Increased expression of glucagon-like peptide-1 and cystic fibrosis transmembrane conductance regulator in the ileum and colon in mouse treated with metformin

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Abstract. Metformin, an oral medication, is prescribed to patients with type 2 diabetes mellitus. Although the efficacy, safety, and low economic burden of metformin on patients have long been recognized, approximately 5% of the patients treated with this drug develop severe diarrhea and discontinue the treatment. We previously reported that 1,000 mg·kg⁻¹·day⁻¹ of metformin induced diarrhea in diabetic obese (db/db) mice and wood creosote (traditional medication for diarrhea) ameliorated the symptoms. In this study, we attempted to elucidate the molecular mechanisms by which metformin induces diarrhea. Cystic fibrosis transmembrane conductance regulator (CFTR) is a key ion (chloride) channel in cyclic adenosine monophosphate (cAMP)-induced diarrhea. Metformin treatment increased bile flow (bile acids and bilirubin) in the ileum of mice. In addition, the treatment was accompanied by an increase in mRNA and protein levels of CFTR in the mucosa of the ileum and colon in both wild-type (C57BL/6J) and db/db mice. Glucagon-like peptide-1 (GLP-1), as well as cholic acid, induces CFTR mRNA expression in human colon carcinoma Caco-2 cells through cAMP signaling. Although wood creosote (10 mg/kg) ameliorated diarrhea symptoms, it did not alter the mRNA levels of Glp-1 or Cftr. Similar to overeating, metformin upregulated GLP-1 and CFTR expression, which may have contributed to diarrhea symptoms in mice. Although we could not identify db/db mouse-specific factors associated with metformin-induced diarrhea, these factors may modulate colon function. Wood creosote may not interact with these factors but ameliorates diarrhea symptoms.

Key words: Metformin, Diarrhea, Glucagon-like peptide-1, Cystic fibrosis transmembrane conductance regulator, Cyclic adenosine monophosphate

METFORMIN is a medication prescribed to treat type 2 diabetes mellitus [1]. It attenuates mitochondrial electron transport systems, which increases the intracellular adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio, and subsequently activates AMP-activated kinase (AMPK) [2]. Active AMPK inhibits gluconeogenesis, stimulates glucose consumption, and improves insulin resistance. Despite the well-established clinical benefits of metformin, its adverse effects are significant. For example, digestive disorders (diarrhea and vomiting) are reported in approximately 30% of the patients who have been prescribed metformin. Moreover, 5% of patients discontinued treatment after experiencing severe symptoms [3].

Retrospective studies have revealed several risk factors for metformin-induced diarrhea, such as daily dose, age, sex, body mass index, and liver and biliary disorders [4]. However, no digestive disorders have been observed in experimental animal models. In the interview form of metformin, diarrhea is documented as an adverse event in mice or rats when it was treated at a half lethal dose (PubChem CID: 14219). Recently, we established a
mouse model of metformin-induced diarrhea in diabetic obese (db/db) mice that shared several risk factors (body mass index and liver and biliary disorders) with human patients [5]. Overeating is a typical feature in db/db mice.

Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride (Cl⁻) channel on the apical plasma membrane that is involved in water absorption and re-flux. An increase in intracellular cyclic AMP (cAMP) levels activates CFTR through protein kinase A-dependent phosphorylation, which leads to an increase in the rate of Cl⁻ ions passage to the luminal side [6]. The accumulation of Cl⁻ ions increases osmotic pressure, which inhibits water absorption and induces water re-flux to the lumen of the alveolus, small intestine, and colon. Cholera toxins and bacterial enterotoxins are examples for the cAMP-mediated diarrhea via the actions of CFTR [7].

After meals, glucagon-like peptide-1 (GLP-1) is processed from proglucagon, the precursor, in the L-cells of the small intestine and then secreted [8]. When GLP-1 binds to its receptor on pancreatic β cells, intracellular cAMP levels increase, which promotes calcium ion-dependent insulin secretion. Because metformin also upregulates GLP-1 secretion in the small intestine [9, 10], the drug may increase cAMP levels in the gastrointestinal tract. Thus, GLP-1 secretion from the L-cells may be a mechanism by which metformin induces diarrhea [11-14].

Here, we demonstrate that peroral treatment with metformin upregulates Glp-1 messenger ribonucleic acid (mRNA) levels and Cftr gene expression in mice. Although metformin induced diarrhea only in db/db mice [5], the mRNA levels of Glp-1 and Cftr in the ileum did not differ between the wild-type and db/db mice. In contrast, Cftr mRNA levels in the colon were higher in db/db mice than in wild-type mice, despite having no difference in Glp-1 mRNA levels. The traditional medication wood creosote ameliorates diarrheal symptoms induced by metformin without affecting Glp-1 or Cftr mRNA levels.

Materials and Methods

Mouse experiments

All experimental procedures were approved by the Animal Committee of Gifu University (H30-040). C57BL/6J and db/db mice (10 W, males) were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under a 12 h light/dark cycle (08:00–20:00).

Metformin hydrochloride (COMBI-BLOCKS, San Diego, CA, USA) and wood creosotes were re-suspended in 0.5% Tween 80 (Kanto Kagaku, Tokyo, Japan). The drugs were orally administrated at 8:00–9:00 am. Recovery of organs was performed under anesthesia with 3% isoflurane (Wako, Osaka, Japan) and they were fixed with 4% paraformaldehyde/phosphate buffer saline (PBS, Wako) for 1 h.

The intestinal mucosal fractions were peeled off from the muscle layers by rubbing two glass slides. The recovered fractions were directly suspended in the RNA isolation buffer RL (Fast Gene RNA Premium kit, Nippon Genetics, Tokyo, Japan) for mRNA analysis and 3 × sodium dodecyl sulfate (SDS) buffer (75 mM Tris-HCl [pH 6.5], 0.3% SDS, 12.5% glycerol, 0.25% bromophenol blue, and 10% 2-mercaptoethanol) for protein analyses.

Quantification polymerase chain reaction (qPCR) and microarray analyses

Human colon adenocarcinoma Caco-2 cells (American Type Culture Collection, ATCC) cultured on 6-well plates and mouse tissues (~10 mg) were used for RNA extraction. cDNA was synthesized from 1 μg total RNA using 40 units of RNase inhibitor (Toyobo, Osaka, Japan), 25 pmol of random primer, 4 μL of 5 × RT buffer, 2 μL of 10 mM deoxynucleotides, and 100 units of ReverTraAce (Toyobo, Osaka, Japan) by incubating at 37°C for 10 min and 42°C for 1 h, followed by denaturation at 99°C for 5 min. The cDNA was amplified in a reaction mixture (20 μL) containing Thunderbird SYBR qPCR Mix (Toyobo) and 3 pmol of each forward and reverse primer using MyiQ (Bio-Rad, Hercules, CA, USA). qPCR analysis was carried out under the following conditions: initial denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 58°C for 30 s, and a final extension at 72°C for 34 s. mRNA levels were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA. The primers used are shown in the Supplementary Table (https://www.researchgate.net/publication/360298386).

Microarray analyses were performed using Mouse GE 4 × 44 K V2 (Agilent Technologies, Santa Clara, CA, USA). The data was analyzed using GeneSpring software (TOMY digital biotechnology, Tokyo, Japan). The raw data were submitted to the Gene Expression Omnibus (GEO) database (Series GSE201039).

Western blotting

Caco-2 cells were cultured onto 12-well plates, and the cells were collected in 2 mL tubes and lysed with 10 volumes of 3 × SDS buffer, and the lysate was heated at 98°C for 10 min and loaded into SDS polyacrylamide gels. The proteins were transferred into polyvinylidene difluoride membranes (Merek, St. Louis, MI, USA). The membrane was blocked with 0.5% skim milk (Wako) and incubated with the antibodies: anti-phospho-cAMP response element-binding protein (CREB) (87G3:
pSer133), and anti-GAPDH (1E6D9), purchased from Cell Signaling Technology (Danvers, MA, USA) and Proteintech (Rosemont, IL, USA), respectively. Anti-CFTR mouse-mono (CF3) and anti-CRTC2 (CREB regulated transcription coactivator 2: NB2-22356SS) were purchased from Novus Biologicals (Centennial, CO, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from MBL (Aichi, Japan) and Abcam Japan (Tokyo, Japan), respectively. Band intensity was measured using a ChemiDoc Image System (BioRad, Hercules, CA, USA). To load the equal amount of protein, signal values of GAPDH were used for the adjustment of sample volumes.

**Immunohistochemistry and immunocytochemistry**

The intestine and colon were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 3 h and washed twice with PBS. We outsourced the paraffin embedding and thin sections to BioGate Inc. (Gifu, Japan). After deparaffinization, the sections were blocked with 20% of BlockOne reagent (Nacalai Tesque, Kyoto, Japan) in PBS/0.05% Tween 20 and subjected to antibody reaction. Fluorescent (Alexa-488 and Alexa-594)-conjugated secondary antibodies were purchased from Abcam Japan (Tokyo, Japan). 4',6-diamidino-2-phenyindole (DAPI) was purchased from Nacalai Tesque. For immunocytochemistry, the cells were fixed with 4% paraformaldehyde in PBS for 15 min.

**Caco-2 assay**

Caco-2 cells were purchased from American Type Culture Collection and maintained in Eagle’s Minimum Essential Medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells that had been differentiated for one week after confluence were treated with metformin, GLP-1, or exendin-4 (~10 nM; Cayman Chemical, Ann Arbor, MI, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from MBL (Aichi, Japan) and Abcam Japan (Tokyo, Japan), respectively. Band intensity was measured using a ChemiDoc Image System (BioRad, Hercules, CA, USA). To load the equal amount of protein, signal values of GAPDH were used for the adjustment of sample volumes.

**Total bilirubin and bile acids measurement**

The mucosa fractions were suspended in five times volumes of PBS and centrifuged (12,000 g, 2 min); the supernatant was used to quantify total bilirubin and bile acids. The specific amount was normalized by protein concentration using the BCA method with bovine serum albumin (BSA) as a standard. Deproteinization was performed as follows: 300 μL of the sample in PBS was mixed with 700 μL of acetonitrile (Kanto Chemical, Tokyo, Japan) and stirred for 1 min. The protein was then removed by centrifugation (12,000 g for 5 min). The supernatant was dried by evaporation at 80°C, and precipitates were resuspended in 100 μL of PBS.

Total bilirubin levels were measured using CicaLiquid T-BIL (Kanto Kagaku). 80 μL of Reagent 1 were plated in a 96-well plate, to which 4 μL of the deproteinization sample, the diluted bilirubin standard, and PBS (as blank) were added, and the plate was incubated at 37°C for 5 min. Then, 20 μL of Reagent 2 were added and incubated at 37°C for 5 minutes, and absorbance (450 nm) was measured using a microplate reader (Glomax Multi Detection System, Promega Corporation, Madison, WI, USA) every minute for ten minutes. Bilirubin amount was calculated by the reduction of the absorbance.

Total bile acid levels were measured using the Total Bile Acids Assay Kit (Diazyme Laboratories, Inc., Poway, CA, USA). 68.5 μL of Reagent 1 were plated in a 96-well plate, to which 1 μL of the deproteinization sample, the diluted bile acid standard, and PBS (as blank) were added, and the plate was incubated at 37°C for 5 min. Then, 22.5 μL of Reagent 2 were added, and the absorbance was immediately measured at 450 nm and denoted as A₀. Each one minute later, the absorbance at 450 nm denoted as Aₜ. Total bile acid levels were determined using the following equation: Sample (μM) = [Sample ΔΔA450/min – Blank ΔΔA450/min]/[Standard ΔΔA450/min – Blank ΔΔA450/min] × Standard (100 μM).

**Statistical analysis**

For all experiments, data is expressed as the mean ± standard deviation (S.D.). The One-way analysis of variance (ANOVA) and the Student’s t-test were used for statistical analysis.
Results

Metformin upregulates GLP-1 and CFTR gene expressions

To examine gastrointestinal abnormalities in metformin-treated mice, C57BL/6J male mice (10 W) were administered a high dose of metformin (1,000 mg/kg) orally. After 3 h (Fig. 1A and Fig. S1A), though diarrhea was not observed in any mouse, an enlarged cecum was commonly observed in the metformin group. A small amount of digested diet was left in the intestine, and yellow-colored sticky fluid was observed in the intestine (especially in the ileum) (Fig. 1B and Fig. S1B), suggesting that metformin treatment may increase bile secretion, which may push out ingested food to the cecum. Hematoxylin-eosin staining of the ileum and colon showed no significant differences in the mucosal fractions (Fig. 1C and Fig. S1C), despite tissue fragility found in the metformin group.

The effects of metformin treatment on the expression of genes related to glucose and ion transport were examined by microarray analysis (Fig. 1D). As the morphological (external) differences were more significant in the ileum than in the colon, the mRNA was purified from the ileal mucosa fractions. Metformin upregulated mRNA expression of glucagon which encodes GLP-1, while the mRNA levels for dipeptidyl peptidase 4 (Dpp4) were downregulated. (DPP4 activity has been reported to be inhibited by metformin [9].) In contrast, a slight decrease in the mRNA levels for gastric inhibitory polypeptide (Gip) and GLP-1 receptor (Glp1r) was observed.

The expression of most glucose transporters was downregulated after metformin treatment. Although the expression of the nuclear receptor FXR (bile acid receptor) was upregulated in the metformin group, the expression of the bile acid transporters (solute carrier family 10 [Slc10a: Ntcp5/7] and apical sodium-dependent bile acid transporter [Asbt]) and organic anion transporting polypeptide B (Oatpb) was downregulated. These results suggest that bile acid reuptake in the small intestine might be impaired, which could explain our previous report that blood bile acid levels did not change significantly in metformin-treated mice [5].

The expression of the diarrhea-related Cl– channel Cftr was upregulated by metformin, while that of sodium–hydrogen exchanger 3 (Nhe3) was downregulated. The expression of the water channel aquaporin-8 was upregulated, whereas that of aquaporin-3 was downregulated. The expression of proinflammatory cytokines was also upregulated by metformin treatment.

Fig. 1  mRNA analyses of mouse ileal mucosa.
A. C57BL/6J mice (10 W, male) were administered metformin orally (1,000 mg/kg) or saline (control). After 3 h, gastrointestinal tracts were recovered under anesthesia. B. Images of the ileal lumen. C. The ileum and colon were fixed and examined by hematoxylin-eosin staining. D. mRNA was purified from the ileal mucosa that was prepared from mice in A and subjected to an array of experiments (n = 2). Possible metformin targets and diarrhea-related ion channels were identified. E. Respective mRNA concentrations were quantified by qPCR. Each level was normalized by Gapdh. n = 6. Means and S.D. are shown. *: p < 0.05, **: p < 0.01. Met: metformin.
To confirm the differences in mRNA expression, qPCR analyses were performed, and the changes in mRNA levels of representative molecules after metformin treatment are shown in Fig. 1E and Fig. S1D. As for pro-inflammatory molecules, only the upregulation of cyclooxygenase-2 gene expression was statistically altered by metformin. In contrast, the mRNA levels of bile acid transports are downregulated. Because GLP-1 and bile acids are associated with cAMP signaling, we decided to focus on CFTR expression.

**Correlation between GLP-1 expression and CFTR expression in the ileum**

Cftr gene expression is regulated by cAMP signaling, and GLP-1 increases intracellular cAMP levels. To examine whether the expression of these two molecules was correlated in the digestive tract, mRNA was extracted from different parts of the tracts of the C57BL/6J mice (Fig. 2A). The mRNA expression of Glp-1 was high in the lower part (ileum) of the small intestine and colon, which was also the case for Cftr mRNA. To examine whether the mRNA levels of ileal Glp-1 and Cftr changed after meals, mice under fed and fasting conditions were prepared. Under fed conditions, high mRNA expression levels of Glp-1 and Cftr were observed (Fig. 2B).

High protein expression of CFTR in the ileal mucosal fraction was also examined (Fig. 2C). Immunohistochemical analyses of ileal sections showed clear signals for CFTR on epithelial cells after metformin treatment (in the control section, faint signals that were emphasized by a longer exposure duration were observed on the internal side of the submucosa) (Fig. 2D).

**GLP-1 induces CFTR in Caco-2 cells**

Recently, Grau-Bové et al., showed a high expression of GLP-1 receptor (GLP1R) in Caco-2 cells [15]. Therefore, we examine whether metformin induces Cftr gene expression in Caco-2 cells. After reaching confluence, the cells were further cultured for one week for differentiation and subjected to treatment. Although metformin (1 mM, the maximum concentration without cytotoxicity) did not upregulate CFTR mRNA (Fig. 3A) or protein (Fig. 3B) levels, GLP-1 induced CFTR expression.

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**Fig. 2** Correlations between GLP-1 and CFTR expression.

A. mRNA was purified from different portions of intestinal tracts of C57BL/6J mice (10 W, male) and subjected to qPCR (Glp-1 and Cftr). n = 4. Means and S.D. are shown. B. Ileum mRNA was prepared from mice under fed (just after lights on, after meal) or 12 h-fasting conditions and subjected to qPCR analyses. C. C57BL/6J mice (10 W, male) were treated with metformin (1,000 mg/kg) or saline (control) orally. After 3 h, ileal mucosa was recovered and lysed in SDS sample buffer followed by heat denaturation, and CFTR protein levels were examined by western blotting (upper). The band intensities for CFTR were normalized using those for GAPDH. *: p < 0.05. D. Ileal sections were stained with an anti-CFTR antibody (red) together with DAPI (nucleus: blue).
GLP-1 upregulates CFTR gene transcription via cAMP signaling in Caco-2 cells. Caco-2 cells were treated with metformin (Met, 1 mM) or GLP-1 (1 μg/mL) for 6 h for mRNA and 12 h for protein measurements. CFTR mRNA/protein levels were analyzed by qPCR (A) or by western blotting (B). n = 3. Means and S.D. are shown. **: p < 0.01. C. CFTR band intensity was normalized to that of GAPDH. Caco-2 cells were treated with metformin or GLP-1 for 1 h, and the CREB phosphorylation levels were examined by western blotting. D. The intracellular localization of CRTC2 and CREB phosphorylation levels were examined by immunocytochemistry. Blue is the nucleus stained by DAPI. E. CFTR promoter (–1260) reporter vector was transferred to Caco-2 cells, and the cells were treated with metformin or GLP-1 for 6 h. n = 3. F. CRE site in the CFTR promoter was disrupted and used in the reporter assays. Caco-2 cells were treated with forskolin (Fsk, 20 μM), metformin (1 mM), and GLP-1 (1 μg/mL) for 24 h. The cell population overexpressing CRTC2 was subjected to a reporter assay without stimulants. G. Caco-2 cells were treated with metformin (Met: 1 mM) or sodium cholate (1 mM). n = 3. The levels of GLP-1 and CFTR mRNA were analyzed by qPCR. H. The protein levels of CFTR were monitored by western blot analyses. The panels were a representative set of triplicate experiments.
As cAMP signaling activates CREB (through protein kinase A [PKA]-dependent phosphorylation) and CRTC2, cAMP-responsive transcriptional coactivator (through dephosphorylation by cAMP-dependent phosphatases followed by nuclear translocation), we examined the status of these factors in Caco-2 cells. Only GLP-1 induced the phosphorylation of CREB and another transcription factor in its family: activating transcription factor-1 (ATF-1) (Fig. 3C). Immunocytochemical analyses revealed that GLP-1 upregulated the level of phospho-CREB in the nucleus, which was associated with the enhanced nuclear accumulation of the CREB specific coactivator CRTC2 (Fig. 3D). The nuclear import of CRTC2 is induced by cAMP-PKA signaling [16]. These alternations were not observed after metformin treatment.

The CFTR promoter-reporter also suggested GLP-1-specific activation of the CFTR promoter in Caco-2 cells (Fig. 3E). We confirmed that GLP-1-mediated upregulation of the CFTR promoter depended on the CRE sequence in its promoter (Fig. 3F). The CRE-disrupted CFTR promoter did not respond to cAMP inducers (forskolin or GLP-1). Overexpression of CRTC2 resulted in the activation of the wild-type CFTR promoter but not the CRE-disrupted promoter. These results suggest that metformin promotes CFTR gene expression by enhancing the production of GLP-1, and GLP-1 activates the CFTR promoter via cAMP signaling.

To examine the involvement of bile acids in the regulation of GLP-1 gene expression, Caco-2 cells were treated with sodium cholate. Although metformin did not upregulate GLP-1 mRNA levels, sodium cholate increased the GLP-1 mRNA levels (Fig. 3G), which was accompanied with the upregulation of CFTR mRNA and protein levels (Fig. 3H), suggesting that the CFTR gene expression might be directly and indirectly regulated by bile acids through GLP-1.

By using the GLP1R agonist Ex-4, we reconfirmed the contribution of GLP1R to the regulation of the CFTR expression. The Ex-4 treatment upregulated the levels of CFTR mRNA, CFTR protein, and CFTR promoter activity in Caco-2 cells (Fig. 4A–C). A discrepancy in the response to Ex-4 (3 nM) between the mRNA and protein assays might be resulted from differences in culture scale. Because metformin downregulated Glp1R mRNA levels (albeit slightly) in the mouse ileum (Fig. 1D), we examined GLP1R mRNA levels in Caco-2 cells. Unlike mouse experiments, GLP1R mRNA expression was not altered by the treatments with metformin, GLP-1, or Ex-4 in Caco-2 cells (Fig. 4D).

**Metformin-induced CFTR expression was not altered between wild-type and db/db mice**

As diarrhea was observed only in db/db mice [5], differences in digestive tracts between wild-type and db/db mice were examined. In addition, we showed that wood creosote (a traditional medication) ameliorates the symptoms of metformin-induced diarrhea. Therefore, the effects of wood creosote were also examined. db/db mice (10 W male) showed loose stools after the treatment with metformin (Fig. 5A), which was ameliorated by the co-treatment with wood creosote. The isolated digestive tracks showed unsegmented feces in the colon of metformin-treated db/db mice (Fig. 5B and Fig. S2A). The administration of wood creosote recovered the segmentation of feces (indicated by triangles). The number of portions of the colon without feces suggested that metformin caused softening of feces (unsegmented) in db/db mice treated with metformin (Fig. 5C).

Although wild-type mice did not ingest diets (8:00 am –), db/db mice consumed diets even after metformin treatment (Fig. S2B), suggesting that overeating might be a

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**Fig. 4** The GLP-1R agonist Ex-4 upregulates GLP-1 expression.

Caco-2 cells were treated with the GLP1R agonist Ex-4 for 3 h. CFTR mRNA and protein levels were analyzed by qPCR (A) or western blotting (B). n = 3. Means and S.D. are shown. *: p < 0.05. The western panels were a representative set of triplicate experiments. **: p < 0.01. C. CFTR promoter (–1260) reporter vector was transferred to Caco-2 cells, and the cells were treated with Ex-4 for 24 h. n = 3. **: p < 0.01. D. GLP-1 receptor (GLP1R) expression was examined by qPCR. Caco-2 cells were treatment with metformin (1 mM), GLP-1 (1 μg/mL), or Ex-4 (10 nM) for 3 h. n = 3. No significant change was observed.
cause for the difference in fecal states in the colon between wild-type (Fig. 1A) and \( \text{db/db} \) mice (Fig. 5B). Conversely, bile flow in the ileum, which was evaluated by measuring the total bilirubin and bile acid levels, was lower in \( \text{db/db} \) mice than in wild-type mice, despite a high induction by metformin-treatment in \( \text{db/db} \) mice (Fig. 5D). Meanwhile, wood creosote might not affect bile flow. On the other hand, an enlarged cecum and the retention of only a small quantity of ingested diets in the small intestine were commonly observed in \( \text{db/db} \) mice treated with metformin alone, and with metformin and wood creosote (Fig. 5B and Fig. S2A). These results suggested that metformin-induced diarrhea may be caused by the differences in colon functions in \( \text{db/db} \) mice. Therefore, mRNA levels of \( \text{Glp-1} \) and \( \text{Cftr} \) were compared in \( \text{db/db} \) mice.

In the ileum, no differences in the mRNA levels of these genes were observed between the wild-type and \( \text{db/db} \) mice (Fig. 5E). Moreover, wood creosotes did not interfere with the effects of metformin on the expression of these genes. Similar to the ileum, \( \text{Glp-1} \) mRNA levels in the colon were not altered in the wild-type and \( \text{db/db} \) mice (Fig. 5F). However, \( \text{Cftr} \) mRNA levels in the colon were higher in \( \text{db/db} \) mice than in wild-type mice, which was not lowered by wood creosote treatment. These results suggest that the upregulation of GLP-1 and CFTR may be responsible for metformin-induced diarrhea, but other factors also contribute to diarrhea symptoms.

The other candidate factors (NHE3, sodium-glucose cotransporter 1 [SGLT1], and glucose transporter 2 [GLUT2]) and the bile acid transporters (ASBT and OATPB) in the ileum (Fig. S2C and D) and colon (Fig. S2E) of \( \text{db/db} \) mice showed no differences in expression between metformin and wood creosote treatments, despite the lowered expression(s) by the metformin treatment. These results again suggested that the combination of several factors may contribute to the symptoms of metformin-induced diarrhea.

**Discussion**

Metformin-induced diarrhea in rodents was recorded in databases and interview forms that documented the results of the preclinical tests. Healthy (wild-type) rodents exhibit diarrhea when treated with a median lethal dose of metformin. Before our previous study using \( \text{db/db} \) mice [5], however, diarrhea was not recorded in rodents treated with metformin at a dose lower
Metformin increases GLP-1/CFTR levels

than 1,000 mg/kg. To elucidate the mechanism by which metformin induces diarrhea, the dose was fixed at 1,000 mg/kg, and early events (~3 h) in the digestive tract were examined.

Similar to the results of GLP-1 blood levels in human clinical examinations [10], Glp-1 mRNA levels (measured as a part of glucagon) were upregulated in the ileum and colon of the mice. In Caco-2 cells, GLP-1 induced CFTR expression through cAMP-dependent transcriptional programs. As CFTR activity and expression are often associated with diarrhea symptoms [7], GLP-1 secretion from the digestive tract is a causation of metformin-induced diarrhea [11-14].

The diarrhea symptoms in our previous study were most severe on the second day, probably because of a decrease in appetite (food consumption) after day 2 [5]. Although wild-type mice did not consume diets during metformin treatment (for 3 h from 8:00 am~), db/db mice consumed diets. Therefore, excess food intake may be another risk factor for metformin-induced diarrhea, and that the mechanisms underlying diarrhea may be similar to those caused by overeating. Further studies are needed to test if dietary restrictions can alleviate diarrhea symptoms.

Although we could not test with effective anti-GLP1R antibodies, Caco-2 cells have been reported to express high levels of GLP-1 receptors [15]. GLP-1 activates the CFTR promoter through cAMP-CREB signaling, which leads to an increase in CFTR protein levels [17]. Metformin did not upregulate CFTR expression in Caco-2 cells, despite their capability of Glp-1 secretion [18], suggesting that metformin indirectly induced CFTR in the ileum. CFTR is a member of the ATP-binding cassette transporter family, and its gating function is regulated by ATP hydrolysis. In addition, the regulatory domain of CFTR inactivates its gating function under basal conditions, and PKA reactivates its gating function through phosphorylation in the regulatory domain of CFTR [19]. GLP-1 may regulate CFTR activity by upregulating its expression and directly activating it via phosphorylation.

Elevated levels of GLP-1 and CFTR in the ileum were commonly observed in metformin-treated mice (wild-type and db/db mice). Similarly, it has been reported that postprandial blood GLP-1 levels are not different between healthy groups and patients with diabetes in human [20]. Therefore, the upregulation of GLP-1 and CFTR expression in the ileum is a cause of metformin-induced diarrhea but is not a sufficient causative factor of the symptoms typically observed in db/db mice. Unidentified factors may contribute to further upregulation of CFTR expression in the colon of db/db mice, and wood creosotes may not cross-react with these factors.

In contrast to CFTR, the activity of NHE3, a Na⁺/H⁺ exchanger, was inhibited by cAMP signaling [21]. The loss of the Nhe3 gene results in Na⁺-dependent diarrhea in mice [22]. Recently, Han et al., reported that metformin inhibited NHE3 activity via enhanced ubiquitination as an action by AMPK, which could be a mechanism of the metformin-induced diarrhea [23]. Activation of CFTR and inactivation of NHE3 may synergistically induce intestinal water loss. In the present study, a lowered expression of Nhe3 mRNA in metformin-treated ileal mucosa was observed. This suggests that metformin may initiate cAMP signaling in the digestive tract through the upregulation of GLP-1 production, and the cooperative regulation with AMPK may also be important for diarrhea as a symptom.

Rong et al. showed that SGLT1-mediated Na⁺/glucose co-transport stimulates NHE3 activity in vivo, which suppresses cholera toxin-induced Na⁺ reflux [24]. Our observation that metformin downregulated Sglt1 mRNA expression might also lead to a decreased NHE3 activity, which may be another cause of metformin-induced diarrhea. However, it was true that the decrease in NHE3 expression was also observed in the metformin-treated wild-type mice.

Similar to SGLT1 and NHE3, metformin treatment decreased the mRNA levels for ASBT and OATPB, apical transporters, in the ileum, which might impair bile acid reabsorption [25]. Then, the retained bile acids might activate the G protein-coupled receptor TGR5 followed by initiating cAMP signaling [26]. Since GLP-1 (Glucagon: Gcg) gene expression is promoted by cAMP signaling [27], GLP-1 may act as a feed forward factor in the mechanisms of metformin-induced diarrhea. Bile acid-mediated GLP-1 release has been demonstrated by ASBT inhibitors in rodents [28], and the inhibitors result in loose passage in human [29]. The carcinostatic agent irinotecan causes diarrhea which is accompanied by the lowered expression of ASBT [26].

However, mRNA levels of ASBT and OATPB were not altered by wood creosote. Moreover, the levels of bile acids as well as bilirubin in the ileal mucosa fractions were lower in db/db mice than in wild-type mice. These results suggest that wood creosote may ameliorate metformin-induced diarrhea by regulating other factors rather than bile acid metabolism. Wood creosotes have been reported to inhibit parasympathetic acetylcholine functions in the colon [30].

Another conflicting observation in rats is that AMPK has been reported to inhibit CFTR activity in the digestive tract [31]. cAMP signals elicited by cholera toxin and bile acids result in fluid accumulation in isolated jejunal loops, which is counteracted by treatment with the AMPK agonists AICAR or metformin. However, the
inhibition of forskolin-stimulated chloride secretion requires a high concentration of metformin (4 mM) [31], which may destroy the plasma membrane of epithelial cells. Moreover, the mutant CFTR (S573C), not wild-type CFTR, is sensitive to AMPK and a possible causation of metformin-induced pancreatitis [32]. These results require reconsideration of the involvement of AMPK in CFTR regulation in the intestinal tract.

DP4 inactivates GLP-1, and DPP4 inhibitors are well-recognized medications for patients with type 2 diabetes. Metformin upregulated and downregulated Glp-1 and Dpp4 expression, respectively (Fig. 1E), which may lead to increased insulin secretion from the pancreas. Active GLP-1 levels in the plasma are higher in the mice co-treated with a DPP4 inhibitor and metformin than in the mice treated with metformin (500 mg/kg) alone [33]. Metformin does not alter the DPP4 activity.

On the other hand, the rate of diarrhea incidence in patients prescribed DPP4 inhibitors is low (~2%) [34, 35], while that of patients prescribed GLP-1 receptor agonists is moderate (~5%) [11-14]. Moreover, the incidence rate of metformin (>1,500 mg/day)-induced diarrhea is not increased in patients co-prescribed with DPP4 inhibitors [34, 35]. These studies suggest that enhanced GLP-1 signaling may be a primary causation of metformin-induced diarrhea, but not sufficient. To examine this point using mice, the dose of metformin may be lowered, because metformin (1,000 mg/kg) in the present study downregulated Dpp4 mRNA expression. Briefly, Gip mRNA levels were not upregulated by metformin in our mouse model, which may agree with the case for plasma GIP levels in human patients [36].

The present study provides new insights into the mechanisms by which metformin improves insulin resistance. Metformin may prevent nutrient absorption (including that of glucose) by accelerating the passage of food in the small intestine. It is important to note that the capacity of the cecum may be different between mice and human, suggesting that the cecum may contribute to the susceptibility of diarrhea. Moreover, metformin-induced diarrhea may share mechanisms with overeating-induced diarrhea. Further studies on the colon, as well as retrospective cohort studies on human patients, are needed to confirm these hypotheses.

Disclosure

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