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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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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 <u>Scope</u>

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

42 Methods and results

We examined CCK secretory activities in a murine CCK-producing cell line STC-1 by exposing the cells to various modified fatty acids produced by gut lactic acid bacteria. The effects of fatty acids on gastric emptying rate as a CCK-mediated function were examined 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 enteroendocrine56 cells.

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.

Recently, fatty acid derivatives (hydroxy- or oxo-fatty acids) produced by gut lactic
acid bacteria (*Lactobacillus plantarum*) [9] have attracted a lot of attention for their
favorable biological functions in both cell and animal models. Specific fatty acid derivatives
can exert anti-inflammatory effects, cytoprotective effects, modify lipid/energy metabolism,
and protect gut barrier function [9-15]. However, no current research is focused on the
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 enteroendocrine cell line, STC-1. We identified two specific structures responsible for 93 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

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

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

109

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

STC-1 cells (a gift from Dr. Hanahan, University of California, San Francisco, CA) were grown in DMEM (Invitrogen, Cat. No. 12100–038) supplemented with 10% fetal bovine serum, 50 IU/mL penicillin, and 500 µg/mL of streptomycin. The cells were incubated in a humidified, 5% CO₂ atmosphere at 37°C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluency. Cells at passage numbers 20–40 were used for experiments.

117 STC-1 cells were seeded in 48-well culture plates at a density of 1.25 × 10⁵ 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 CaCl₂, 1.2 mM MgCl₂, 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 × 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

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

134

135 Measurement of cytotoxicity in STC-1 cells

The cytotoxic effects of the fatty acids on STC-1 cells were determined by measuring the release of lactate dehydrogenase (LDH) from the cell into the supernatant. STC-1 cells were exposed to test agents, as described above. LDH was measured using a cytotoxicity detection kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Cytotoxicity was calculated as the relative release (%) of LDH after exposure to the test 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 ± 2°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

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

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

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

181

182 Gastric emptying rate (%)

183 = [{the amount of phenol red administrated (mg) – the amount of phenol red remaining in

184 the stomach (mg)}/the amount of phenol red administrated (mg)] x 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 (10-0x0-6c, 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 common а

202 " α , β ,-unsaturated carbonyl" or "enone" structure (Fig. 1E).

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

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

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 μ 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 µmol/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 µM 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 µL 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 gut275 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 α , β ,-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

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

320 ingestion of substrate fatty acids for gut lactic acid bacteria may provide beneficial effects

mouse colon [9], as gut microbiota is located primarily in the distal intestine. Continuous

319

321 through increased gut hormone secretion induced by specific luminal fatty acids, such as

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

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

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.

452 Figure legends

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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 μ 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.

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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 µM). 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

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 μ M) or 13-oxo-9c,15c-18:2 (100 μ 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 µmol/kg), 13-oxo-9c,15c-18:2 489 (84.3 µmol/kg), or 2t,4t-decadienal (164.2 µmol/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 µmol/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 µM 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 µmol/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).











Graphic abstract

