Title

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

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Statement of Author's Contributions to Manuscript

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

Abstract

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

Keywords

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

1. Introduction

The secretion of cholecystokinin (CCK) is triggered by dietary components through the activation of specific receptors (Psichas et al., 2015). Intracellular second messengers such as calcium (Ca^{2+}) and cyclic adenosine mono phosphate mediate these stimulus-induced CCK secretions (Psichas et al., 2015). It is well known that secreted CCK is recognized by CCK receptors (CCK1R and CCK2R) to regulate gastrointestinal motility, food intake suppression, and pancreatic enzyme secretion (Chandra and Liddle, 2009; Dockray, 2009). In addition, the activation of CCK receptors inhibits the inflammatory response via vagus nerve and nicotinic receptor signaling (Luyer et al., 2005). Further, CCK blocks lipopolysaccharide-induced proinflammatory cytokine production in pulmonary interstitial macrophages (Li et al., 2007), suggesting the contribution of CCK in protective immunity during food or unexpected toxicant ingestion.

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

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

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

2. Materials and Methods

2.1 Cell culture

STC-1 cells (derived from murine duodenum, a gift from Dr. Hanahan, University of California, San Francisco, CA) and GLUTag cells (derived from murine colon, a gift from Dr. D. J. Drucker, University of Toronto, Canada) were grown in Dulbecco's modified Eagle's medium (Thermo scientific, IL, USA) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, and 500 µg/ml streptomycin. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C and were routinely subcultured with trypsinization until subconfluence (80-90% in a T75 flask).

2.2 Measurement of CCK secretion from STC-1 cells

STC-1 cells were grown in 48-well plates for 2–3 days until they reached 80–90% confluence. Before exposure to test agents, the cells were washed twice with HEPES buffer (140 mM NaCl, 4.5 mM KCl, 20 mM HEPES, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose, and 0.1% BSA; pH 7.4) to remove the medium. The cells were incubated with $ZnCl_2$, $CaCl_2$, $CuCl_2$, and $CdCl_2$ (all from Wako Pure Chemical Industries, Osaka, Japan) for 60 min at 37°C. β-conglycinin peptone (βconP), a peptic hydrolysate of soy protein, was used as positive control for CCK secretion as reported previously (Nakajima et al., 2012; Nishi et al., 2003). All test substances were dissolved in HEPES buffer. The cells were treated with TRPA1 antagonist (HC-030031; Sigma-Aldrich, MO, USA), MEK inhibitor (U0126; Merck Millipore, MA, USA) or zinc chelator {*N*,*N*,*N*',*N*'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN); Dojindo Laboratories, Kumamoto, Japan} for 20 min before exposing to the test substances. After the incubation, the supernatants were collected and remaining cells were removed by centrifugation at 800 × g for 5 min at 4°C. The CCK concentration in the supernatants was measured using a commercial enzyme immunoassay (EIA) kit (Phoenix Pharmaceuticals, Belmont, CA) according to the manufacture's instructions. The primary antiserum provided in this kit cross-reacts 100% with sulfated and non-sulfated CCK (26-33), CCK-33 (porcine), caerulein, gastrin-1 (human), and big gastrin-1 (human). The antiserum also cross-reacts 12.6% with CCK (30-33) and 0% with pancreatic polypeptide (human) and vasoactive intestinal peptide (human, porcine, and rat). Although the antibody cross-reacts with gastrin, we selected this EIA kit because gastrin is not expressed at a detectable level in STC-1 cells (McLaughlin et. al. 1999). The coefficients of intra- and inter-assay variation were < 5% and < 14%, respectively.

2.3 Measurement of cytotoxicity in STC-1 cells

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

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

2.4 Intracellular Ca^{2+} and Zn^{2+} imaging

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

2.5 Animals

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

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

2.6 Acetaminophen test

Overnight-fasted rats were orally administered $ZnCl_2$ at 0.5 mg/kg body weight or its vehicle (8 ml/kg body weight, saline) with acetaminophen (100 mg/kg body weight). Blood (60 µL) was collected from the tail vein into 1.5 -ml tube containing 1.5 U/tube heparin at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min after the administration of the test solution. The blood samples were centrifuged at 2,300 × g for 10 min at 4°C, and the supernatants were collected. The acetaminophen concentration was measured with an acetaminophen detection kit (Kanto Chemical Co. Inc., Tokyo, Japan) according to the manufacturer's protocol with some modification. Briefly, plasma (5 µl) was mixed with Reagent A (25 µl) followed by centrifugation (8,400 x g, 2 min). The supernatant (20 µl) was transferred to fresh tube, and then the sample was boiled for 10 min. After addition of Reagent B (50 µl), the concentration of acetaminophen was calculated based on spectrophotometrically measurement at 600 nm.

2.7 Measurements of gastric emptying rate and portal CCK level

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

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

calculated as follows:

Gastric emptying rate (%) = [{the amount of phenol red administered (mg) – the amount of phenol red remained in the stomach (mg)} / the amount of phenol red administered (mg)] × 100

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

2.8 Statistical analysis

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

3. Results

3.1 Extracellular zinc triggers CCK release in STC-1 cells

Extracellular zinc application resulted in significantly increased CCK secretion from STC-1 cells, especially, 100 μM $ZnCl_2$ showed the highest CCK-releasing activity among the various concentrations tested (Fig. 1A). The CCK secretion induced by 100 μM $ZnCl_2$ was much higher than that induced by βconP. The peptone has potent CCK-releasing activity in STC-1 cells (Nakajima et al., 2012) and rats (Nishi et al., 2003). The CCK-releasing activity of zinc was weakened at higher doses (200-400μM) more than 100 μM (Fig. 1B). Extracellular calcium significantly increased CCK secretion at concentrations higher than 5 mM (Fig. 1C), while heavy metal ions such as copper and cadmium induced CCK secretion at 100 -μM dosage with potency equivalent to that of zinc (Fig. 1D). $ZnCl_2$ and $CdCl_2$ at 100 μM caused a small increase

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

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

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

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

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

3.4 Zinc induces Gastric emptying through CCK release in rats

The American Institute of Nutrition recommends the use of zinc carbonate at 1.65 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 $ZnCl_2$ (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).

4. Discussion

Although the stimulatory effects of macronutrients on CCK secretion are well known, the effects of micronutrients have been less well studied. Here, we found that extracellular zinc stimulates CCK secretion in STC-1 cells. Recently, intracellular Zn^{2+} signaling has been reported to have a role in the secretion of hormones such as insulin and glucagon (Liu et al., 2015; Solomou et al., 2015). Zn^{2+} signal induces intracellular Ca^{2+} mobilization (Hershfinkel et al., 2001; Hu et al., 2009), which is one of the major second messengers for exocytosis including gut hormone secretion (Psichas et al., 2015). Involvement of intracellular Ca^{2+} signal in CCK secretion from enteroendocrine cells has been established in previous studies (Liou et al., 2011; Nakajima et al., 2014; Psichas et al., 2015). To our knowledge, this is the first study demonstrating that intracellular Zn^{2+} (possibly transported into the cells) induced the influx of extracellular

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

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

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

olive oil (0.5 ml/mouse, Liou et al., 2011), and a bitter taste compound (phenylthiocarbamide; 10–30 mg/kg body weight, Jeon et al., 2008) to stimulate CCK secretion. Surprisingly, a physiological amount of $ZnCl_2$ (0.5 mg/kg body weight, supplied 0.24 mg zinc) significantly delayed gastric emptying through CCK signaling (Fig. 4), indicating that luminal zinc has high potential for reducing gastric emptying through CCK secretion from CCK-producing cells.

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

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

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Legend to Figure

Figure 1. Extracellular zinc stimulates CCK secretion from STC-1 cells through the increase of intracellular Zn^{2+}.

Dose-dependent effects of extracellular zinc on CCK secretion (A, B) in STC-1 cells (n = 4). βconP (5 mg/ml) was used as a positive control. (C) Calcium and (D) other heavy metal ions (30 and 100 μM) were tested on CCK secretion (n = 4). (E) The effect of heavy metal ions on LDH release in STC-1 cells (n = 4). (F) Intracellular Zn^{2+} (494 nm) level was increased after exposure to extracellular zinc (n = 3). (G) 30 μM $ZnCl_2$ or 70 mM KCl was applied to STC-1 cells with or without pretreatment of intracellular Zn^{2+} chelator (n = 4). Values are means ± SEM. Data were analyzed by one-way ANOVA with Tukey's *post-hoc* test. * $P < 0.05$ vs. control (Fig. 1A-E), # $P < 0.05$ between vehicle and TPEN treatment (Fig. 1G).

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

Effect of extracellular zinc (30 and 100 μM) on intracellular Ca^{2+} levels (Δ340 nm/380 nm ratio) in Fura-2 loaded (A) STC-1 cells (n = 3) or (B) GLUTag cells (n = 5). (C) Pretreatment with TPEN (10 μM) blocked zinc-induced intracellular Ca^{2+} mobilization (n = 3). (D) Zinc-induced intracellular Ca^{2+} mobilization in the presence (1.2 mM $CaCl_2$) or absence of extracellular calcium (n = 3). (E) The effect of zinc on CCK secretion in the presence (1.2 mM $CaCl_2$) or absence of extracellular calcium (n = 4). Values are means ± SEM. Data were analyzed by one-way ANOVA with Tukey's *post-hoc* test. * $P < 0.05$ vs. control, # $P < 0.05$ between the presence and absence of extracellular calcium (Fig. 2E).

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

(A) Intracellular Ca^{2+} mobilization in response to zinc in the presence or absence of TRPA1 antagonist, HC-030031 (100 μM) (n = 3, 4). CCK secretion in response to zinc in the presence or absence of 100 μM HC-030031 (B) or 10 μM U0126 (C) (n = 4). Values are means ± SEM. Data were analyzed by one-way or two-way ANOVA with Tukey's *post-hoc* test. * $P < 0.05$ vs. control, # $P < 0.05$ between the presence and absence of HC-030031 (Fig. 3B).

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

(A) Rats were orally administered saline or $ZnCl_2$ (0.5 mg/kg body weight, supplied 0.24 mg zinc/kg body weight) with gastric emptying marker (acetaminophen, 100

mg/kg body weight). Tail vein blood was collected at 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min (n = 5, 6). (B) Area under the curve (AUC) of changed acetaminophen concentration (n = 6). Portal zinc (C) and CCK/gastrin (D) levels after the administration of $ZnCl_2$ (0.5 mg/kg body weight, supplied 0.24 mg zinc/kg body weight) (n = 6, 7). (E) Zinc-delayed gastric emptying was canceled by intraperitoneal injection of CCK1R antagonist (devazepide, 0.5 mg/kg body weight) (n = 7, 8). Correlation between the plasma CCK/gastrin level and the gastric emptying rate in the solo zinc administered group (F) and devazepide co-administered group (G) (n = 7, 8). Values are means ± SEM. Data were analyzed by one-way or two-way ANOVA with Student's *t*-test. * $P < 0.05$ vs. control.

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

Increased intracellular Zn^{2+} was observed after the exposure of extracellular zinc in CCK-producing cells. Intracellular Ca^{2+} mobilization was abolished under the chelation of zinc and the inhibition of TRPA1, suggesting that intracellular zinc induced calcium 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.

Figure1

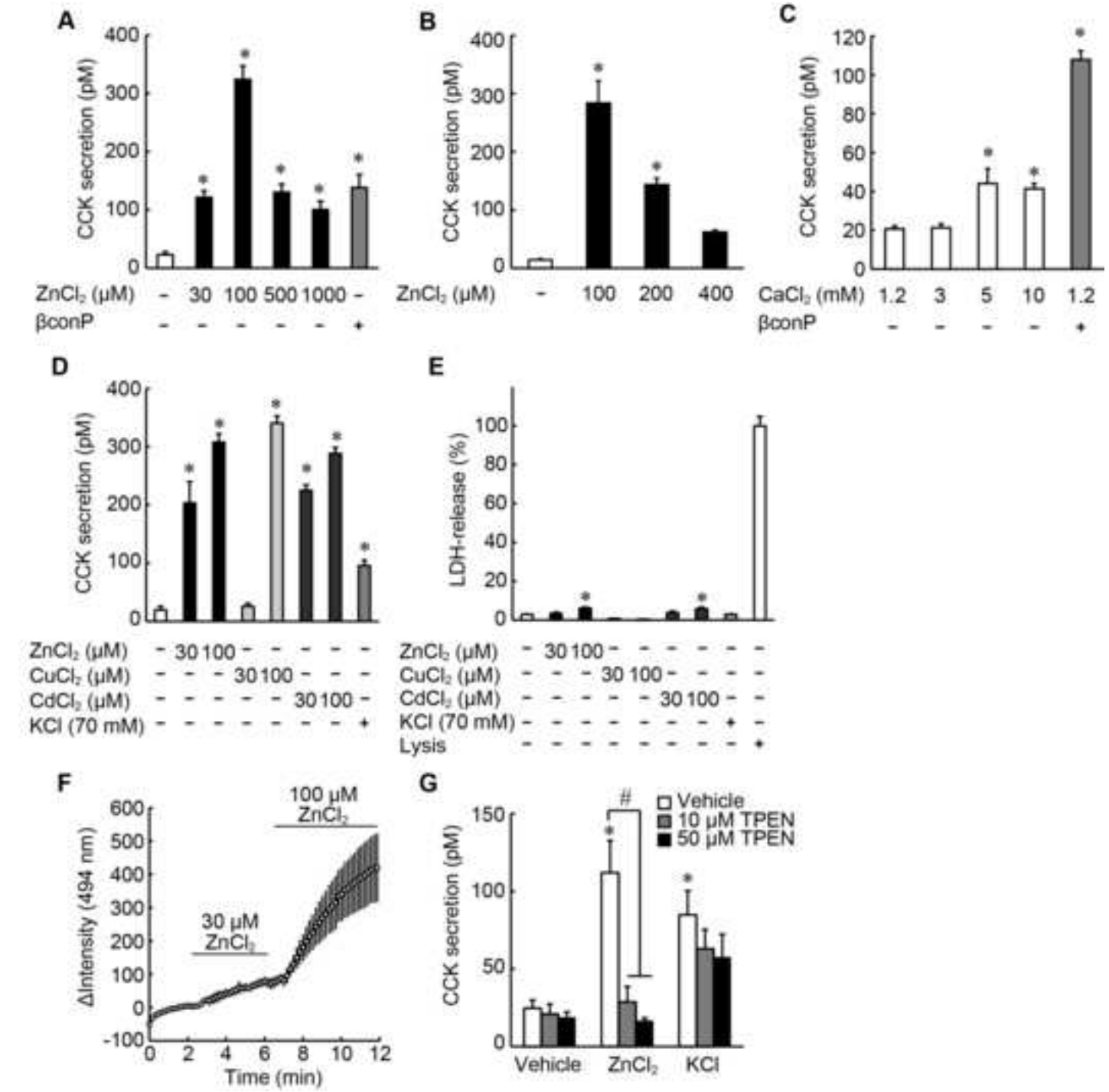
Figure 1
A
CCK secretion (pM)
ZnCl2 (μM) – 30 100 500 1000 –
βconP – – – – – +
B
CCK secretion (pM)
ZnCl2 (μM) – 100 200 400
C
CCK secretion (pM)
CaCl2 (mM) 1.2 3 5 10 1.2
βconP – – – – +
D
CCK secretion (pM)
ZnCl2 (μM) – 30 100 – – – – –
CuCl2 (μM) – – – 30 100 – – –
CdCl2 (μM) – – – – – 30 100 –
KCl (70 mM) – – – – – – – +
E
LDH-release (%)
ZnCl2 (μM) – 30 100 – – – – – –
CuCl2 (μM) – – – 30 100 – – – –
CdCl2 (μM) – – – – – 30 100 – –
KCl (70 mM) – – – – – – – + –
Lysis – – – – – – – – +
F
ΔIntensity (494 nm)
100 μM ZnCl2
30 μM ZnCl2
Time (min)
G
CCK secretion (pM)
Vehicle
10 μM TPEN
50 μM TPEN
Vehicle
ZnCl2
KCl

Figure2

Figure 2

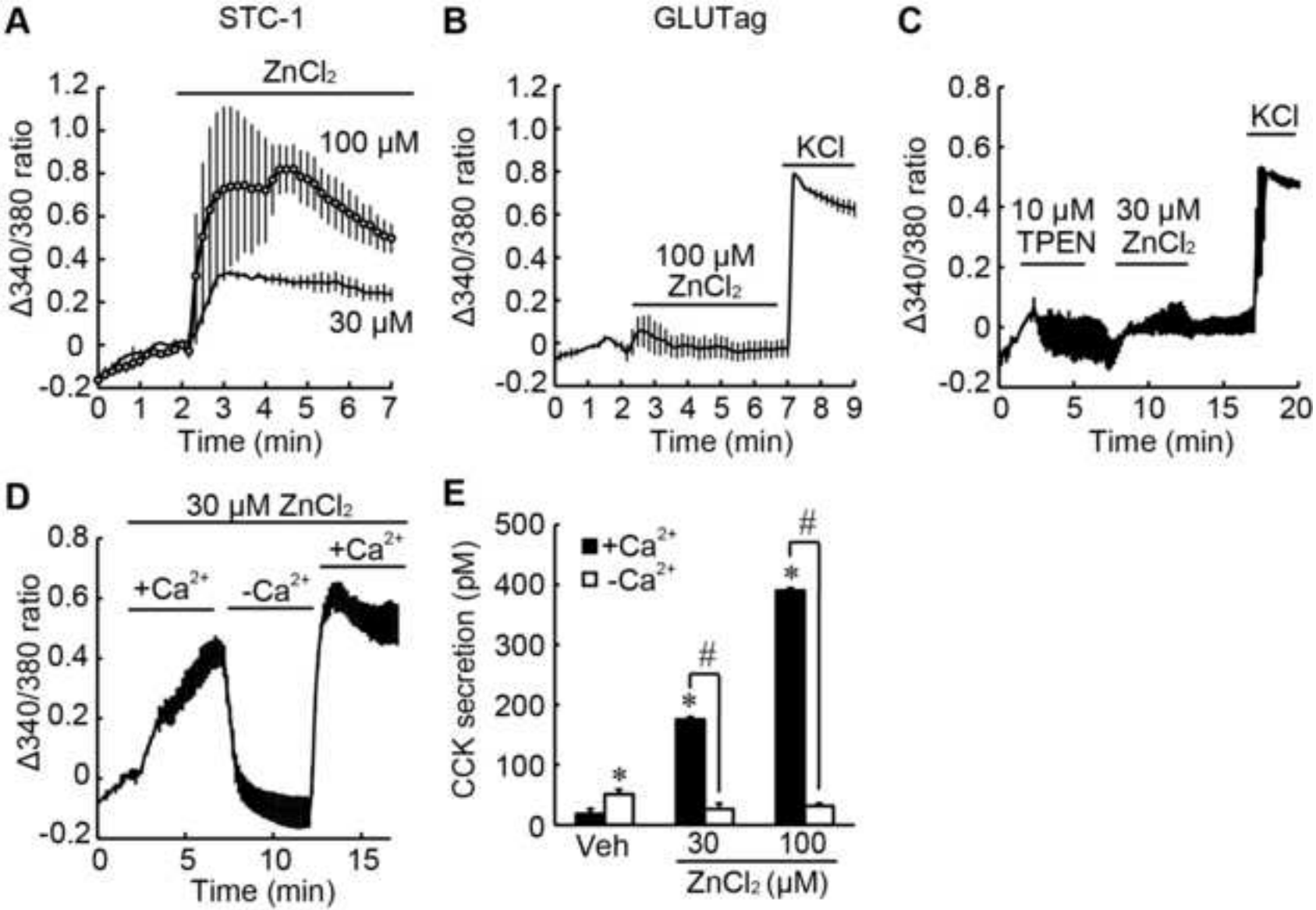

Figure3

Figure 3

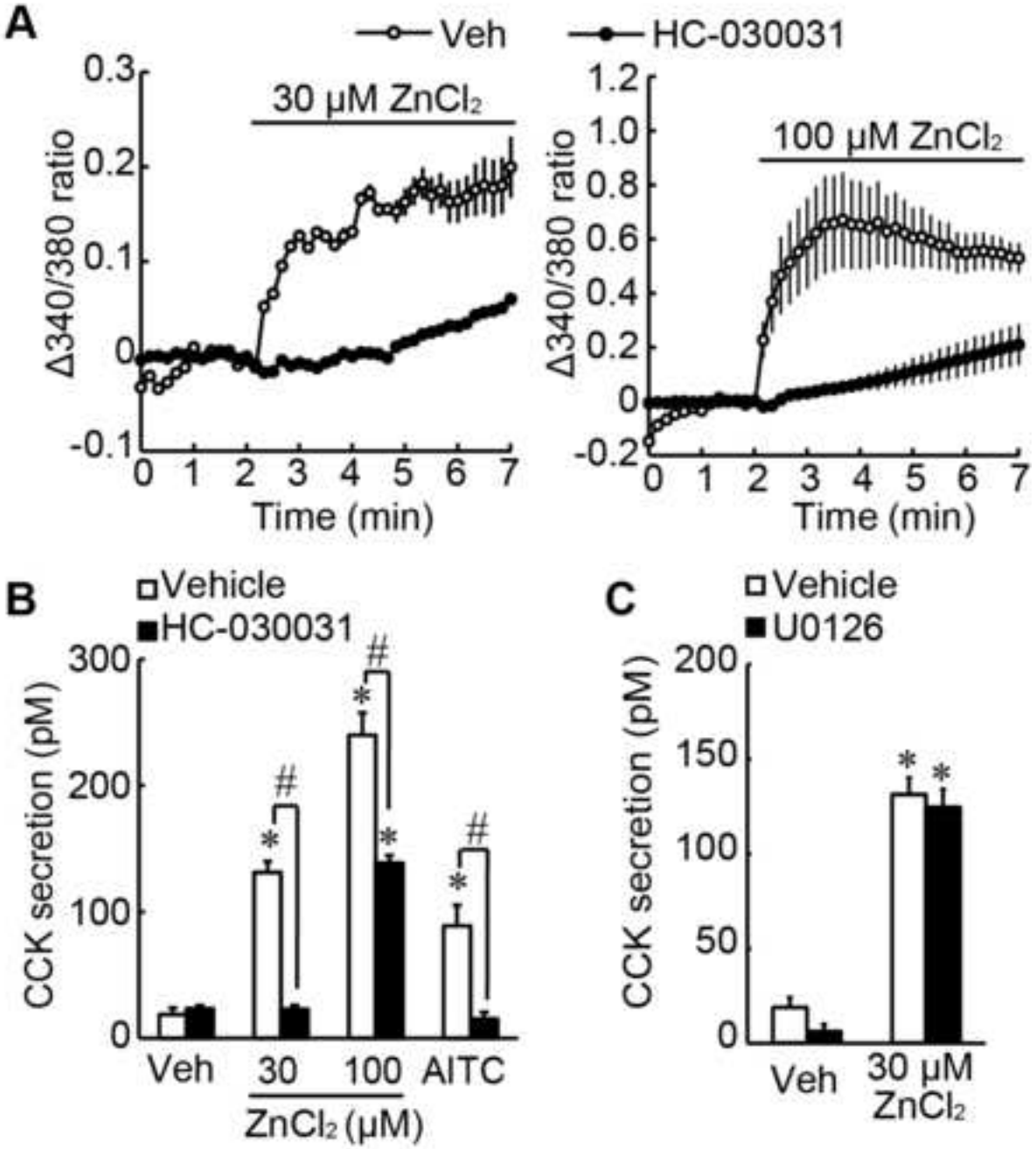

Figure4

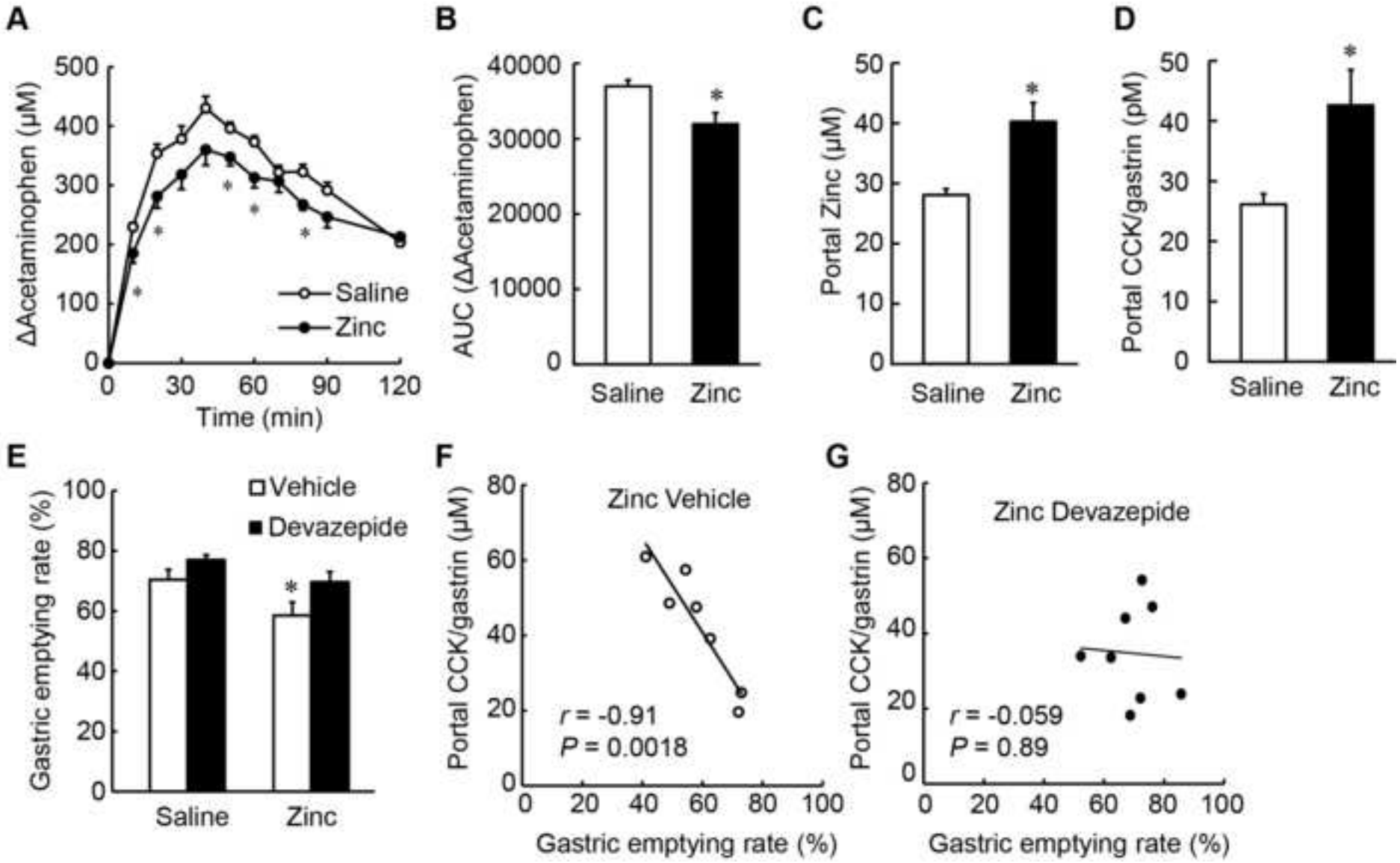
Figure 4
A
ΔAcetaminophen (μM)
500
400
300
200
100
0
0
30
60
90
120
Time (min)
Saline
Zinc
B
AUC (ΔAcetaminophen)
40000
30000
20000
10000
0
Saline
Zinc
C
Portal Zinc (μM)
50
40
30
20
10
0
Saline
Zinc
D
Portal CCK/gastrin (pM)
50
40
30
20
10
0
Saline
Zinc
E
Gastric emptying rate (%)
100
80
60
40
20
0
Vehicle
Devazepide
Saline
Zinc
F
Portal CCK/gastrin (μM)
Zinc Vehicle
r = -0.91
P = 0.0018
0
20
40
60
80
100
Gastric emptying rate (%)
G
Portal CCK/gastrin (μM)
Zinc Devazepide
r = -0.059
P = 0.89
0
20
40
60
80
100
Gastric emptying rate (%)

Figure5

Figure 5