Instructions for use

Title

2-Arachidonoyl glycerol suppresses gastric emptying via the cannabinoid receptor 1-cholecystokinin signaling pathway in mice

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Abbreviated title

2-Arachidonoyl glycerol suppresses gastric emptying via CB1 and CCK in mice

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1 **Abstract**

2   2-Monoacylglycerol (2-MAG) is one of the digestion products of dietary lipids.

3 We recently demonstrated that a 2-MAG, 2-arachidonoyl glycerol (2-AG) potently

4 stimulated cholecystokinin (CCK) secretion via cannabinoid receptor 1 (CB1) in a murine

5 CCK-producing cell line, STC-1. CCK plays a crucial role in suppressing postprandial

6 gastric emptying. To examine the effect of 2-AG on gastric emptying, we performed

7 acetaminophen and phenol red recovery tests under oral or intraperitoneal administration

8 of 2-AG in mice. Orally administered 2-AG (25 mg/kg) suppressed the gastric emptying

9 rate in mice, as determined by the acetaminophen absorption test and phenol red recovery

10 test. Intraperitoneal administration of a cholecystokinin A receptor antagonist (0.5 mg/kg)

11 attenuated the gastric inhibitory emptying effect. In addition, both oral (10 mg/kg) and

12 intraperitoneal (0.5 mg/kg) administration of a CB1 antagonist counteracted the 2-AG-

13 induced gastric inhibitory effect. Furthermore, intraperitoneal 2-AG (25 mg/kg)

14 suppressed gastric emptying. These results indicate that 2-AG exhibits an inhibitory effect

15 on gastric emptying in mice, possibly mediated by stimulating both CCK secretion via

16 CB1 expressed in CCK-producing cells and acting on CB1 expressed in the peripheral

17 nerves. Our findings provide novel insights into the 2-MAG-sensing mechanism in

18 enteroendocrine cells and the physiological role of 2-MAG.

Abbreviations

20	2-AG	2-arachidonoyl glycerol
21	2-MAG	2-monoacylglycerol(s)
22	2-OG	2-oleoyl glycerol
23	AEA	anandamide
24	20:4n-6	arachidonic acid
25	CB1	cannabinoid receptor 1
26	CMC	carboxymethyl cellulose
27	CCK	cholecystokinin
28	CCK-A	cholecystokinin A
29	FA	fatty acid(s)
30	GLP-1	glucagon-like peptide-1
31	TAG	triacylglycerol(s)

Introduction

Gastric emptying occurs after meal ingestion and primarily affects the subsequent digestion and absorption of nutrients in the intestine. Luminal nutrients such as lipids, proteins/peptides, and carbohydrates in the small intestine (rather than the stomach contents) are critical for delaying gastric emptying via neuroendocrine pathways such as enteroendocrine and vagal (Luttikhold *et al.* 2013; Hellström *et al.* 2006). Delayed gastric emptying facilitates efficient digestion and absorption of nutrients. Accordingly, the rate of gastric emptying markedly affects postprandial glycemia and lipidemia (Westphal *et al.* 2004; Muramatsu *et al.* 2014; Phillips *et al.* 2015), thereby suppressing the rate of gastric emptying and contributing to the attenuation of postprandial hyperglycemia and/or hyperlipidemia.

In the small intestine, dietary triacylglycerols (TAG) are hydrolyzed to fatty acids (FA) and 2-monoacylglycerols (2-MAG). In contrast to FA (Hunt and Knox 1968; McLaughlin *et al.* 1999), although it has been suggested that 2-oleoyl glycerol (2-OG) inhibits gastric emptying via glucagon-like peptide-1 (GLP-1) secretion (Lauffer *et al.* 2009; Hansen *et al.* 2011; Hansen *et al.* 2012), studies on the inhibitory effects of 2-MAG on gastric emptying are limited.

In a previous study (Marzo *et al.* 2008), intraperitoneal administration of anandamide (AEA), one of the endogenous cannabinoids (endocannabinoids) that regulate food intake and energy balance through cannabinoid receptor 1 (CB1) in the brain and the peripheral tissues (Ueda *et al.* 2011; Gendaszewska-Darmach *et al.* 2019; Di Marzo and Matias 2005), suppressed gastric emptying in mice; this inhibitory effect was counteracted by the intraperitoneal injection of a CB1 antagonist. These results suggest that CB1 activation can suppress gastric emptying; however, the site of action

56   and its relationship with gastrointestinal hormone secretion remain unknown.

57   We have recently demonstrated that one of 2-MAG and endocannabinoids, 2-

58   arachidonoyl glycerol (2-AG), potently stimulates cholecystokinin (CCK) secretion via

59   CB1 in the murine enteroendocrine cell line, STC-1 (Ochiai *et al.* 2021). CCK, a

60   gastrointestinal hormone produced by enteroendocrine 'I cells' located in the upper small

61   intestine (Dockray 2012; Rehfeld 2000), plays a major role in suppressing gastric

62   emptying (Liddle *et al.* 1986). Furthermore, CB1 is reportedly expressed in mouse

63   duodenal CCK-producing cells (Argueta *et al.* 2019; Sykaras *et al.* 2012). Accordingly,

64   we hypothesized that 2-AG suppresses gastric emptying via CB1 activation and CCK

65   secretion in enteroendocrine cells.

66   Herein, to examine this hypothesis, we assessed the effects of orally administered

67   2-AG on the gastric emptying rate in mice using the acetaminophen absorption test and

68   the phenol red recovery method. In addition, we investigated the molecular and signaling

69   mechanisms involved in the 2-AG-induced effects.

70

71   **Materials and Methods**

72   *Animals and diet*

73   Male C57BL/6J mice (6-week-old) were purchased from Japan SLC

74   (Hamamatsu, Japan) and individually housed in a temperature- and humidity-controlled

75   room (22 ± 2°C, 55 ± 5%), maintained on a 12 h light-dark cycle (8:00-20:00 light period).

76   All animals had free access to water and were fed a laboratory chow containing 49.9%

77   carbohydrate, 24.8% protein, and 4.6% fat (CE-2, CLEA Japan Inc., Tokyo). Experiments

78   were performed after an acclimatization period of ≥ 1 week. Mice were fasted overnight

79   the day before the experiment. The study was approved by the Hokkaido University

80　　Animal Committee, and animals were maintained according to the guidelines for the care

81　　and use of laboratory animals at Hokkaido University (permission no. 19-0064).

82

83　　*Reagents*

84　　　　Arachidonic acid (20:4n-6) and acetaminophen were purchased from Sigma-

85　　Aldrich (St. Louis, MO, USA). 2-AG was purchased from Cayman Chemical (Ann Arbor,

86　　MI, USA). Devazepide, a cholecystokinin-A (CCK-A) receptor antagonist, was donated

87　　by ML Laboratories (Liverpool, UK), and SR141716A, a selective CB1 antagonist, was

88　　purchased from Tocris Bioscience (Ellisville, MO, USA). Unless otherwise specified, all

89　　other reagents were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka,

90　　Japan).

91

92　　*Acetaminophen test*

93　　　　Test agents were suspended in a vehicle composed of saline, containing 1.5%

94　　w/v carboxymethyl cellulose (CMC), 1% w/v acetaminophen, and 2% ethanol.

95　　Acetaminophen (100 mg/kg body weight) was used as an absorbable marker to assess the

96　　gastric emptying rate (Heading *et al.* 1973; Maida *et al.* 2008). Lipid (2-AG or 20:4n-6;

97　　25 mg/kg body weight)-containing test suspensions or the vehicle were orally

98　　administered at a dose of 10 mL/kg body weight. Tail vein blood samples were collected

99　　before (0 min) and 15, 30, 60, and 120 min after administration. Blood samples were

100　　immediately mixed with heparin (final concentration, 50 IU/mL; Nacalai Tesque, Inc.,

101　　Kyoto, Japan) and placed on ice. Plasma was separated by centrifugation at 2300 × *g* for

102　　10 min at 4°C, then stored at −80°C until analysis. Plasma acetaminophen concentrations

103　　were measured using an acetaminophen detection kit (Kanto Chemical Co., Inc., Tokyo,

104   Japan).

105   In a separate experiment, we compared the effects of oral and intraperitoneal 2-

106   AG on gastric emptying. Mice were divided into control, oral 2-AG, and intraperitoneal

107   2-AG groups. Control mice were intraperitoneally administered sterile saline (10 mL/kg)

108   containing 1.5% CMC and 2% ethanol immediately after oral administration of 10 mL/kg

109   acetaminophen (100 mg/kg) dissolved in the same vehicle. The oral 2-AG group was

110   intraperitoneally administered the same sterile saline solution (10 mL/kg; same as the

111   control group) immediately after orally administering 2-AG (25 mg/kg) suspended in the

112   acetaminophen solution. Finally, the intraperitoneal 2-AG group was intraperitoneally

113   administered 2-AG (25 mg/kg) suspended in sterilized saline containing 1.5% CMC and

114   2% ethanol (10 mL/kg) immediately after oral administration of acetaminophen solution.

115   Tail vein blood samples were collected, and plasma acetaminophen concentrations were

116   determined as described above.

117

118   *RT-PCR*

119   RNA was isolated from the mouse jejunum using a Fast Gene^{TM} RNA Premium

120   Kit (NIPPON Genetics, Tokyo, Japan) according to the manufacturer's instructions.

121   cDNA was prepared from 1 µg RNA using ReverTra Ace® qPCR RT Master Mix with

122   gDNA Remover (TOYOBO, Osaka, Japan) and subjected to PCR using primers based on

123   the mouse CB1 mRNA sequence (GenBank accession number NM001355020; forward

124   primer   5'-CCACCTTCCGTACCATCACC-3',   reverse   primer   5'-

125   AACCAACGGGGAGTTGTCTC-3')   and   mouse   glyceraldehyde-3-phosphate

126   dehydrogenase (GAPDH) mRNA sequence (GenBank accession number NM008084;

127   forward   primer   5'-TCACCACCATGGAGAAGGC-3',   reverse   primer   5'-

128 GCTAAGCAGTTGGTGGTGCA-3'). PCR conditions were as follows: 95°C for 2 min,

129 followed by 35 cycles of 95°C for 30 s, 63.3°C for 30 s, and 72°C for 30 s. In addition,

130 PCR products were separated by 1.5% agarose gel electrophoresis and visualized using

131 Midori Green Advance DNA stain (NIPPON Genetics, Tokyo, Japan).

132

133 *Phenol red test*

134   2-AG (25 mg/kg body weight) or the vehicle (1.5% w/v CMC and 2% ethanol

135 in saline) was orally administered (10 mL/kg body weight). The suspensions contained

136 phenol red (5 mg/kg body weight), as a non-absorbable marker, in addition to

137 acetaminophen (100 mg/kg body weight) to assess the gastric emptying rate (Feldman

138 and Gibaldi 1968; Nishimukai *et al.* 2003). Portal blood was collected into a syringe

139 containing heparin (final concentration 50 IU/mL), aprotinin (final concentration 500

140 KIU/mL), and dipeptidyl peptidase-IV inhibitor (final concentration 50 μM, Millipore,

141 MA, U.S.A.), 15 min after oral administration from mice under isoflurane anesthesia

142 (MSD K.K., Tokyo, Japan). Plasma was separated and stored as described previously.

143 Immediately after the procedure, mice were euthanized by exsanguination. The stomach

144 was removed after clamping both the distal end of the esophagus and the proximal end of

145 the duodenum. The stomach content was flushed twice with cold saline, and the washout

146 solution was then collected. The debris was removed by centrifugation at 8400 × *g* for 10

147 min at 4°C. After adding 1 N NaOH to the supernatant (1/10 volume of the supernatant),

148 the concentration of phenol red was spectrophotometrically measured at 560 nm. The

149 gastric emptying rate was calculated as follows:

150   Gastric emptying rate (%) = [{the amount of phenol red administered (mg) – the

151 amount of phenol red remaining in the stomach (mg)}/the amount of phenol red

152 administered (mg)] × 100

153

154 *Statistical analyses*

155   All values are expressed as mean ± SEM. Statistical analyses were performed

156 using JMP Pro version 14.0.0 software (SAS Institute, Inc., Cary, NC, USA). As described

157 in figure legends, significant differences among groups were determined using Student's

158 *t*-test to compare two groups, whereas Dunnett's post hoc test was employed to compare

159 multiple groups. Statistical significance was set at $p < 0.05$.

160

161 **Results**

162 *Oral administration of 2-AG delayed gastric emptying*

163   We first examined the effects of orally administered 2-AG or 20:4n-6 on gastric

164 emptying in fasted mice using an absorbable marker, acetaminophen (Fig. 1). In all groups,

165 plasma acetaminophen concentrations in tail vein blood increased immediately after oral

166 administration of test liquids, gradually decreasing after 15 or 30 min. Compared with the

167 vehicle, oral administration of 20:4n-6 significantly reduced the appearance of

168 acetaminophen in the tail vein blood at 15 min. In addition, oral administration of 2-AG

169 (25 mg/kg) significantly lowered acetaminophen levels after 15 min.

170

171 *Involvement of CCK-A receptor in the effect of 2-AG*

172   Next, we examined the involvement of the CCK-A receptor in mediating the

173 effects of oral 2-AG following the intraperitoneal administration of devazepide, a CCK-

174 A receptor antagonist. Compared with the control/vehicle treatment, a single oral

175 administration of 2-AG significantly reduced acetaminophen levels at 15 min (similar to

176 the result shown in Fig. 1) and 30 min (Fig. 2a). Conversely, oral 2-AG did not

177 significantly reduce acetaminophen levels following treatment with intraperitoneal

178 devazepide compared with orally administered vehicle (Fig. 2b).

179

180 *Involvement of CB1 in the effect of 2-AG*

181   2-AG is a ligand for CB1, an endocannabinoid receptor (Ueda *et al.* 2011;

182 Gendaszewska-Darmach *et al.* 2019). As determined by conventional PCR, single bands

183 were detected at the expected DNA size (182 bp) for CB1 in the mouse jejunum (Fig. 3a).

184   To elucidate the involvement of CB1, we next performed the acetaminophen test

185 by administering SR141716A, a selective CB1 antagonist. A single oral administration of

186 2-AG significantly reduced acetaminophen concentrations at 15 min compared with the

187 control group (Fig. 3b); however, no significant difference in acetaminophen levels was

188 observed between control and 2-AG groups treated orally with SR141716A (Fig. 3c).

189 Next, we examined the effect of the intraperitoneally administered SR141716A.

190 Treatment with intraperitoneal SR141716A suppressed the oral 2-AG-induced decrease

191 in acetaminophen levels at 15 min (Fig. 3d and e).

192

193 *Measurement of gastric emptying rate by phenol red test*

194   To further investigate the involvement of CB1 in the inhibitory effect of 2-AG

195 on gastric emptying rate, we performed experiments using an unabsorbable marker,

196 phenol red. We observed that the gastric emptying rate was significantly lower in the oral

197 2-AG group than in the control/vehicle group (Fig. 4a), thus further supporting the gastric

198 inhibitory effect of 2-AG. And no significant differences between the control and 2-AG

199 groups were observed following intraperitoneal treatment with SR141716A (Fig. 4b).

200   Similar to the phenol red test result, plasma acetaminophen levels in the portal vein were

201   significantly lower in the oral 2-AG group than in the control/vehicle group (Fig. 4c). In

202   contrast, plasma acetaminophen levels did not significantly differ between control and 2-

203   AG groups following intraperitoneal administration of SR141716A (Fig. 4d).

204

205   *Effect of intraperitoneal 2-AG*

206   Next, we examined the effect of intraperitoneal 2-AG (Fig. 5), given that

207   intraperitoneal administration of the CB1 antagonist attenuated 2-AG-induced effects

208   (Fig. 3 and 4). At an identical dose (25 mg/kg), both oral and intraperitoneal

209   administration of 2-AG significantly attenuated the elevation of plasma acetaminophen

210   concentrations at 15 min. Notably, intraperitoneal administration induced a considerably

211   greater and sustained (~30 min) reduction than oral administration. At 120 min, the

212   acetaminophen concentration in the intraperitoneal 2-AG group was significantly higher

213   than the control group.

214

215   **Discussion**

216   Notably, FA suppress gastric emptying in a carbon chain length-dependent

217   manner by stimulating the secretion of gastrointestinal hormones such as CCK (Hunt and

218   Knox 1968; McLaughlin *et al.* 1999); however, the effect of 2-MAG on gastric emptying

219   remains to be clarified. Accordingly, the objective of the present study was to examine

220   the effect of 2-AG, which is composed of 20:4n-6 and glycerol, on gastric emptying in

221   mice and to elucidate the mechanism underlying its effect. We observed that oral

222   administration of 2-AG (25 mg/kg) suppressed gastric emptying in mice. Furthermore,

223   the involvement of CCK and cannabinoid receptor CB1 was determined using antagonists

224 against CCK-A and CB1 receptors. These results provide novel insights into the 2-MAG-

225 sensing mechanism in enteroendocrine cells and afford a better understanding of the

226 physiological role of 2-MAG.

227 Oral administration of 2-AG (25 mg/kg) significantly reduced acetaminophen

228 levels at 15 min, similar to 20:4n-6 (25 mg/kg; Fig. 1); however, the time at which the

229 acetaminophen concentration peaked tended to differ between 2-AG (15 min) and 20:4n-

230 6 (30 min). In our previous recent study using the murine enteroendocrine cell line, STC-

231 1 (Ochiai *et al.* 2021), 2-AG was shown to stimulate CCK secretion via CB1, whereas

232 20:4n-6 stimulated CCK secretion via G protein-coupled receptor 120 (GRP120).

233 Although further studies are warranted to elucidate the mechanism underlying the effect

234 of 20:4n-6 *in vivo*, the present results (Fig. 1) suggest that 20:4n-6-rich TAG could

235 effectively exert inhibitory effects on gastric emptying.

236 The dose of 25 mg/kg 2-AG used in the present study was not supraphysiological,

237 as described below. According to previous reports (Hansen *et al.* 2012; Hansen and Vana

238 2019), westerners consume approximately 100 g of lipids (TAG) per day, equivalent to

239 33 g of lipids per meal on the consumption of three meals per day. Therefore, ingesting

240 33 g of lipid in a single meal could provide a maximum of 11 g of 2-MAG in the small

241 intestine. Accordingly, 11 g/55 kg body weight equals 200 mg/kg body weight, which is

242 considerably higher than the dose (25 mg/kg body weight) used in the present study.

243 Furthermore, a previous study has demonstrated enhanced GLP-1 secretion in humans

244 weighing 73-97 kg by administering 2 g of 2-OG into the jejunum (Hansen *et al.* 2011),

245 thus supporting the notion that the dose employed in the present study, i.e., 25 mg/kg (2

246 g/80 kg body weight), is within the physiological range.

247 CCK is mainly recognized by CCK-A receptors expressed on peripheral nerves

248 and inhibits gastric emptying (Herranz 2003). Treatment with a CCK-A receptor

249 antagonist, devazepide, reversed the gastric inhibitory effect of oral 2-AG (Fig. 2),

250 suggesting the involvement of CCK signaling. Our previous finding (Ochiai *et al.* 2021)

251 that 2-AG promoted CCK secretion in the CCK-producing cell line, STC-1 supports that

252 orally given 2-AG acts on CCK-producing enteroendocrine cells, namely, I cells, to

253 promote CCK secretion.

254   2-AG is an endocannabinoid, and previous reports have suggested that the

255 endocannabinoid system regulates food intake and energy balance through CB1 in the

256 brain and peripheral nerves (Di Marzo and Matias 2005; Sharkey and Pittman 2005; Osei-

257 Hyiaman *et al.* 2006). In the present study, we detected that CB1 was expressed in the

258 jejunum of mice (Fig. 3a). This result is consistent with previous findings that revealed

259 CB1 expression throughout the mouse gastrointestinal tract (Casu *et al.* 2003). CB1

260 expressed in the intestinal tract is likely involved in mediating the effect of oral 2-AG on

261 gastric emptying, as oral treatment with a CB1 antagonist, SR141716A, counteracted the

262 effect of oral 2-AG (Fig. 3b and c). Furthermore, CB1 expression has been confirmed in

263 murine CCK-producing cells (Argueta *et al.* 2019; Sykaras *et al.* 2012). We recently

264 demonstrated that 2-AG potently stimulates CCK secretion via CB1 expressed in the

265 CCK-producing cell line, STC-1 (Ochiai *et al.* 2021). Therefore, 2-AG might stimulate

266 CCK secretion by acting on CB1 expressed in CCK-producing enteroendocrine cells,

267 thereby suppressing gastric emptying.

268   Intraperitoneal CB1 antagonists also reversed the gastric inhibitory effect of oral

269 2-AG in the acetaminophen and phenol red tests (Fig. 3d, e, and 4). These findings suggest

270 that after absorption by intestinal epithelial cells, 2-AG suppresses gastric emptying by

271 acting on CB1 expressed on the basolateral side of CCK-producing cells or in peripheral

272 nerves. Consistent with this notion, intraperitoneal 2-AG potently suppressed gastric

273 emptying compared with oral 2-AG (Fig. 5). Furthermore, in a previous study (Marzo *et*

274 *al.* 2008), intraperitoneal AEA-suppressed gastric emptying was reversed following

275 intraperitoneal administration of a CB1 antagonist.

276   Treatment with a CCK-A receptor or CB1 antagonist did not completely

277 counteract the 2-AG-induced gastric emptying inhibition (Fig. 2, 3, and 4). These results

278 may be attributed to the insufficient inhibition of CCK-A or CB1 under experimental

279 conditions (although the doses used in the present study were selected based on previous

280 reports (Chen *et al.* 2012; Hira *et al.* 2015; Madsen *et al.* 2009; Orio *et al.* 2011; Marzo

281 *et al.* 2008)) or the involvement of additional mechanisms. A previous study (Burdyga

282 2004) has shown that vagal afferent neurons expressing CCK-A receptors also express

283 CB1; the expression of CB1 in the nodose ganglia is increased by fasting and inhibited

284 by CCK. Additionally, the CCK-A receptor antagonist, lorglumide, blocks the loss of CB1

285 expression in afferent neurons after refeeding (Burdyga 2004). Based on the results of

286 these previous reports and those noted in the present study, the following pathways are

287 potential mechanisms of action of 2-AG: When CCK-A receptors are inhibited, CB1

288 activation by 2-AG in peripheral nerves contributes to the suppression of gastric emptying.

289 On inhibiting CB1 on the peripheral nerves and the basolateral side of intestinal epithelial

290 cells, CCK release by 2-AG via activation of CB1 expressed on the apical side of I cells

291 contributes to suppressing gastric emptying. Treatment with a combination of the CCK-

292 A receptor antagonist and an oral or intraperitoneal CB1 antagonist could clarify these

293 possibilities in future studies.

294   Compared with oral administration (Fig. 5), intraperitoneal administration of 2-

295 AG, at the same dose (25 mg/kg), suppressed gastric emptying more potently and

296 continuously (~30 min). As the result of potent inhibition of gastric emptying, the plasma

297 acetaminophen concentration peaked much later (at 60 min) than other groups (peaked at

298 15 min). Accordingly, plasma acetaminophen concentration at 120 min was maintained

299 higher in the intraperitoneal 2-AG group than the other two groups. There are two possible

300 reasons for the more potent effect of intraperitoneal administration of 2-AG than oral 2-

301 AG. First, only a certain portion of orally administered liquid flows into the small

302 intestine over a short period, and the stomach and intestinal fluids dilute the liquid.

303 Second, only a fraction of 2-AG is transferred to the extracellular side of the basolateral

304 membrane, as 2-AG is degraded by hydrolytic enzymes such as monoacylglycerol lipase

305 expressed in the cell membrane and cytoplasm of intestinal epithelial cells (Dinh *et al.*

306 2002a; Dinh *et al.* 2002b; Blankman *et al.* 2007) and resynthesized into TAG

307 intracellularly (Mu and Høy 2004). Further studies are needed to confirm the presence of

308 2-AG in the lumen after oral administration and the extracellular transfer after absorption.

309 As rapid gastric emptying contributes to postprandial hyperglycemia and

310 lipidemia, the suppression of gastric emptying is a promising target for preventing or

311 reducing glucose intolerance and dyslipidemia. The limitation of the current study is that

312 the gastric inhibitory effect of 2-AG was not compared with that of other 2-MAG because

313 we primarily focused on *in vivo* effect of 2-AG. Although beyond the scope of our current

314 study, comparing their effects would provide valuable insights into the physiological role

315 of 2-MAG and warrant future investigation. Additionally, further studies are required to

316 verify the effects of dietary lipid-derived 2-MAG, including 2-AG, on postprandial blood

317 glucose levels and these diseases.

318 In conclusion, oral administration of 2-AG suppressed gastric emptying in mice,

319 as assessed by the acetaminophen and phenol red tests. Furthermore, studies using CCK-

320   A receptor or CB1 antagonists suggested the involvement of CCK and CB1 expressed on

321   peripheral nerves and CCK-producing enteroendocrine cells. These results demonstrate

322   that oral 2-AG suppresses gastric emptying in mice via the CB1-CCK and/or CB1

323   signaling pathways. Thus, our findings indicate a novel physiological interaction between

324   2-AG and neuroendocrine systems that regulate the gastric emptying rate.

325

326   **Author Contributions**

327   K.O. and T.H. conceived and designed the study and wrote the first draft of the

328   manuscript; K.O. performed the experiments; K.O. and T.H. analyzed the data. K.O., R.H.,

329   M.S., S.T., and T.H. contributed to and approved the final draft of the manuscript.

330

331   **Conflict of Interest**

332   The authors declare no conflict of interest.

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Figure legends

Fig. 1 Effects of oral administration of 2-AG or 20:4n-6 on gastric emptying. 2-AG or 20:4n-6 (25 mg/kg) was orally administered to fasted mice along with acetaminophen. Blood samples were collected from the tail vein for up to 120 min, and plasma acetaminophen concentrations were measured. Values are expressed as changes from the basal (0 min) concentration of plasma acetaminophen (ΔAcetaminophen) and as the mean ± SEM (n = 6). Plots with asterisks (*) show significant differences compared with the control group at the same time point ($^{*}p < 0.05$ and $^{**}p < 0.01$, Dunnett's test). 2-AG, 2-arachidonoyl glycerol; 20:4n-6, arachidonic acid

Fig. 2 Effects of 2-AG on gastric emptying in the absence (a) or presence (b) of the CCK-A receptor antagonist (devazepide). Devazepide (0.5 mg/kg) or vehicle (10% TWEEN80 + 10% DMSO in sterilized saline) was intraperitoneally injected at a dose of 5 mL/kg, 15 min before the oral administration of 2-AG (0 or 25 mg/kg) and acetaminophen (100 mg/kg) in fasted mice. Values are expressed as changes from the basal (0 min) concentration of plasma acetaminophen (ΔAcetaminophen) and as the mean ± SEM (n = 6). Plots with asterisks (*) show significant differences compared with the control group at the same time point ($^{*}p < 0.05$, Student's *t*-test). NS indicates that there was no significant difference between treatments. 2-AG, 2-arachidonoyl glycerol; Dvz, devazepide; CCK, cholecystokinin

Fig. 3 Expression of CB1 in the mouse jejunum (a) and effects of 2-AG on gastric emptying in the absence (b, d) or presence (c, e) of CB1 receptor antagonist (SR141716A). (a) Total RNA was extracted from the mouse jejunum and then subjected to RT-PCR with

specific CB1 or GAPDH primers. PCR products were separated in agarose gel and visualized by Midori Green Advance DNA stain. (b, c) 2-AG (25 mg/kg) was orally administered to fasted mice with SR141716A (10 mg/kg) or vehicle (saline containing 1.5% w/v CMC, 1% w/v acetaminophen, 10% TWEEN80, 10% DMSO, and 2% ethanol). (d, e) SR141716A (0.5 mg/kg) or vehicle (10% TWEEN80 + 10% DMSO in sterile saline) was intraperitoneally injected at a dose of 5 mL/kg, 15 min before the oral administration of 2-AG (0 or 25 mg/kg) and acetaminophen in fasted mice. Values are expressed as changes from the basal (0 min) concentration of plasma acetaminophen (ΔAcetaminophen) and as the mean $\pm$ SEM (n = 5-6). Plots with asterisks (*) show significant differences compared with the control group at the same time point ($^{*}p < 0.05$ and $^{**}p < 0.01$, Student's t-test). NS indicates that there was no significant difference between the treatments. 2-AG, 2-arachidonoyl glycerol

Fig. 4 Effects of 2-AG on the gastric emptying rate in the absence (a, c) or presence (b, d) of SR141716A (phenol red recovery method). 2-AG (25 mg/kg) was orally administered to fasted mice along with phenol red and acetaminophen, 15 min after the intraperitoneal injection of SR141716A (0.5 mg/kg) or vehicle (10% TWEEN80 + 10% DMSO in sterilized saline). Stomach contents and portal blood were collected 15 min after oral administration. (a, b) The gastric emptying rate was determined by measuring luminal phenol red collected from the stomach. (c, d) Acetaminophen levels in the portal plasma were measured. Values are expressed as the mean $\pm$ SEM (n = 5-6). Plots with asterisks (*) show significant differences compared to the control group ($^{*}p < 0.05$ and $^{**}p < 0.01$, Student's t-test). NS indicates that there was no significant difference between the treatments. 2-AG, 2-arachidonoyl glycerol

Fig. 5 Effect of intraperitoneal administration of 2-AG on gastric emptying. 2-AG (25 mg/kg) was orally or intraperitoneally coadministered (10 mL/kg) to fasted mice with oral acetaminophen. Values are expressed as changes from the basal (0 min) concentration of plasma acetaminophen (ΔAcetaminophen) and as the mean ± SEM (n = 6). Plots with asterisks (*) show significant differences compared to the control group at the same time point ($^*p < 0.05$ and $^{**}p < 0.01$, Dunnett's test). 2-AG, 2-arachidonoyl glycerol

Fig. 1

Fig. 2

(a)

(b)

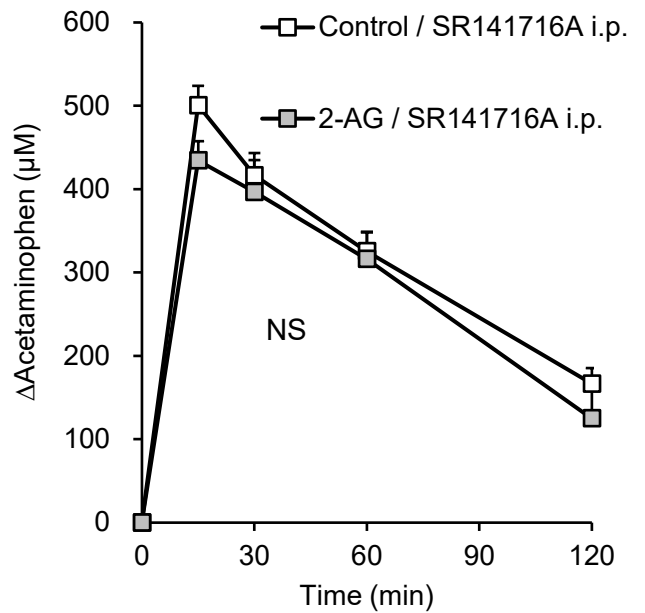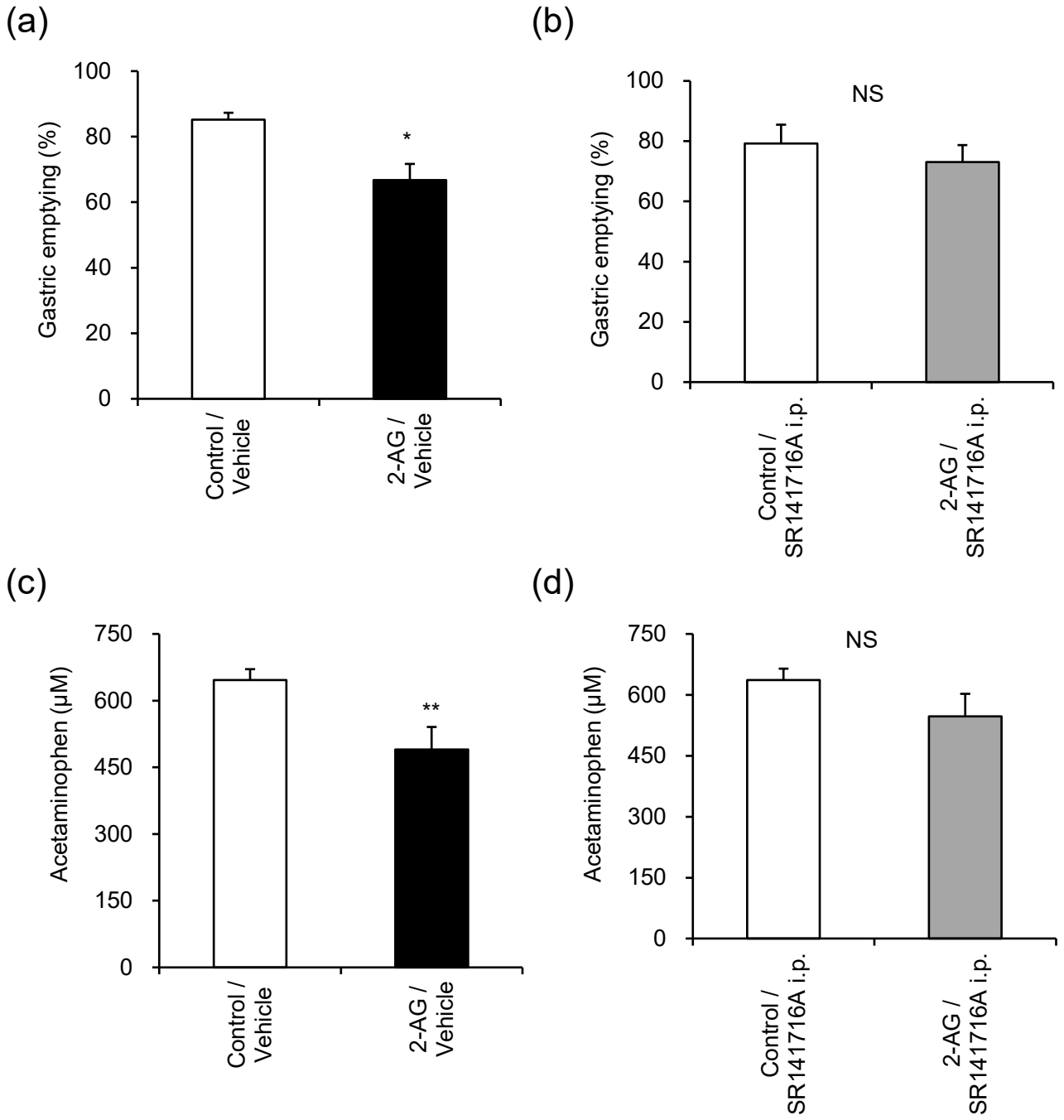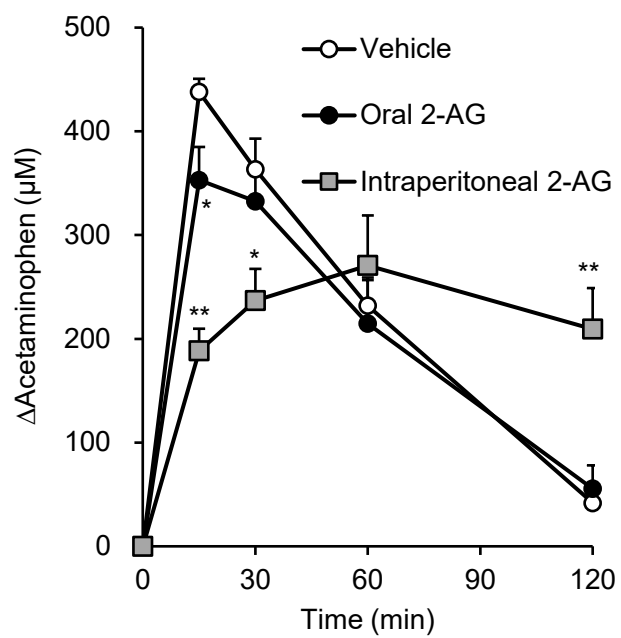# Fig. 3

Fig. 4

(a)

(b)

(c)

(d)
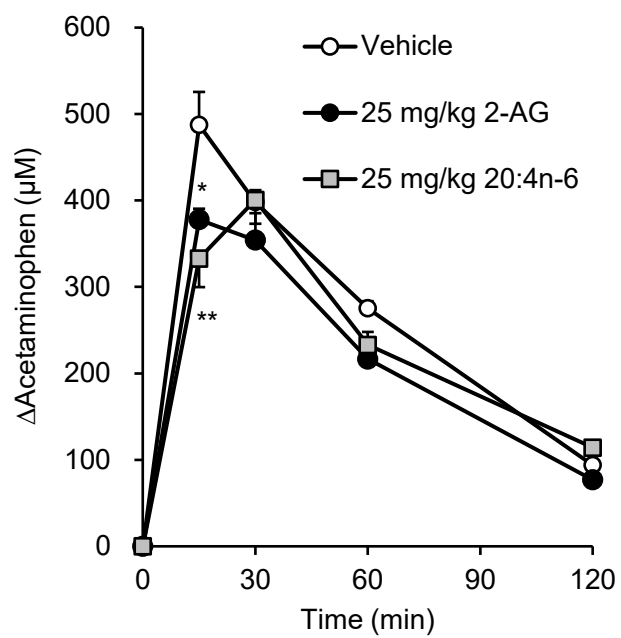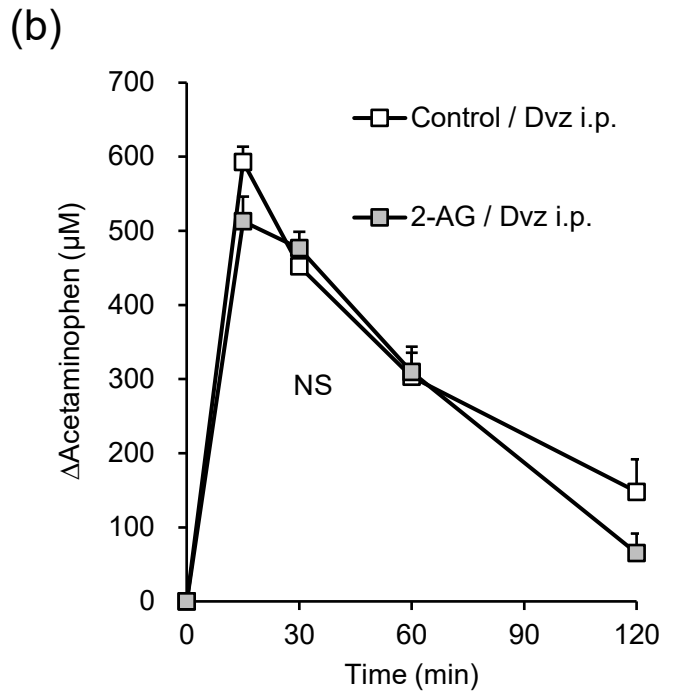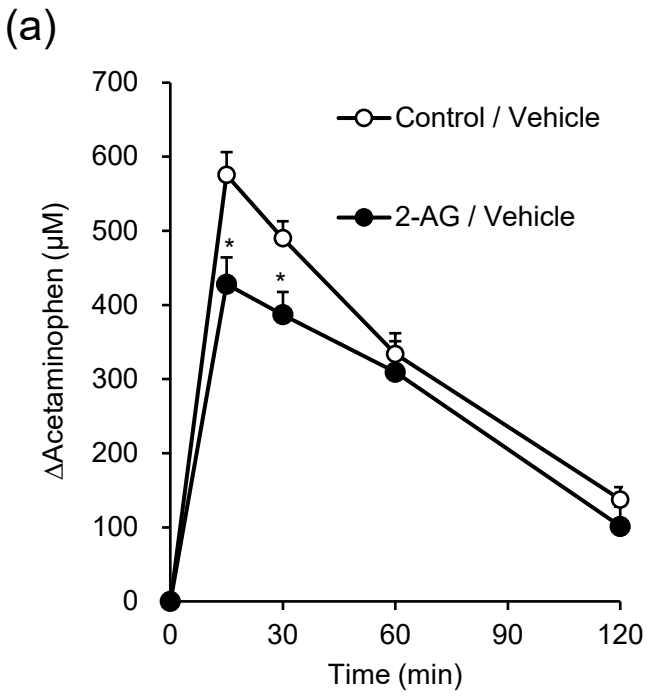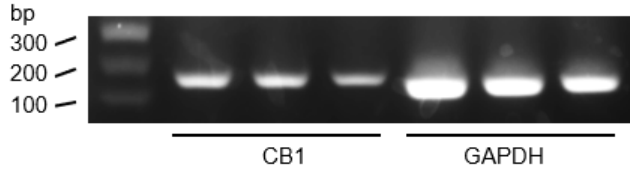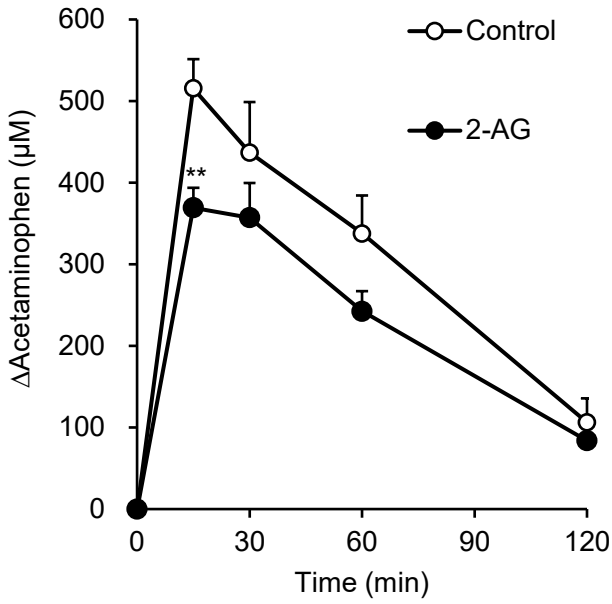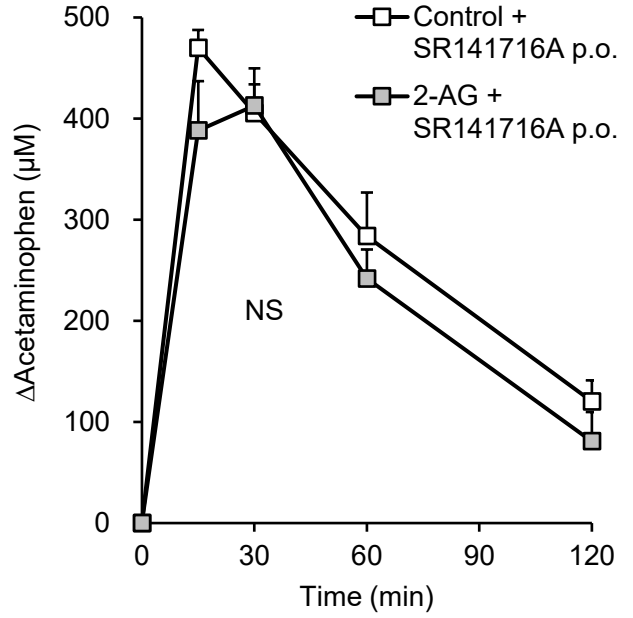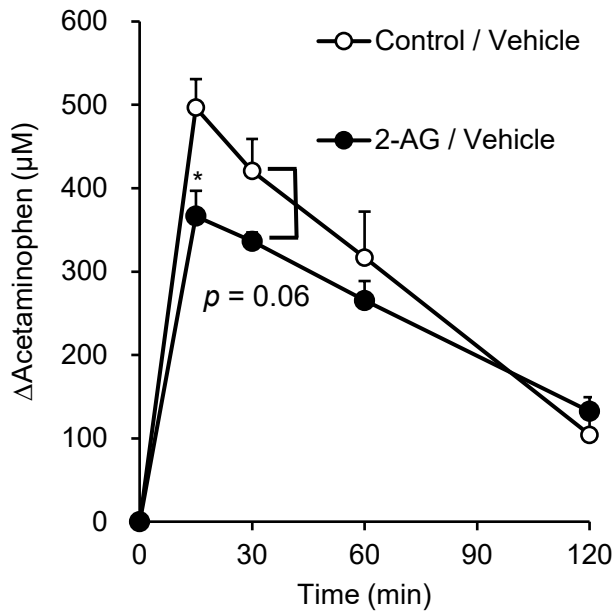
Fig. 5

Title

2-Arachidonoyl glycerol suppresses gastric emptying via the cannabinoid receptor 1-cholecystokinin signaling pathway in mice

Journal

Lipids

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