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1 **Title**

2 Zinc directly stimulates cholecystokinin secretion from enteroendocrine cells and
3 reduces gastric emptying in rats

4

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21 **Statement of Author's Contributions to Manuscript**

22 S. N., T. H., H.I. and H. H. designed research; S.N. and H. I. conducted research and
23 analyzed data; S.N. and T. H. wrote the paper. T. H. had primary responsibility for
24 final content. All authors read and approved the final manuscript.

25

26 **Abstract**

27 Zinc, an essential mineral element, regulates various physiological functions such as
28 immune responses and hormone secretion. Cholecystokinin (CCK), a gut hormone, has
29 a role in protective immunity through the regulation of gastrointestinal motility, appetite,
30 and inflammatory response. Here, we examined the effect of zinc on CCK secretion in
31 STC-1 cells, an enteroendocrine cell line derived from murine duodenum, and in rats.
32 Extracellular zinc triggered CCK secretion accompanied with increased intracellular
33 Ca^{2+} and Zn^{2+} mobilization in STC-1 cells. Zinc-induced CCK secretion was abolished
34 in the absence of intracellular Zn^{2+} or extracellular calcium. Upon inhibition of transient
35 receptor potential ankyrin 1 (TRPA1), extracellular zinc failed to increase intracellular
36 Ca^{2+} and subsequent CCK secretion. In rats, oral zinc administration decreased gastric
37 emptying through the activation of CCK signaling. These results suggest that zinc is a
38 novel stimulant for CCK secretion through the activation of TRPA1 related to
39 intracellular Zn^{2+} and Ca^{2+} mobilization.

40

41 **Keywords**

42 cholecystokinin, zinc, gastric emptying, transient receptor potential ankyrin 1 (TRPA1)

43 **1. Introduction**

44 The secretion of cholecystokinin (CCK) is triggered by dietary components through
45 the activation of specific receptors (Psichas et al., 2015). Intracellular second
46 messengers such as calcium (Ca²⁺) and cyclic adenosine mono phosphate mediate these
47 stimulus-induced CCK secretions (Psichas et al., 2015). It is well known that secreted
48 CCK is recognized by CCK receptors (CCK1R and CCK2R) to regulate gastrointestinal
49 motility, food intake suppression, and pancreatic enzyme secretion (Chandra and Liddle,
50 2009; Dockray, 2009). In addition, the activation of CCK receptors inhibits the
51 inflammatory response via vagus nerve and nicotinic receptor signaling (Luyer et al.,
52 2005). Further, CCK blocks lipopolysaccharide-induced proinflammatory cytokine
53 production in pulmonary interstitial macrophages (Li et al., 2007), suggesting the
54 contribution of CCK in protective immunity during food or unexpected toxicant
55 ingestion.

56 Zinc is an essential mineral element regulating various physiological functions such
57 as enzyme activity, protein synthesis, neuronal activity, and immune function (Haase
58 and Rink, 2014; Hagemeyer et al., 2015; McClung et al., 2007). In addition, cytoplasmic
59 and vesicular zinc in pancreatic beta cells play an important role in insulin secretion and
60 sensitivity in response to increased glucose (Bellomo et al., 2011; Chimienti et al.,
61 2006). Decreased zinc uptake of beta cells in *Slc30a8* (zinc transporter 8)-deficient mice
62 causes impaired glucose homeostasis and insulin secretion (Nicolson et al., 2009).

63 Transient receptor potential (TRP) ankyrin 1 (TRPA1), a member of the TRP family,
64 was identified as a zinc sensor in sensory neurons and is related to nocifensive behavior
65 of mice (Hu et al., 2009). Interestingly, TRPA1 is abundantly expressed in
66 CCK-producing cells, and is involved in CCK secretion induced by peroxidized lipids
67 and pungent component such as unsaturated aldehydes and allyl isothiocyanate (AITC),
68 respectively (Cho et al., 2014; Nakajima et al., 2014; Purhonen et al., 2008). Among
69 mineral elements, the role of extracellular calcium on CCK secretion has been
70 elucidated (Macleod, 2013; Nakajima et al., 2012); however, it is still unclear whether
71 zinc has ability for stimulating CCK release through TRPA1 in CCK-producing cells.

72 Therefore, in the present study, we examined whether zinc shows CCK-releasing
73 activity through the activation of TRPA1 in the murine duodenal enteroendocrine cell
74 line, STC-1. Further, we tested the effect of single oral zinc administration on gastric
75 emptying that is controlled by CCK in rats.

76

77 **2. Materials and Methods**

78 *2.1 Cell culture*

79 STC-1 cells (derived from murine duodenum, a gift from Dr. Hanahan, University
80 of California, San Francisco, CA) and GLUTag cells (derived from murine colon, a gift
81 from Dr. D. J. Drucker, University of Toronto, Canada) were grown in Dulbecco's
82 modified Eagle's medium (Thermo scientific, IL, USA) supplemented with 10% fetal
83 bovine serum, 50 IU/ml penicillin, and 500 µg/ml streptomycin. The cells were cultured
84 in a humidified 5% CO₂ atmosphere at 37°C and were routinely subcultured with
85 trypsinization until subconfluence (80-90% in a T75 flask).

86

87 *2.2 Measurement of CCK secretion from STC-1 cells*

88 STC-1 cells were grown in 48-well plates for 2–3 days until they reached 80–90%
89 confluence. Before exposure to test agents, the cells were washed twice with HEPES
90 buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM CaCl₂, 1.2 mM MgCl₂,
91 10 mM D-glucose, and 0.1% BSA; pH 7.4) to remove the medium. The cells were
92 incubated with ZnCl₂, CaCl₂, CuCl₂, and CdCl₂ (all from Wako Pure Chemical
93 Industries, Osaka, Japan) for 60 min at 37°C. β-conglycinin peptone (βconP), a **peptic**
94 **hydrolysate of soy protein**, was used as positive control for CCK secretion as reported
95 previously (Nakajima et al., 2012; Nishi et al., 2003). **All test substances were dissolved**
96 **in HEPES buffer. The cells were treated with** TRPA1 antagonist (HC-030031;
97 Sigma-Aldrich, MO, USA), MEK inhibitor (U0126; Merck Millipore, MA, USA) **or**
98 **zinc chelator** {*N,N,N,N*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN); Dojindo
99 Laboratories, Kumamoto, Japan} **for 20 min before exposing to the test substances.**
100 After the incubation, the supernatants were collected and remaining cells were removed
101 by centrifugation at 800 × g for 5 min at 4°C. The CCK concentration in the
102 supernatants was measured using a commercial enzyme immunoassay (EIA) kit
103 (Phoenix Pharmaceuticals, Belmont, CA) according to the manufacture's instructions.
104 The primary antiserum provided in this kit cross-reacts 100% with sulfated and
105 non-sulfated CCK (26-33), CCK-33 (porcine), caerulein, gastrin-1 (human), and big
106 gastrin-1 (human). The antiserum also cross-reacts 12.6% with CCK (30-33) and 0%
107 with pancreatic polypeptide (human) and vasoactive intestinal peptide (human, porcine,
108 and rat). Although the antibody cross-reacts with gastrin, we selected this EIA kit
109 because gastrin is not expressed at a detectable level in STC-1 cells (McLaughlin et al.
110 1999). The coefficients of intra- and inter-assay variation were < 5% and < 14%,
111 respectively.

112

113 *2.3 Measurement of cytotoxicity in STC-1 cells*

114 Release of lactate dehydrogenase (LDH) from STC-1 cells was measured to

115 evaluate the cytotoxic effect of the test agents. After exposure of the cells to the test
116 agents for 60 min, the supernatants were collected and centrifuged as described above.
117 The concentration of LDH was measured using a cytotoxicity detection kit (Roche,
118 Basel, Switzerland) following the manufacture's manual. The cytotoxic effect was
119 calculated as the relative LDH release (%) after exposure to the test agents as compared
120 to total LDH (set as 100%) released by treatment with lysis reagent.

121

122 *2.4 Intracellular Ca²⁺ and Zn²⁺ imaging*

123 A total of 3×10^5 cells were grown on 0.025% poly-L-lysine-coated coverslips (1.3
124 cm²) in 24-well plates for 24–48 h after seeding. The cytoplasmic Ca²⁺ and Zn²⁺ in the
125 cells were determined with a Ca²⁺ indicator (Fura-2-AM; Thermo scientific) and a Zn²⁺
126 indicator (Fluozin-3-AM; Thermo scientific) dissolved in HEPES buffer containing
127 0.005% Pluronic F-127 (Sigma-Aldrich), respectively. **Fluozin-3 has been demonstrated**
128 **to be specific to Zn²⁺ compared to other divalent cations (Devinney et al., 2005).** After
129 loading the ion indicator for 20 min, the coverslip was washed with HEPES buffer and
130 mounted into the coverslip folder to set onto the stage of microscope. For Ca²⁺
131 measurement, the fluorescence intensity was measured at an emission wavelength of
132 510 nm, and excitation wavelengths of 340 and 380 nm. For Zn²⁺ measurement, the
133 fluorescence intensity was measured at an emission wavelength of 494 nm, and an
134 excitation wavelength of 516 nm. Images were captured at 10 -s intervals using a video
135 image analysis system consisting of a 12 -bit C7780-22 ORCA3CCD camera
136 (Hamamatsu Photonics, Hamamatsu, Japan) and a fluorescence microscope (IX71;
137 Olympus, Tokyo, Japan). Images were analyzed with AquaCosmos software
138 (Hamamatsu Photonics). After stabilization of basal fluorescence, the cells were
139 exposed to the test agents. The data were expressed as changes in fluorescence intensity
140 from basal levels before exposure to the test agents (0 min) in 20–30 individual cells
141 from 3–4 independent cultures. The cell viability was assessed by exposing cells to 70
142 mM KCl after a challenge with the test agents.

143

144 *2.5 Animals*

145 Male Sprague-Dawley rats (7 -weeks old) were purchased from Japan SLC
146 (Hamamatsu, Japan). The diet consisted of 250 g/kg casein, 602.5 g/kg sucrose, 50 g/kg
147 soybean oil, 50 g/kg cellulose, 35 g/kg mineral mixture (AIN-93G), 10 g/kg vitamin
148 mixture (AIN-93G), and 2.5 g/kg choline bitartrate. The experiments were performed in
149 a temperature-controlled room maintained at $23 \pm 2^\circ\text{C}$ with a 12-h light-dark cycle
150 (8:00–20:00, light period). The rats were able to access the diet, except during the

151 gastric emptying test, and they had free access to water throughout the experiment. The
152 study was approved by the Hokkaido University Animal Committee, and the animals
153 were maintained in accordance with the guidelines for the care and use of laboratory
154 animals of Hokkaido University.

155

156 2.6 Acetaminophen test

157 Overnight-fasted rats were orally administered ZnCl₂ at 0.5 mg/kg body weight or
158 its vehicle (8 ml/kg body weight, saline) with acetaminophen (100 mg/kg body weight).
159 Blood (60 µL) was collected from the tail vein into 1.5 -ml tube containing 1.5 U/tube
160 heparin at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min after the administration of the
161 test solution. The blood samples were centrifuged at 2,300 × g for 10 min at 4°C, and
162 the supernatants were collected. The acetaminophen concentration was measured with
163 an acetaminophen detection kit (Kanto Chemical Co. Inc., Tokyo, Japan) according to
164 the manufacturer's protocol with some modification. Briefly, plasma (5 µl) was mixed
165 with Reagent A (25 µl) followed by centrifugation (8,400 × g, 2 min). The supernatant
166 (20 µl) was transferred to fresh tube, and then the sample was boiled for 10 min. After
167 addition of Reagent B (50 µl), the concentration of acetaminophen was calculated based
168 on spectrophotometrically measurement at 600 nm.

169

170 2.7 Measurements of gastric emptying rate and portal CCK level

171 Rats were orally administered 0.5 mg/kg zinc or 8 ml/kg body weight saline at 15
172 min after intraperitoneal injection of devazepide (0.5 mg/kg body weight, ML
173 Laboratories, Liverpool, UK) or its vehicle (0.5 ml/kg body weight, 10% DMSO and
174 10% Tween-80 in saline). Phenol red (5 mg/kg body weight) was orally administered to
175 rats immediately after zinc or vehicle administration as a non-absorbable marker
176 (Feldman and Gibaldi, 1968). Rats were anesthetized with isoflurane (Pfizer Inc., NY,
177 USA) at 15 min after phenol-red administration. Portal vein blood (1 mL) was collected
178 into a syringe containing aprotinin (540 KIU/ml) / heparin (50 IU/ml). The blood
179 samples were centrifuged at 2,300 × g for 10 min at 4°C, and then the supernatants were
180 collected into fresh tubes. The zinc concentration in the portal blood was determined
181 using a Zn test (Wako Pure Chemical Industries).

182 The gastric content including phenol red was collected into a 50 -mL tube by
183 flushing with cold saline. The content was centrifuged at 8,400 × g for 10 min at 4°C
184 and the supernatant was collected and adjusted to 10 ml with saline. After adding 1 N
185 NaOH to the supernatant (1/30 volume of the supernatant), the concentration of phenol
186 red was measured spectrophotometrically at 560 nm. The gastric emptying rate (%) was

187 calculated as follows:

188 Gastric emptying rate (%) = [$\{$ the amount of phenol red administered (mg) – the
189 amount of phenol red remained in the stomach (mg) $\}$ / the amount of phenol red
190 administered (mg)] \times 100

191 To measure CCK in portal plasma, the plasma was pretreated as reported previously
192 (Rehfeld, 1998). Briefly, two volumes of 99.5% EtOH were added to one volume of
193 plasma. The mixture was centrifuged at $9,300 \times g$ for 10 min at 4°C followed by
194 incubation on ice for 30 min. The supernatant was transferred to a new tube, and
195 evaporated with a centrifugal vacuum concentrator. The dried concentrate was dissolved
196 in one volume of ELISA buffer. The plasma CCK concentration was measured with an
197 EIA kit (Phoenix Pharmaceuticals). The primary antiserum cross-reacts 100% with
198 sulfated and non-sulfated CCK (26-33), CCK-33 (porcine), CCK (27-33), caerulein,
199 gastrin-1 (human) and big gastrin-1 (human), and this antiserum cross-reacts 12.8%
200 with CCK (30-33). The coefficients of intra- and inter-assay variation were within
201 5–10% and <15%, respectively. Because the primary antibody cross-reacts with gastrin,
202 the result was expressed as CCK/gastrin.

203

204 *2.8 Statistical analysis*

205 Results are expressed as the mean \pm SEM. Statistical significance was assessed
206 using one-way or two-way ANOVA and significant differences were determined by
207 Tukey's *post-hoc* test or Student's *t*-test as indicated in each figure legend. The Pearson
208 product-moment correlation coefficient was determined, $P < 0.05$ was considered
209 significant.

210

211 **3. Results**

212 *3.1 Extracellular zinc triggers CCK release in STC-1 cells*

213 Extracellular zinc application resulted in significantly increased CCK secretion from
214 STC-1 cells, especially, 100 μ M ZnCl₂ showed the highest CCK-releasing activity
215 among the various concentrations tested (Fig. 1A). The CCK secretion induced by 100
216 μ M ZnCl₂ was much higher than that induced by β conP. The peptone has potent
217 CCK-releasing activity in STC-1 cells (Nakajima et al., 2012) and rats (Nishi et al.,
218 2003). **The CCK-releasing activity of zinc was weakened at higher doses (200-400 μ M)
219 more than 100 μ M (Fig. 1B).** Extracellular calcium significantly increased CCK
220 secretion at concentrations higher than 5 mM (Fig. 1C), while heavy metal ions such as
221 copper and cadmium induced CCK secretion at 100 μ M dosage with potency
222 equivalent to that of zinc (Fig. 1D). ZnCl₂ and CdCl₂ at 100 μ M caused a small increase

223 in LDH release from STC-1 cells when compared to control treatment (Fig. 1E). The
224 intracellular Zn^{2+} levels were dose-dependently increased by treatment with
225 extracellular zinc (Fig. 1F). To clarify whether intracellular Zn^{2+} is required for CCK
226 secretion, STC-1 cells were treated with TPEN, a cell membrane-permeable zinc
227 chelator, before the extracellular zinc application. Extracellular zinc-induced CCK
228 secretion was abolished by the pretreatment with TPEN (Fig. 1G). **The deletion of**
229 **intracellular Zn^{2+} by TPEN treatment was confirmed in intracellular Zn^{2+} imaging**
230 **experiment (data not shown).**

231

232 *3.2 Intracellular Ca^{2+} is involved in extracellular zinc-induced CCK secretion*

233 Exposure to extracellular zinc dose-dependently induced intracellular Ca^{2+}
234 mobilization in STC-1 cells (Fig. 2A), but not in colon-derived GLUTag cells (Fig. 2B).
235 Extracellular zinc-induced intracellular Ca^{2+} mobilization was abolished by
236 pretreatment with TPEN (Fig. 2C), but the treatment did not affect the KCl-induced
237 response. Because extracellular calcium is a main source of Ca^{2+} for triggering CCK
238 secretion (Psichas et al., 2015), the effect of zinc on CCK secretion was tested in the
239 absence of extracellular calcium. Zinc-induced intracellular Ca^{2+} mobilization and CCK
240 secretion were abolished by the removal of extracellular calcium (Fig. 2D, E).

241

242 *3.3 TRPA1 is involved in zinc-induced intracellular Ca^{2+} mobilization and CCK* 243 *secretion*

244 The TRPA1 channel is expressed in STC-1 cells and mediates CCK secretions
245 induced by AITC and unsaturated aldehyde (Nakajima et al 2014; Purhonen et al., 2008).
246 Zinc has been demonstrated to increase intracellular Ca^{2+} through the activation of the
247 intracellular domain of TRPA1 (Hu et al., 2009). Based on these previous findings, we
248 examined whether zinc-induced CCK secretion was mediated by TRPA1. Intracellular
249 Ca^{2+} mobilization induced by 30 μ M and 100 μ M zinc was abolished by pretreatment
250 with the TRPA1 antagonist, HC-030031 (Fig. 3A). The CCK secretions induced by
251 extracellular zinc and AITC (Sigma-Aldrich) were reduced by the antagonist treatment
252 (Fig. 3B). Extracellular signal-regulated kinase is known to contribute to CCK-releasing
253 (Guilmeau et al., 2003); however, treatment with MEK inhibitor (U0126) did not affect
254 zinc-induced CCK secretion (Fig. 3C).

255

256 *3.4 Zinc induces Gastric emptying through CCK release in rats*

257 The American Institute of Nutrition recommends the use of zinc carbonate at 1.65
258 g/kg diet (supplied 0.858 g zinc/kg diet) in diets for experimental rodents (Reeves et al.,

1993). Because the amount of food intake in rats (200–300 g body weight) is around 20–30 g (containing 11.7–17.6 mg of zinc) per day, zinc intake is calculated to be approximately 11.7–17.6 mg/day in rats with a body weight of 200–300 g (58.5 mg/kg body weight/day). The oral dose of ZnCl₂ (0.5 mg/kg body weight, supplied 0.24 mg zinc/kg body weight) examined in the gastric emptying test was about 1/100 of the daily intake of zinc. Significant effects of zinc ($P < 0.05$) and time ($P < 0.05$) in the acetaminophen test were determined by two-way ANOVA; however, there was no significant interaction between zinc and time ($P = 0.99$) (Fig. 4A). Lower acetaminophen levels in peripheral plasma were observed at 10, 20, 50, 60, and 80 min after oral administration in zinc-treated rats than in saline-treated rats (Fig. 4A). The area under the curve of the peripheral acetaminophen level in the zinc-treated group was significantly lower than that in vehicle-treated group (Fig. 4B).

In zinc-treated rats, portal zinc and CCK/gastrin levels were significantly higher than those in saline-treated rats (Fig. 4C, D). Although no significant interaction between zinc and devazepide was observed ($P = 0.49$), significant effects of zinc ($P < 0.05$) and devazepide ($P < 0.05$) on gastric emptying were determined by two-way ANOVA (Fig. 4E). The phenol red (non-absorbable marker) test confirmed the inhibitory effect of oral zinc administration on the gastric emptying rate (Fig. 4E). However, upon treatment with the CCK1R antagonist devazepide, oral zinc did not reduce the gastric emptying rate (Fig. 4E). The gastric emptying rate in the zinc/vehicle-treated group was negatively correlated to the portal CCK/gastrin levels ($r = -0.91$, $P = 0.0018$); however, no such correlation was observed in the zinc/devazepide-treated group (Fig. 4F, G).

282

283 **4. Discussion**

284 Although the stimulatory effects of macronutrients on CCK secretion are well
285 known, the effects of micronutrients have been less well studied. Here, we found that
286 extracellular zinc stimulates CCK secretion in STC-1 cells. Recently, intracellular Zn²⁺
287 signaling has been reported to have a role in the secretion of hormones such as insulin
288 and glucagon (Liu et al., 2015; Solomou et al., 2015). Zn²⁺ signal induces intracellular
289 Ca²⁺ mobilization (Hershinkel et al., 2001; Hu et al., 2009), which is one of the major
290 second messengers for exocytosis including gut hormone secretion (Psichas et al., 2015).
291 Involvement of intracellular Ca²⁺ signal in CCK secretion from enteroendocrine cells
292 has been established in previous studies (Liou et al., 2011; Nakajima et al., 2014;
293 Psichas et al., 2015). To our knowledge, this is the first study **demonstrating** that
294 intracellular Zn²⁺ (possibly transported into the cells) induced the influx of extracellular

295 calcium and subsequent CCK secretion in CCK-producing cells as illustrated in Fig. 5.
296 Although TPEN chelates not only zinc but also copper (Hyun et al., 2001), the
297 incubation buffer did not contain copper in the present study. Further, we confirmed the
298 deletion of intracellular zinc by TPEN, which supports the intracellular Zn^{2+} is required
299 for extracellular zinc-induced CCK secretion.

300 Notably, it has been demonstrated that intracellular zinc activates the cytosolic
301 domain of TRPA1 (Hu et al., 2009). In CCK-producing cells, TRPA1 agonists induce
302 intracellular Ca^{2+} mobilization and subsequent CCK secretion (Kurogi et al., 2012;
303 Nakajima et al., 2014; Purhonen et al., 2008). In the present study, we identified zinc as
304 a one of the physiological stimulants for CCK secretion through the activation of
305 TRPA1 (Fig. 3B). Furthermore, other metal ions (Cu^{2+} and Cd^{2+}) reportedly activate
306 TRPA1 (Gu and Lin, 2010), which supports that TRPA1 contributes to heavy metal
307 ion-induced CCK secretion. These results suggest that intracellular Zn^{2+} signaling is
308 related to intracellular Ca^{2+} signaling and CCK secretion through TRPA1 activation in
309 CCK-producing cells. In the intestinal epithelium, TRPA1 is mainly expressed in CCK-
310 and 5-HT-producing cells (Cho et al., 2014; Nozawa et al., 2009). TRPA1 agonists such
311 as unsaturated aldehydes and AITC regulate gastrointestinal motility through serotonin
312 secretion (Hira et al., 2015; Nozawa et al., 2009). Although it is still unclear whether
313 serotonin secretion is also induced by zinc through the activation of TRPA1, the present
314 results suggest that gastrointestinal motility is modulated by zinc administration through
315 increased CCK secretion. Although the ELISA assay used in this study cross-reacts with
316 gastrin according to the manufacturer's manual, the results obtained by using specific
317 CCK1R antagonist (Fig. 4E) strongly support the notion that oral zinc induces CCK
318 secretion. Furthermore, extracellular zinc increased intracellular Ca^{2+} in STC-1 cells
319 derived from murine duodenum, but not in GLUTag cells derived from murine colon
320 (Fig. 2A, B), supporting that proximal enteroendocrine cells are responsible for luminal
321 zinc-sensing.

322 The basal zinc level in blood is maintained at 10–30 μ M (Chen et al., 2000; Iwaya et
323 al., 2011), and the luminal zinc level after meal ingestion has been found to reach
324 approximately 100 μ M (Cragg et al. 2002). In the present study, zinc at 100 μ M had
325 higher ability for stimulating CCK release than dietary peptide and calcium (5-10 mM)
326 in STC-1 cells (Fig. 1A–C). Although it is still unclear why higher dose of zinc (\geq 200
327 μ M) had lower potency to stimulate CCK secretion, these results suggest that the
328 physiological level of zinc is optimal for potent stimulation of CCK release. Generally,
329 relatively high doses are required for dietary factors such as dietary peptide (50
330 mg/ml/rat, Sufian et al., 2006), dietary protein (0.5 g/kg body weight, Chen et al., 2012),

331 olive oil (0.5 ml/mouse, Liou et al., 2011), and a bitter taste compound
332 (phenylthiocarbamide; 10–30 mg/kg body weight, Jeon et al., 2008) to stimulate CCK
333 secretion. Surprisingly, a physiological amount of ZnCl₂ (0.5 mg/kg body weight,
334 supplied 0.24 mg zinc) significantly delayed gastric emptying through CCK signaling
335 (Fig. 4), indicating that luminal zinc has high potential for reducing gastric emptying
336 through CCK secretion from CCK-producing cells.

337 Zinc deficiency causes immune dysfunctions such as impaired inflammatory
338 response (Wong et al., 2015), exacerbated experimental colitis induced by dextran
339 sulfate sodium (Iwaya et al., 2011), and abnormal T cell development (Blewett et al.,
340 2012). On the other hand, over intake of zinc causes toxicities in testes and liver (Abban
341 et al., 2003, Turgut et al., 2003). In addition, high doses of zinc (150–200 μM) causes
342 cell death in rat primary cortical neurons and astrocytes (Kim and Koh, 2002; Ryu et al.,
343 2002). Thus, zinc homeostasis is important for maintenance of various physiological
344 functions. Interestingly, dietary components such as amino acids (histidine and
345 methionine) promote zinc absorption in the duodenum through complex formation with
346 zinc (Lönnerdal, 2000). In addition, impaired pancreatic function causes lower serum
347 zinc concentration (Milnerowicz et al., 2009), which may be related to the loss of CCK
348 signaling for pancreatic enzyme secretion (Chandra and Liddle, 2009). Taken together
349 with our results, these findings suggest that gastrointestinal functions regulated by CCK
350 could be involved in luminal zinc absorption. Furthermore, it has been demonstrated
351 that CCK exerts anti-inflammatory effects in macrophages, B cells, and dendritic cells
352 (Cevik-Aras and Ekstrom., 2010; Li et al., 2007; Li et al., 2011; Miyamoto et al., 2012).
353 Dietary zinc possibly contributes to such immune function through CCK secretion.

354 In summary, we demonstrated that zinc stimulates CCK secretion in enteroendocrine
355 STC-1 cells. TRPA1 mediates zinc-induced CCK secretion through the influx of
356 extracellular calcium. Further, single oral administration of zinc delays gastric emptying
357 via CCK signaling in rats. These results reveal a novel physiological role of zinc in the
358 enteroendocrine system and gastrointestinal motility that can affect zinc homeostasis in
359 response to meal ingestion.

360

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364

365

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566

567

568 **Legend to Figure**

569 **Figure 1. Extracellular zinc stimulates CCK secretion from STC-1 cells through**
570 **the increase of intracellular Zn²⁺.**

571 **Dose-dependent effects of extracellular zinc on CCK secretion (A, B) in STC-1 cells (n**
572 **= 4).** βconP (5 mg/ml) was used as a positive control. **(C) Calcium and (D) other heavy**
573 **metal ions** (30 and 100 μM) were tested on CCK secretion (n = 4). **(E)** The effect of
574 heavy metal ions on LDH release in STC-1 cells (n = 4). **(F)** Intracellular Zn²⁺ (494 nm)
575 level was increased after exposure to extracellular zinc (n = 3). **(G)** 30 μM ZnCl₂ or 70
576 mM KCl was applied to STC-1 cells with or without pretreatment of intracellular Zn²⁺
577 chelator (n = 4). Values are means ± SEM. Data were analyzed by one-way ANOVA
578 with Tukey's *post-hoc* test. * *P* < 0.05 vs. control (Fig. 1A-E), # *P* < 0.05 between
579 vehicle and TPEN treatment (Fig. 1G).

580

581 **Figure 2. Extracellular calcium is required for zinc-induced CCK secretion.**

582 Effect of extracellular zinc (30 and 100 μM) on intracellular Ca²⁺ levels (Δ340 nm/380
583 nm ratio) in Fura-2 loaded (A) STC-1 cells (n = 3) or (B) GLUtag cells (n = 5). (C)
584 Pretreatment with TPEN (10 μM) blocked zinc-induced intracellular Ca²⁺ mobilization
585 (n = 3). (D) Zinc-induced intracellular Ca²⁺ mobilization in the presence (1.2 mM
586 CaCl₂) or absence of extracellular calcium (n = 3). (E) The effect of zinc on CCK
587 secretion in the presence (1.2 mM CaCl₂) or absence of extracellular calcium (n = 4).
588 Values are means ± SEM. Data were analyzed by one-way ANOVA with Tukey's
589 *post-hoc* test. * *P* < 0.05 vs. control, # *P* < 0.05 between the presence and absence of
590 extracellular calcium (Fig. 2E).

591

592 **Figure 3. Involvement of TRPA1 in zinc-induced CCK secretion.**

593 (A) Intracellular Ca²⁺ mobilization in response to zinc in the presence or absence of
594 TRPA1 antagonist, HC-030031 (100 μM) (n = 3, 4). CCK secretion in response to zinc
595 in the presence or absence of 100 μM HC-030031 (B) or 10 μM U0126 (C) (n = 4).
596 Values are means ± SEM. Data were analyzed by one-way or two-way ANOVA with
597 Tukey's *post-hoc* test. * *P* < 0.05 vs. control, # *P* < 0.05 between the presence and
598 absence of HC-030031 (Fig. 3B).

599

600 **Figure 4. Zinc administration delays gastric emptying with increased plasma CCK**
601 **level.**

602 (A) Rats were orally administered saline or ZnCl₂ (0.5 mg/kg body weight, supplied
603 0.24 mg zinc/kg body weight) with gastric emptying marker (acetaminophen, 100

604 mg/kg body weight). Tail vein blood was collected at 0, 10, 20, 30, 40, 50, 60, 70, 80,
605 90, and 120 min (n = 5, 6). (B) Area under the curve (AUC) of changed acetaminophen
606 concentration (n = 6). Portal zinc (C) and CCK/gastrin (D) levels after the
607 administration of ZnCl₂ (0.5 mg/kg body weight, supplied 0.24 mg zinc/kg body
608 weight) (n = 6, 7). (E) Zinc-delayed gastric emptying was canceled by intraperitoneal
609 injection of CCK1R antagonist (devazepide, 0.5 mg/kg body weight) (n = 7, 8).
610 Correlation between the plasma CCK/gastrin level and the gastric emptying rate in the
611 solo zinc administered group (F) and devazepide co-administered group (G) (n = 7, 8).
612 Values are means ± SEM. Data were analyzed by one-way or two-way ANOVA with
613 Student's *t*-test. * *P* < 0.05 vs. control.

614

615 **Figure 5. Proposed mechanism of extracellular zinc-induced CCK secretion in**
616 **CCK-producing cells.**

617 Increased intracellular Zn²⁺ was observed after the exposure of extracellular zinc in
618 CCK-producing cells. Intracellular Ca²⁺ mobilization was abolished under the chelation
619 of zinc and the inhibition of TRPA1, suggesting that intracellular zinc induced calcium
620 influx through the activation of TRPA1 for CCK secretion.

High lights

3 to 5 bullet points (maximum 85 characters (including spaces) per bullet point).

- Zinc directly stimulates cholecystokinin (CCK) secretion from CCK-producing cells.
- Extracellular calcium is required for zinc-induced CCK secretion.
- Transient receptor potential ankyrin 1 (TRPA1) mediates zinc-induced CCK secretion.
- Zinc delays gastric emptying through the CCK secretion in rats.

Figure 1

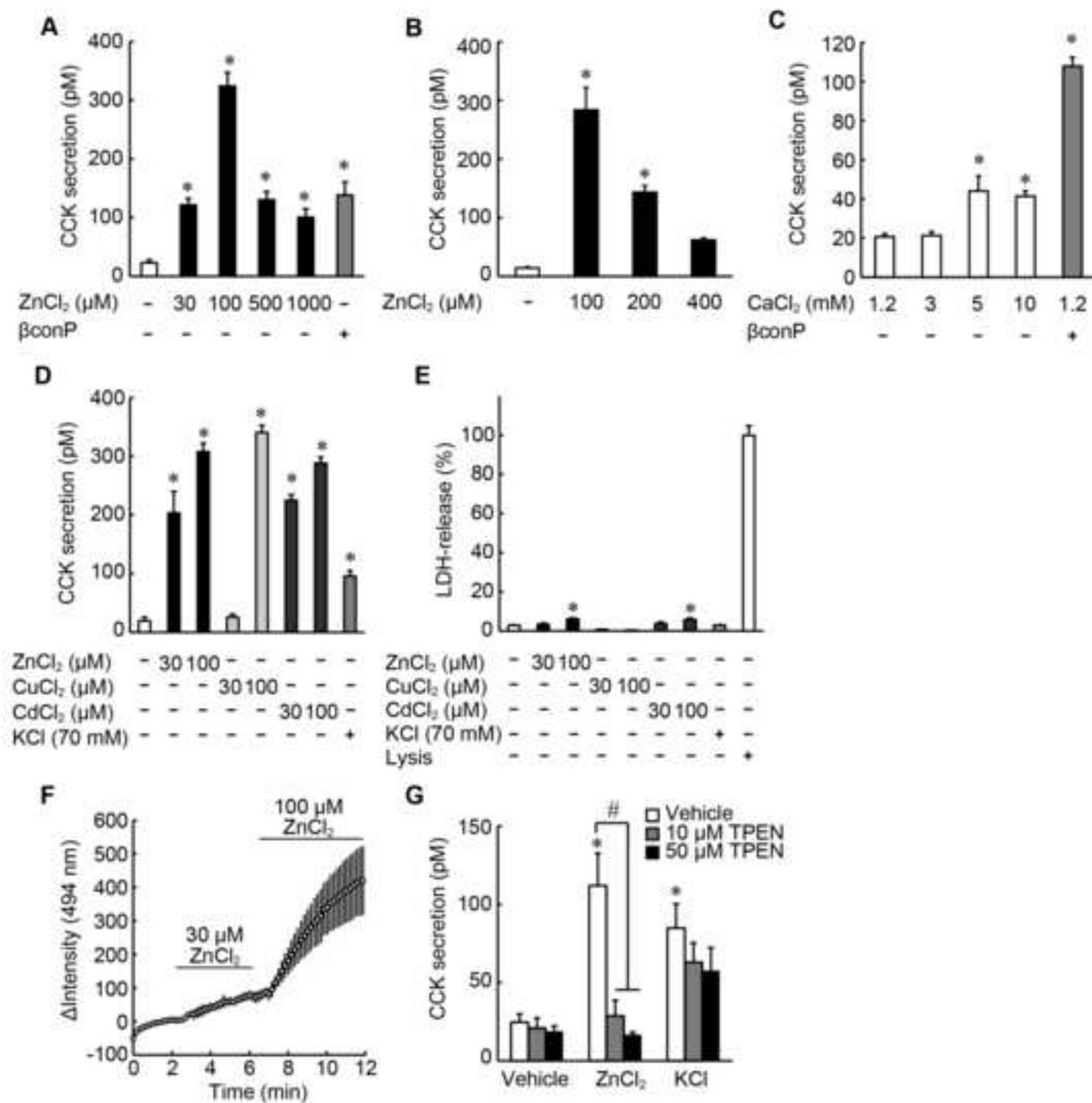


Figure 2

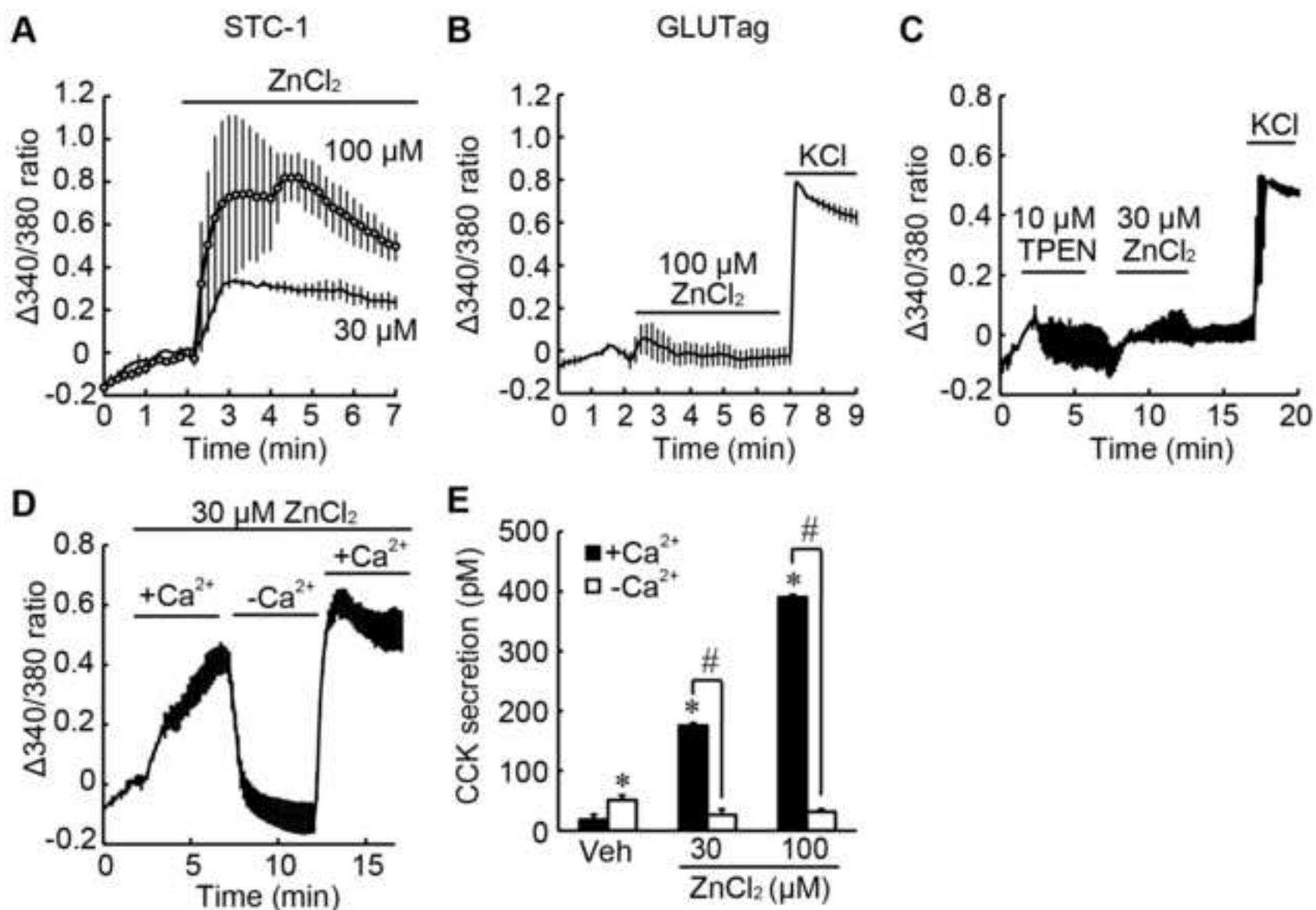


Figure 3

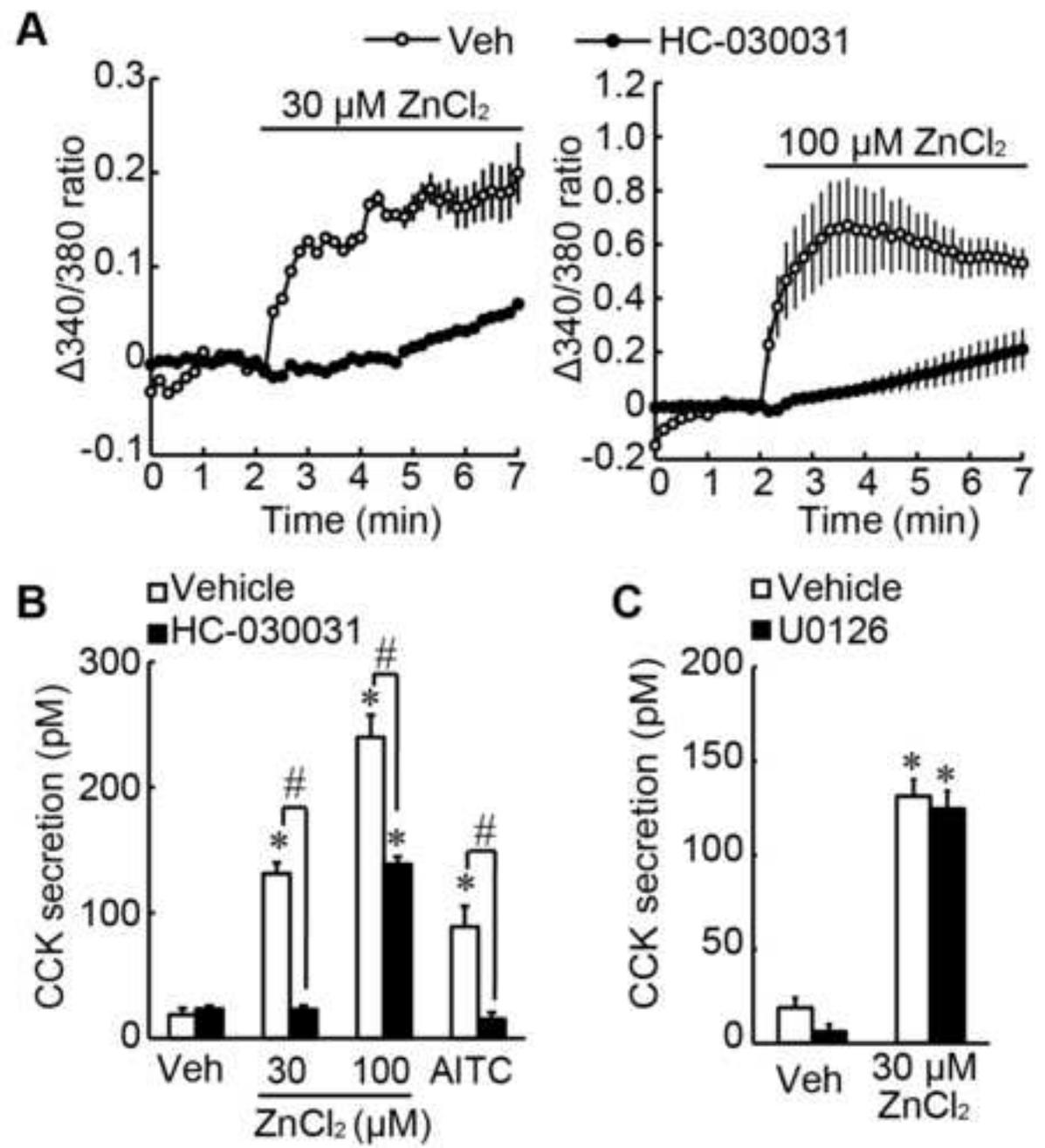


Figure 4

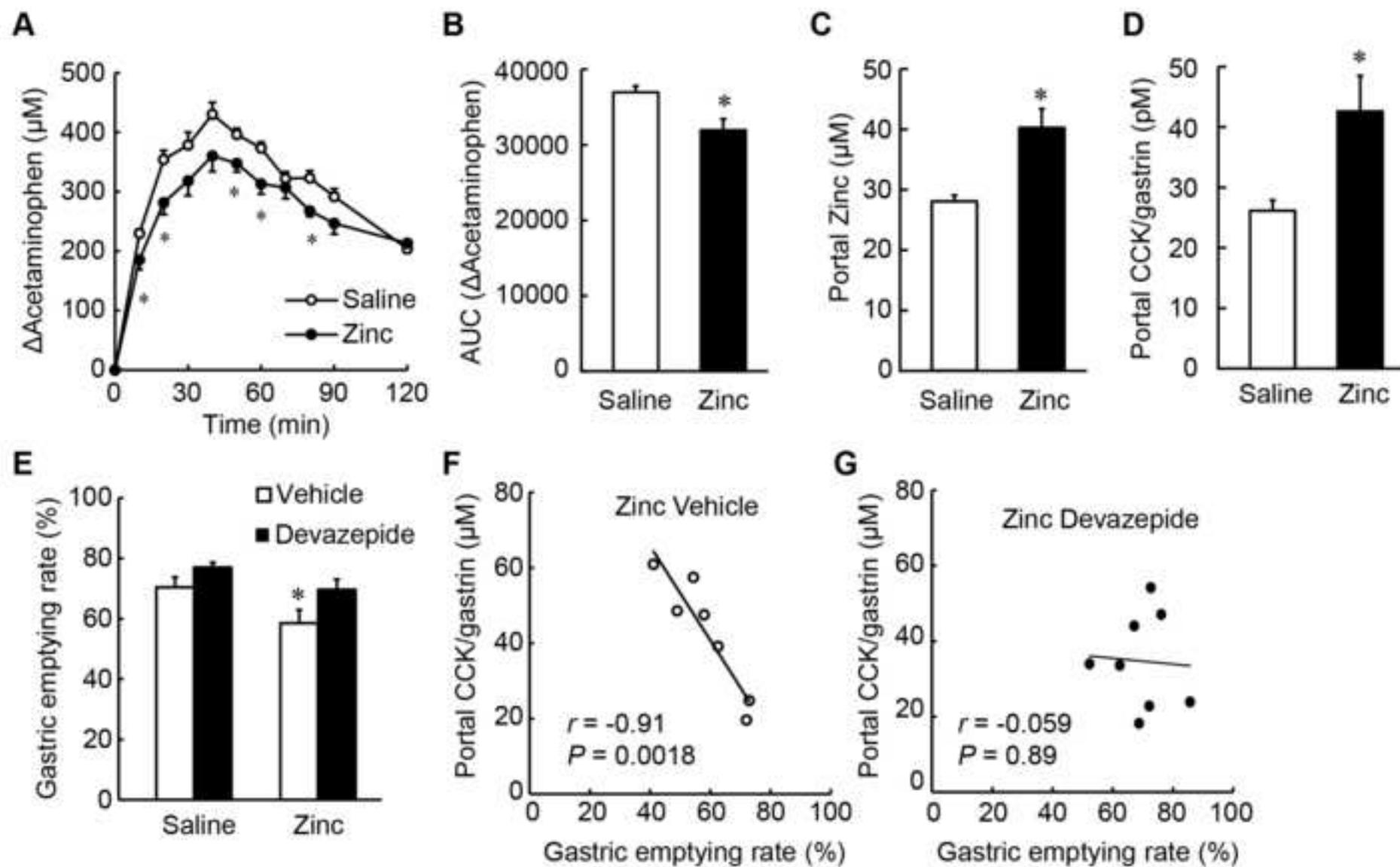


Figure 5

