Phloretin suppresses GLP-1 secretion by modulating SCFA production by gut microbiome

（フロレチンは腸内細菌叢での短鎖脂肪酸産生を変化させ GLP-1 分泌を抑制する）

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Abstract

We previously found that glucagon-like peptide 1 (GLP-1) secretion by co-administration of maltose plus an α-glucosidase inhibitor miglitol (maltose/miglitol) was suppressed by a GLUT2 inhibitor phloretin in mice. In addition, maltose/miglitol inhibited glucose-dependent insulinotropic polypeptide (GIP) secretion through a mechanism involving short chain fatty acids (SCFAs) produced by microbiome. However, it remains unknown whether phloretin suppresses GLP-1 secretion by modulating SCFAs. In this study, we examined the effect of phloretin on SCFA release from microbiome *in vitro* and *in vivo*. In *Escherichia coli*, acetate release to the medium was suppressed by phloretin, when cultured with maltose/miglitol. In mice, phloretin inhibited maltose/miglitol-induced SCFA increase in the portal vein. In addition, alpha methyl-d-glucose (αMDG), a poor substrate for GLUT2, significantly increased GLP-1 secretion when co-administered with phloridzin in mice, suggesting that GLUT2 is not essential for glucose/phloridzin-induced GLP-1 secretion. αMDG increased portal SCFA levels, thereby suppressing GIP secretion in mice, suggesting that αMDG is metabolizable not for mammals, but for microbiota. In conclusion, phloretin is suggested to suppress maltose/miglitol-induced GLP-1 secretion via inhibiting SCFAs produced by microbiome. (175words)

Keywords phloretin, short chain fatty acids, glucagon-like peptide 1, gut microbiome

1. Introduction

Incretin is a hormone secreted from enteroendocrine cells on feeding, and potentiates glucose-induced insulin secretion [1] [2]. The main incretins comprise glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) secreted from enteroendocrine L-cells and K-cells, respectively. Especially, GLP-1 can ameliorate defective insulin secretion of diabetic patients and is an important molecular target for treating type 2 diabetes mellitus. Indeed, dipeptidyl peptidase-4 inhibitors (DPP4is) that inhibit GLP-1 inactivation and GLP-1 receptor agonists have been used worldwide for its treatment [3-5]. GLP-1 secretion is induced in response to oral nutrient ingestion. Among the nutrients, carbohydrate is a major stimulator for its release.

We previously elucidated the mechanism of sugar-induced GLP-1 secretion from L-cells, and found that ingestion of test meal supplemented with a disaccharide sucrose with an α glucosidase inhibitor (αGI) acarbose evokes GLP-1 secretion in humans [6]. We also presented in mice that oral administration of a disaccharide maltose plus another αGI miglitol evokes GLP-1 secretion at 30 min after loading [7]. Interestingly, maltose/miglitol-induced GLP-1 secretion was not suppressed by a sodium-glucose cotransporter (SGLT) inhibitor phloridzin [7], but by a glucose transporter 2 (GLUT2) inhibitor phloretin. We also found that stimulatory effect of two αGIs (acarbose and miglitol) on maltose-induced GLP-1 secretion differs quantitatively; more potent in miglitol than in acarbose. This is shown to be due at least in part to activation of SGLT3 in duodenal enterochromaffin (EC) cells by miglitol [7], while the other mechanism may also contribute to the difference.

In contrast to GLP-1, secretion of the other incretin GIP was shown to be suppressed, but not potentiated, by maltose/miglitol. We identified that un-digested, unabsorbed maltose in gut lumen is utilized by gut microbiome to produce SCFAs, which suppress GIP secretion through activating their receptor (GPR41) [8]. Intriguingly, not only maltose/miglitol-induced

suppression in GIP secretion, but also the increase in GLP-1 secretion were abolished in germ-free mice. Therefore, we assumed that GLP-1 secretion, as well as GIP secretion, is regulated by SCFAs produced by gut microbiome. Taken together with our finding that phloretin suppressed maltose/miglitol induced GLP-1 secretion [7], we hypothesized that phloretin may suppress maltose/miglitol-induced GLP-1 secretion by regulating SCFA production in gut microbiome.

In the present study, we examined the effect of phloretin on bacterial growth and SCFA production in *Escherichia coli* (*E. coli*), and its effect on plasma SCFA levels in the portal vein after maltose/miglitol administration in mice. Accordingly, we concluded that phloretin suppresses SCFA release from gut microbiome, thereby inhibiting maltose/miglitol-induced GLP-1 secretion. In addition, using α-methylglucopyranoside (αMDG), a glucose derivative but an unpreferred substrate for GLUT2, we showed that GLUT2 is not essential for glucose/phloridzin-induced GLP-1 secretion, despite that phloretin is well known to be a GLUT2 inhibitor.

2. Materials and methods

2.1. Reagents

Phloridzin and phloretin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-MDG was purchased from Tokyo Chemical Industry, Ltd. (Tokyo, Japan). Diprotin A was purchased from Peptide Institute, Inc. (Osaka, Japan). Miglitol was kindly provided by Sanwa Kagaku Kenkyusho Co. Ltd. (Nagoya, Japan).

2.2. Culture of *Escherichia coli* (*E. coli*)

For monitoring bacterial growth and acetate release into the medium, DH5α, an *E. coli* K12 strain, was cultured in M9 minimum medium (Merck KGaA, Darmstadt, Germany). For this aim, a single colony of DH5α was pre-cultured overnight in LB medium. The following day, DH5α was resuspended (at OD_{600} 0.1) in M9 minimum medium supplemented with or without 0.5 % (*w/v*) maltose, 2 *mM* miglitol, and 200 *μM* phloretin, and was cultured at 37 °C with shaking at 180 rpm. Bacterial growth was monitored at the timepoints depicted in Fig. 1A. Aliquots (100 μl) of the culture medium were collected at 6, 8, 10, and 12 hr after initiating culture, and were subjected to acetate measurement.

2.3. Measurement of acetate in M9 medium after culturing *E. coli*

To assess the production of acetate by DH5α, the aliquots of the culture medium were centrifuged at 15,000 rpm at 4 °C for 10 min and subjected to measuring acetate concentration using an acetic acid test kit (R-Biopharm AG, Darmstadt, Germany).

2.4. Animal experiments

C57BL/6J mice were used for measuring incretin secretion. The mice were maintained on normal standard chow diet (CE-2) (12.1 % kcal from fat) (CLEA Japan Inc., Tokyo, Japan)

and kept with a 12hr light/dark cycle. All animal studies were approved by the Animal Care and Use Committee of Chiba University.

2.5. Incretin secretion in *in vivo*

Sixteen-hour fasted mice were subjected to studies of incretin secretion. In brief, we collected blood samples at 30 min after disaccharide (maltose) plus α-GI (miglitol, 10 mg/kg), and at 30 min after glucose (2 g/kg) plus a SGLT-inhibitor phloridzin (500 mg/kg) or a GLUT2-inhibitor phloretin (500 mg/kg) administration. α-MDG (2 g/kg) plus phloridzin (500 mg/kg) were administered orally. At 30 min after ingestion, portal vein samples were collected under deep anaesthesia with 2 ~ 2.5 % isoflurane, and subjected to measurement of blood glucose, plasma GLP-1, and plasma GIP.

2.6. Measurement of GLP-1 and GIP

For measurement of GLP-1 and GIP, the blood sample from the portal vein was immediately mixed with EDTA (final 0.15% *w/v*) and Diprotin A (final 3 mmol/l). Concentrations of plasma GLP-1 and GIP were measured using Glucagon Like Peptide-1 (active) ELISA (Millipore, Billerica, MA, USA) and Rat/Mouse GIP (total) ELISA (Millipore), as previously described [9].

2.7. Measurement of SCFAs

To measure plasma SCFAs levels, the blood was collected from the portal vein and plasma sample was separated by centrifugation and plasma levels of the SCFAs (acetate, propionate, butyrate, valerate, iso-butyrate, and iso-valerate) in the portal vein were measured as previously described [10].

2.8. **Statistics**

Results are expressed as means ± SEM. Differences between two groups were assessed using the unpaired two-tailed Student's *t*-test unless otherwise noted. Data sets involving more than two groups were assessed by One-way ANOVA or Two-way ANOVA (GraphPad Prism software, version 7.0). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phloretin inhibited acetate release from *E. coli* when cultured in a maltose-containing medium.

Maltose/miglitol induced-GLP-1 secretion was significantly suppressed by phloretin [7]. At that time, we interpreted that phloretin inhibits GLUT2 in L-cells, directly suppressing GLP-1 secretion. In the present study, we raised another possibility that GLP-1 secretion may be suppressed by phloretin via its action on gut microbiome. To test this, we examined whether phloretin affects bacterial growth of an *E. coli* K-12 strain DH5α in the medium containing maltose, maltose/miglitol, or maltose/miglitol/phloretin. DH5α grew in the medium containing maltose as the only carbon source, and the growth was not suppressed by miglitol (Fig.1A). Contrary to our expectation, phloretin suppressed the growth only marginally in the end of logarithmic phase, suggesting that phloretin has little impact on the growth of *E. coli* by maltose.

Next, we cultured *E. coli* in the medium containing maltose, maltose/miglitol, or maltose/miglitol/phloretin, and measured acetate concentrations in the medium over time (at 6, 8, 10, and 12 hr) (Fig.1B). When DH5α was cultured in the maltose-containing medium, the acetate concentrations in the medium showed a transient rise, peaking at 10 hours after initiating culture, suggesting that acetate generated by *E. coli* was released into the medium, and was taken up again by *E. coli* to be utilized as an energy source. The timepoint of acetate depletion in the medium (12 hr) coincided with the cessation of bacterial growth, suggesting that the growth cessation is attributable to depletion of the carbon source in the medium, as was reported previously [11]. The presence of miglitol did not affect the acetate release. However, when *E. coli* was cultured with phloretin, acetate appeared in the medium with a similar time course to that without phloretin, but with much lower peak levels, suggesting that phloretin may change maltose metabolic pathway in *E. coli* or acetate release from *E. coli*.

3.2. Phloretin significantly attenuated the increase in SCFAs levels in the portal vein at 30 min after oral maltose/miglitol administration in mice.

Considering that not only *E. coli*, but a wide variety of gut microbiome reside in gut lumen in mice and humans, we next measured plasma SCFA levels in mouse portal vein to see if phloretin affects SCFA production in *in vivo*. For this aim, we orally administrated maltose/miglitol with or without phloretin, and measured SFCA levels (Fig. 2) in the portal vein at 30 min after loading. We previously reported that oral administration of maltose/miglitol to mice increased the plasma levels of SCFAs in the portal vein including acetate, propionate, and butyrate [8]. Interestingly, phloretin suppressed the plasma levels of most SCFAs, but increased that of acetate. Although this result of mice *in vivo* was discordant with that of *E. coli*, we think the result in mice is likely to represent the actual gut condition in humans.

3.3. Phloretin did not attenuate the maltose/miglitol-induced suppression of GIP secretion in mice.

Since phloretin suppressed the SCFAs production by microbiome in mice *in vivo*, we postulated that GIP secretion might be de-suppressed by co-administration of phloretin with maltose/miglitol. There was no difference in blood glucose between vehicle and phloretin-treated group (Fig. 3A). However, contrary to our expectation, while plasma GIP levels in the portal vein significantly suppressed by phloretin compared with control (vehicle) (Fig. 3B), suggesting that the increased acetate production by phloretin may contribute to the suppression of plasma GIP levels.

3.4. αMDG, a glucose derivative barely transported by GLUT2, increased GLP-1 and portal SCFA levels, but decreased GIP in the portal vein in mice.

We previously considered that suppression of maltose/miglitol-induced GLP-1 secretion by phloretin is mediated by its inhibitory action on GLUT2 in L-cells. GLUT2 protein is known to be expressed at the basolateral membrane of the intestinal absorptive epithelial cells. However, it has been reported that after meal ingestion, when the concentration of glucose levels increases, GLUT2 protein is recruited to the apical membrane to allow bulk of glucose absorption [12] [13]. On the other hand, Röder and co-workers reported that high luminal glucose concentrations administrated by glucose gavage (4g/kg) did not affect GLUT2 protein expression in brush border membrane fractions [14]. Therefore, the concept for GLUT2 translocation into apical membrane by glucose gavage remains elusive.

To clarify the involvement of GLUT2 translocation into the apical membrane in glucose/phloridzin-induced GLP-1 secretion, we utilized αMDG, a glucose derivative that is a preferred substrate of SGLTs, but not of GLUT2. When we treated the mice with αMDG/phloridzin, blood glucose levels in the portal vein were lower than those of control (vehicle) (Fig.4A), suggesting that phloridzin-induced glycosuria may contribute to this as was seen in the mice treated by glucose/phloridzin.

Intriguingly, αMDG/phloridzin significantly induced GLP-1 secretion (Fig. 4B), suggesting that GLUT2 is not essential for glucose/phloridzin-induced GLP-1 secretion. In addition, αMDG is a non-metabolizable glucose derivative, and cannot be used as the substrate for the production of ATP via glycolysis and oxidative phosphorylation in mammalian. However, as αMDG/phloridzin induced GLP-1 secretion at 30 min after oral loading, we postulated that αMDG may be used by gut microbiota as an energy substrate. Then, we measured SCFA levels in the portal vein of mice after αMDG/phloridzin administration. As expected, several SCFAs in the portal vein were significantly increased (Fig.4C), indicating that αMDG is metabolizable as an energy substrate in some gut microbiota.

Finally, we evaluated GIP secretion at 30 minutes after αMDG/phloridzin administration, since we previously found that glucose/phloridzin suppresses GIP secretion through the action of gut microbiota ([8]. Consistent with the results of SCFAs, GIP secretion was significantly decreased (Fig.4D). Taken together, these results suggest that αMDG, when co-administered with phloridzin, alters incretion secretion through the SCFAs produced by gut microbiota, although αMDG cannot be utilized by the host.

4. Discussion

We previously found that maltose administration with miglitol, an α-glucosidase inhibitor, induced GLP-1 secretion, which was inhibited by a GLUT2 inhibitor phloretin [7]. At that time, we considered the possible involvement of GLUT2 of L-cells in the secretion. This is compatible with the report by Mace *et al.* [15], in which GLUT2 was shown to participate in glucose-induced GLP-1 secretion from the isolated loops of rat small intestine. However, we later found that maltose/miglitol suppressed GIP secretion, which was mediated by SCFAs produced in gut microbiome [8]. Therefore, in the present study, we hypothesized that the suppression of maltose/miglitol-induced GLP-1 by phloretin is mediated by its action on gut microbiome.

Unlike mammalians, gut microbiome can transport oligodextrins including maltose through several sugar transporters. Among various gut microbiota, transport system of maltose is the best characterized in *E. coli*. Maltose transport system of *E. coli* consists of three components; an outer membrane porin (LamB, also called maltoporin) [16] [17], a periplasmic maltose-binding protein, MalE [18] [19], and an inner membrane ABC transporter, MalFGK2. Maltose is also known to be transported by the trehalose phosphoenolpyruvate (PEP)-sugar phosphotransferase (PTS) system [20]. Therefore, if phloretin inhibits any of these apparatuses for maltose transport in *E. coli*, maltose entry to cytoplasm is considered to be decreased. However, the maltose-induced growth of *E. coli* was not suppressed by phloretin in our study, suggesting that maltose transport to cytoplasm and its utilization is not inhibited by phloretin. By contrast, phloretin suppressed the acetate release from *E. coli*, when grown in the medium containing maltose as the only carbon source. Furthermore, phloretin did not affect the temporal profile of acetate appearance in the medium, suggesting that phloretin does not inhibit the uptake of maltose, but affects acetate release in *E. coli*.

Phloretin is a dihydrochalcone contained in an apple tree leaves [21]), and is a strong inhibitor of GLUT2. Therefore, phloretin has been widely used as a GLUT2 blocker in many pharmacological studies. In addition, phloretin has been reported to have anti-oxidative, anti-inflammatory, anti-microbial, anti-allergic, anticarcinogenic, anti-thrombotic, and hepatoprotective actions in mammalians [22]. By contrast, actions of phloretin on gut microbiome have been studied so far. Nevertheless, in *Plasmodium falciparum*, phloretin was reported to suppress acetate export through a lactate/H^+ symporter PfFNT [23]. Accordingly, phloretin may suppress acetate release from *E. coli* by inhibiting FocA, a bacterial gene that has the highest structural homology to PfFNT in Plasmodium, although we could not elucidate this possibility in our current study.

Recent studies have clarified that metabolites produced from diet by gut microbiome diversely influence energy homeostasis in the hosts [24]. SCFAs are products made of indigestible diet by fermentation in gut microbiome. A growing body of evidence suggests that SCFAs affect not only microbiome but also host metabolism via activation of its receptor G-protein coupled receptors, including GPR41 and GPR43. GPR41 and GPR43 are activated by propionate, butyrate and acetate [25] [26]. GPR43 activation by SCFAs induced GLP-1 from L-cells [27] [28] [29]. Therefore, phloretin-induced suppression of SCFA production is likely to contribute to the decreased GLP-1 secretion. However, the mechanism for further suppression of maltose/miglitol-induced GIP suppression by phloretin remains unknown in this study. We speculate that the increase, but not the decrease in acetate by phloretin may contribute to the suppression of GIP. However, we need to investigate further the mechanism underlying the relationship between specific SCFAs and incretin secretion in the future. In addition, interestingly, αMDG that were not metabolized in the host significantly induced GLP-1, while GIP secretion was inhibited along with increased the production of SCFAs. Thus, our present results demonstrate that oral compounds administered to the hosts with a certain aim

to elicit pharmacological actions may act also on the gut microbiome in different manners, exerting unexpected physiological responses to the hosts via an indirect host/microbiome circuit.

relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Fig. 1. Bacterial growth and acetate release of *E. coli*.

Growth curve (1) and acetate concentrations in the medium (2) of *E. coli* cultured in different medium. Circles in white, yellow, red, and green denote OD_{600} at indicated time-points of vehicle, maltose, maltose/miglitol, and maltose/miglitol/phloretin, respectively. (1) Bars denote means ± SEM (n = 4). (2) **P < 0.01 by one-way ANOVA with Tukey's *post hoc* analysis (n = 4).

Fig. 2. Effect of phloretin on plasma SCFAs.

Plasma SCFAs (A) (n = 6-7) levels in the portal vein are shown. *P <0.05, ***P <0.001 by unpaired-Student t-test.

Fig. 3. Effect of phloretin on blood glucose and plasma levels of GIP in the portal vein.

Blood glucose (A) (n = 6-8), and plasma GIP (B) (n = 6-8) levels in the portal vein are shown. ***P <0.001 by unpaired-Student t-test.

Fig. 4. Effect of αMDG on blood glucose, plasma levels of GLP-1, SCFAs, and GIP in the portal vein.

Levels of blood glucose (A), GLP-1 (B), SCFAs (C), and GIP (D) in the portal vein (n = 4-9) are shown. *P <0.05, **P < 0.01, by unpaired-Student t-test.

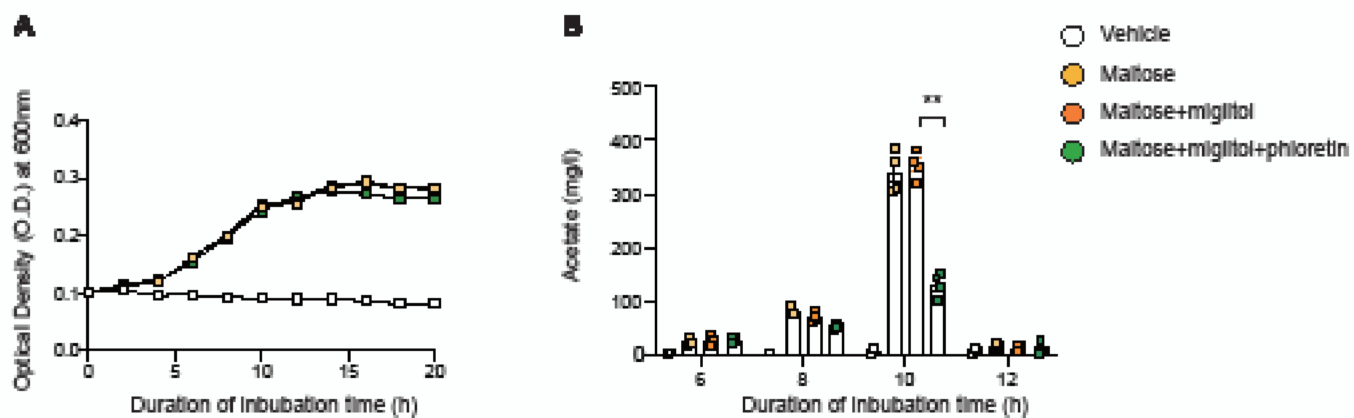

Fig. 1 Ma *et al*.

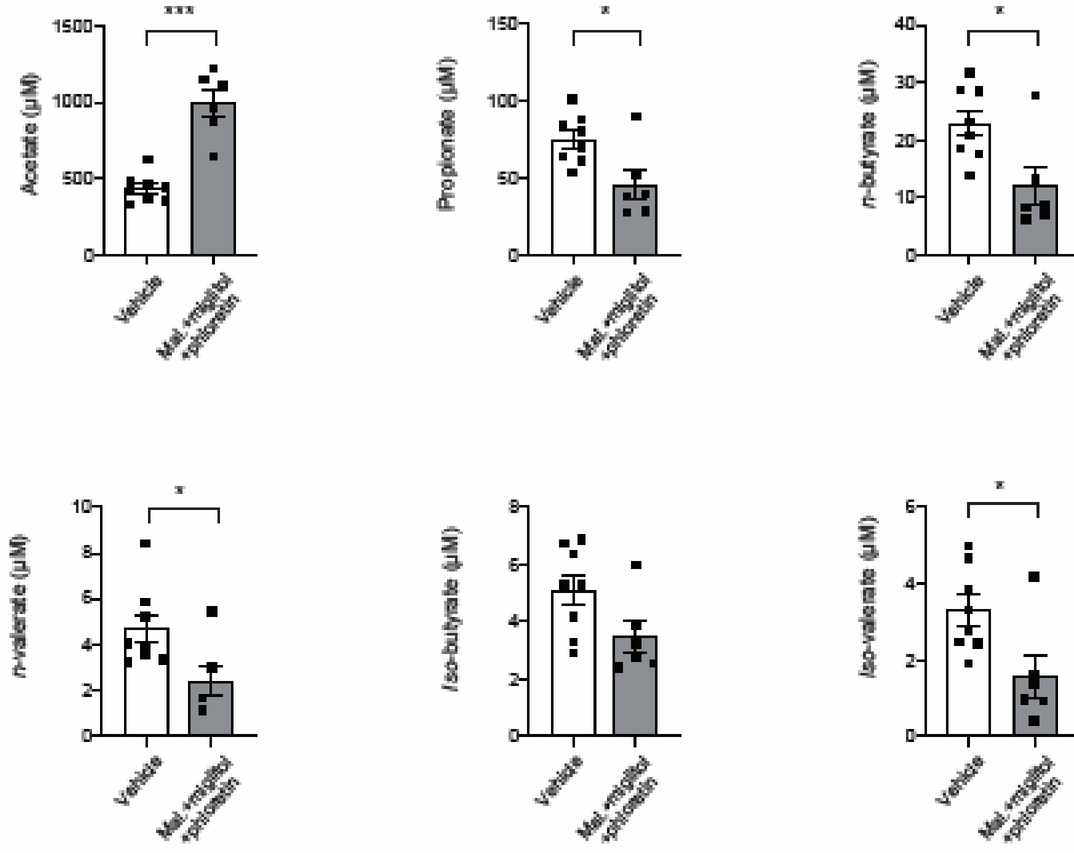

Fig. 2 Ma *et al*.

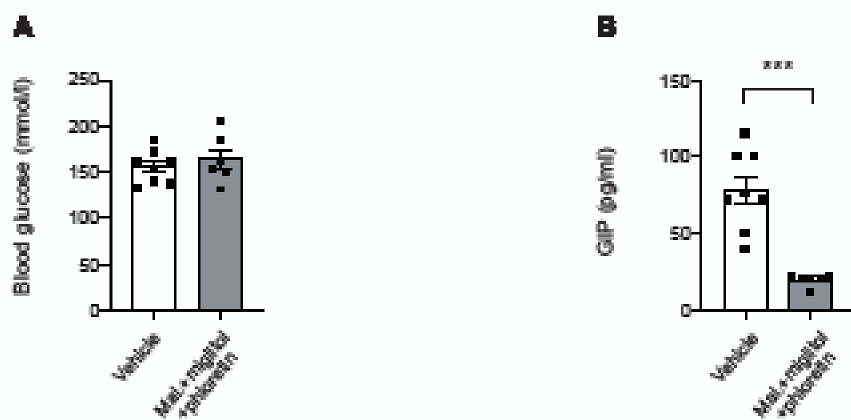

Fig. 3 Ma *et al.*

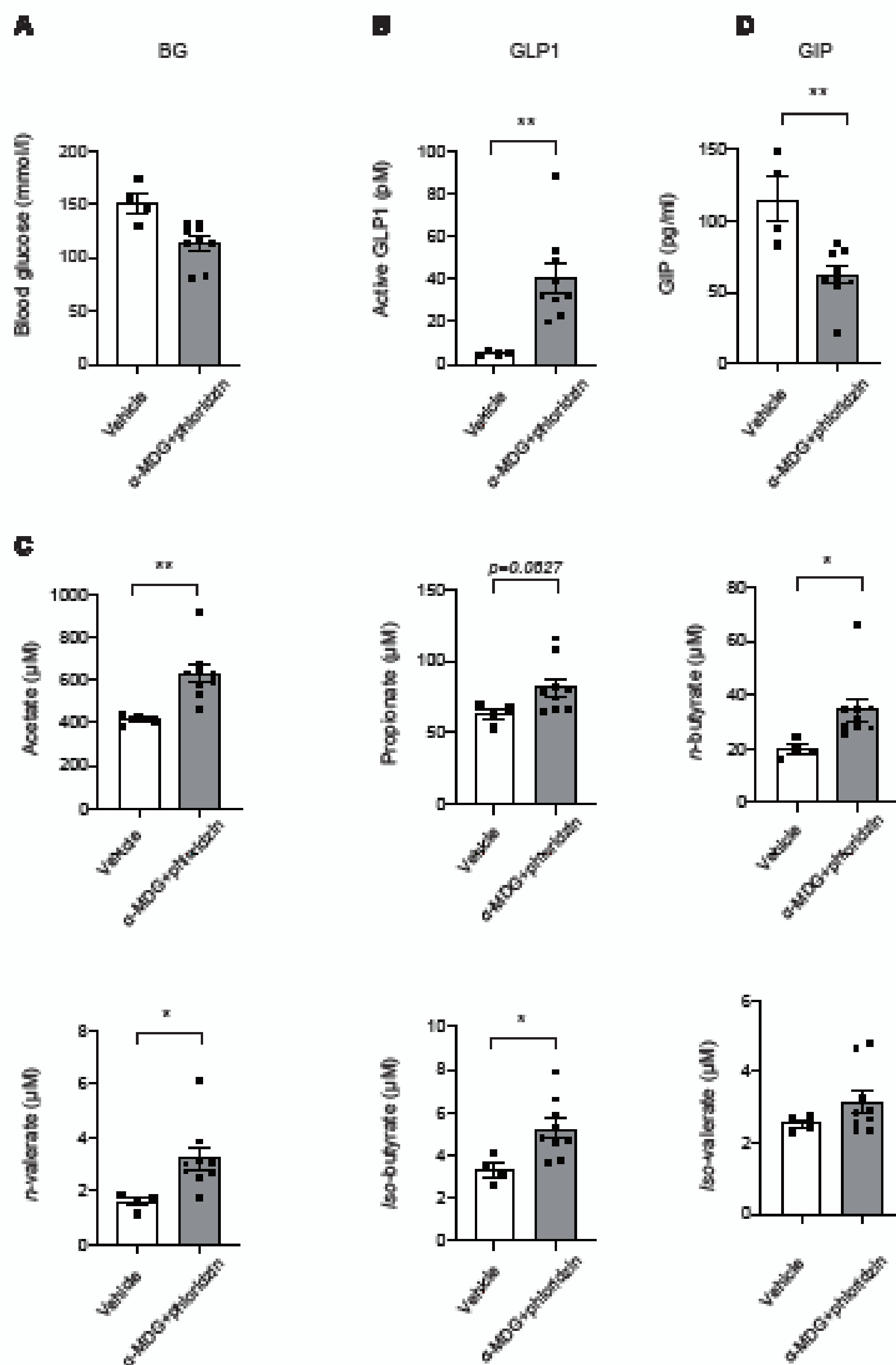

Fig. 4 Ma *et al*.

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