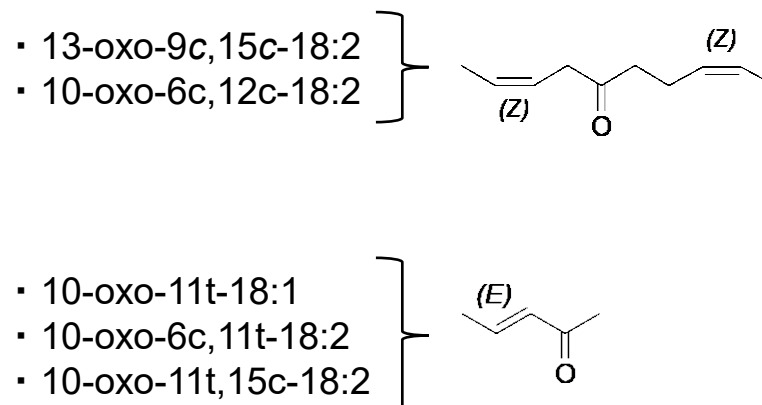
498 Dunnett's test).

499

500 **Fig. 5.**

501 CCK secretion in STC-1 cells and gastric emptying rate in rats in response to oral  
502 administration of 13-oxo-9c,15c-18:2 and structurally related fatty acids.

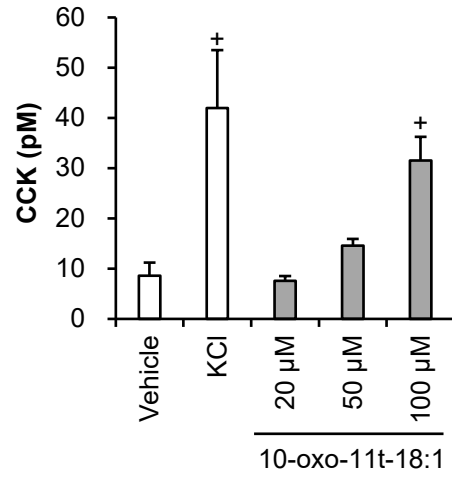
503 STC-1 cells were exposed 100  $\mu$ M of fatty acids (13-oxo-9c,15c-18:2, 12-oxo-9c-18:1 or  
504 13-hydroxy-9c,15c-18:2) for 60 min, and the concentrations in the supernatant were  
505 measured (A). Values are expressed as means and SEM (n=3-4). Plus (+) signs indicate  
506 significant differences in concentration compared to the vehicle treatment ( $P < 0.05$  by  
507 Dunnett's test). Fatty acids (160  $\mu$ mol/kg) or vehicle were orally administered to rats, and  
508 blood samples were collected from the tail vein. Acetaminophen concentrations were  
509 measured in the plasma. Values are expressed as mean and SEM (n=5-6). Asterisks (\*)  
510 indicate significant differences in concentration compared to the vehicle treatment at each  
511 time point ( $P < 0.05$  by Dunnett's test).

**Figure 1****A****B****C****D****E**

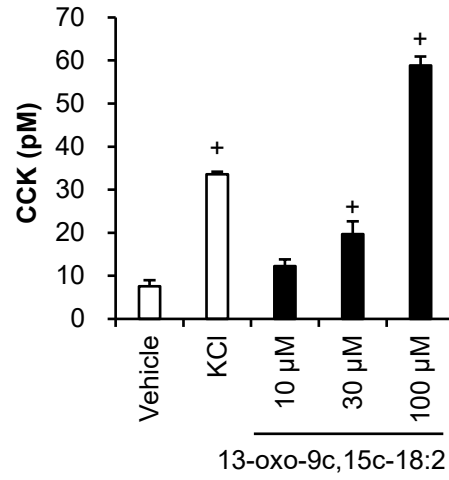


**Figure 2**

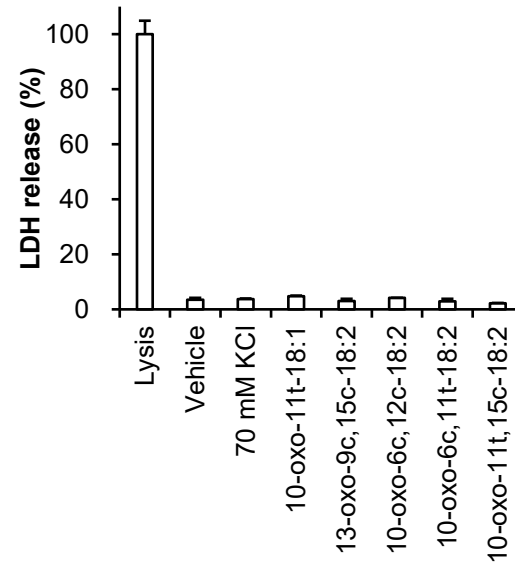
**A**



**B**

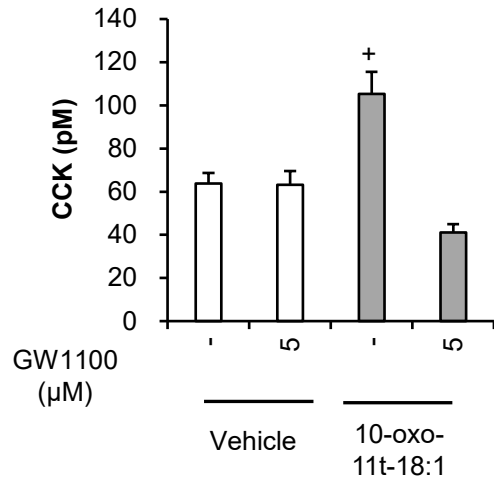


**C**

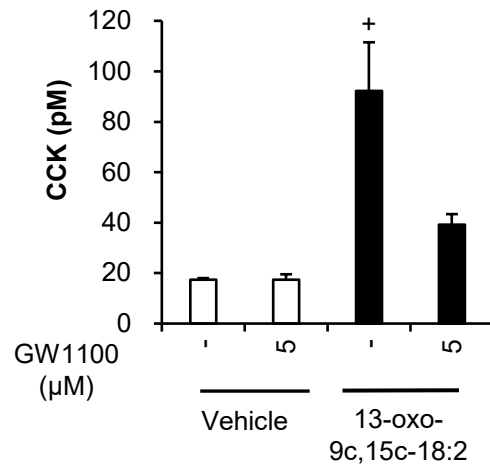


# Figure 3

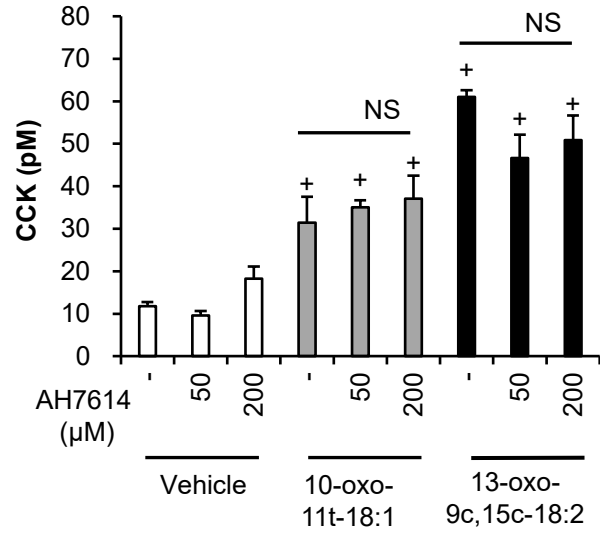
## A



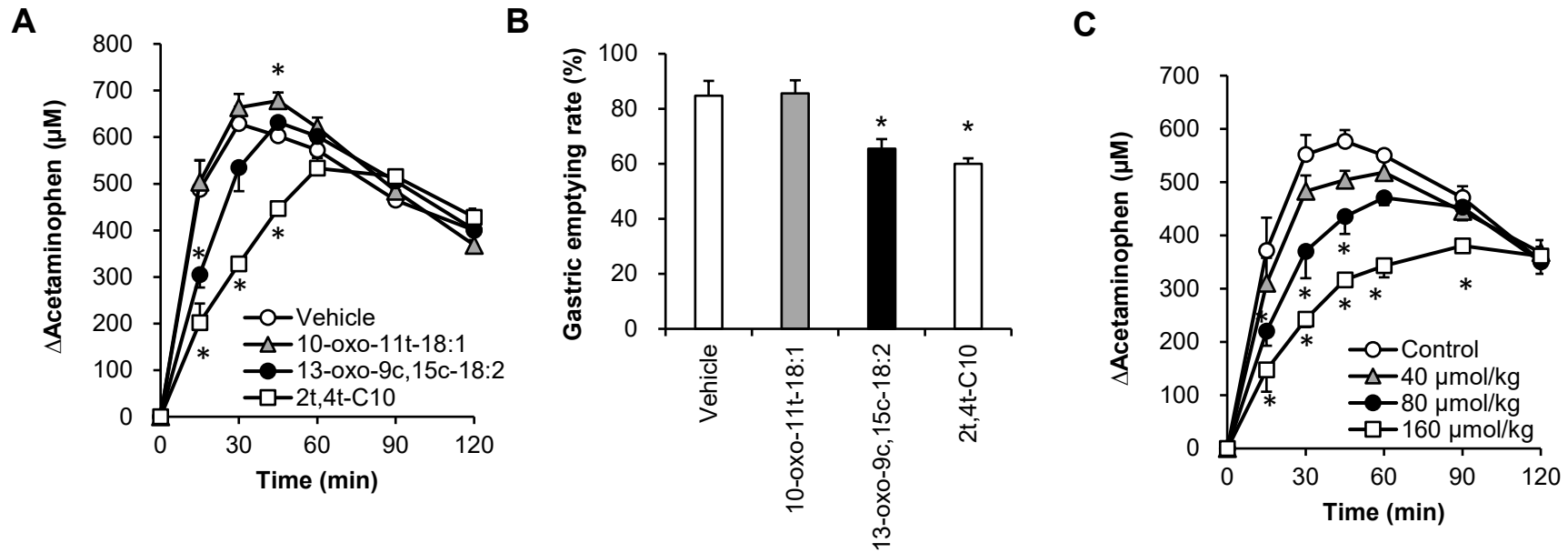
## B



## C

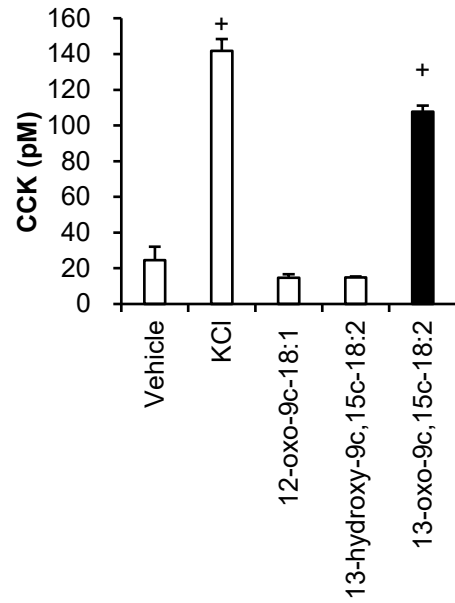


**Figure 4**

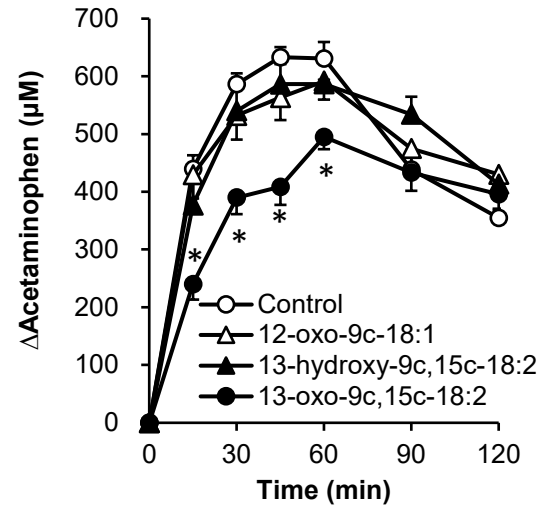


**Figure 5**

**A**



**B**



# Graphic abstract

