Impairment of intestinal monocarboxylate transporter 6 function and expression in diabetic rats induced by combination of high-fat diet and low dose of streptozocin. Involvement of butyrate-PPARγ activation.

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Butyrate-PPARγ activation impaired MCT6 in diabetic rats

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Abbreviations:

BCRP, breast cancer resistance protein; Caco-2, human colorectal adenocarcinoma cell line; CYP450, cytochrome P450; FBG, fasting glucose in blood; FINS, fasting insulin; G-6PDH, glucose-6-phosphate dehydrogenase; G-6-P, D-Glucose-6-phosphate; Gly-Sar, glycylsarcosine; HBSS, Hank’s
balanced salt solution; HOMA-IR, homeostasis model assessment - insulin resistance; MCT6, monocarboxylate transporter 6; MRP4, multidrug resistance protein 4; NKCC1, sodium-potassium-2chloride cotransporter 1; PEPTs, oligopeptide transporters; PPARγ, peroxisome proliferator-activated receptor-gamma; P-GP, P-glycoprotein; OAT2, organic anion transporter 2; OATPs, organic anion-transporting polypeptides; OCTs, organic cation transporters; SCFA, short chain fatty acids; SPIP, single-pass intestinal perfusion; STZ, streptozocin; TC, total cholesterol; TG, triglyceride; TGZ, troglitazone.
Abstract

Generally, diabetes remarkably alters expression and function of intestinal drug transporters. Nateglinide and bumetanide are substrates of monocarboxylate transporter 6 (MCT6). We aimed to report that diabetes downregulated function and expression of intestinal MCT6 and to investigate the possible mechanism in diabetic rats induced by combination of high-fat diet and low dose of streptozocin. The results indicated that diabetes significantly decreased oral plasma exposure of nateglinide. The plasma peak concentration and area under curve in diabetic rats were 16.9% and 28.2% of control rats, respectively. Diabetes significantly decreased protein and mRNA expressions of intestinal MCT6 and oligopeptide transporter 1 (PEPT1), but upregulated peroxisome proliferator-activated receptor-gamma (PPARγ) protein level. Single-pass intestinal perfusion (SPIP) demonstrated that diabetes prominently decreased absorption of nateglinide and bumetanide. MCT6 inhibitor bumetanide, but not PEPT1 inhibitor glycylsarcosine, significantly inhibited intestinal absorption of nateglinide in rats. Co-administration with bumetanide remarkably decreased oral plasma exposure of nateglinide in rats. High concentrations of butyrate were detected in intestine of diabetic rats. In Caco-2 cells, bumetanide and MCT6 knockdown remarkably inhibited uptake of nateglinide. Butyrate concentration-dependently downregulated function and expression of MCT6 but increased PPARγ expression. The decreased expressions of MCT6 by PPARγ agonist troglitazone or butyrate were reversed by both PPARγ knockdown and PPARγ antagonist GW9662. Four-week butyrate treatment significantly decreased oral plasma concentrations of nateglinide in rats, accompanied by significantly higher intestinal PPARγ and lower MCT6 protein levels. In conclusion, diabetes impaired expression and function of intestinal MCT6 partly via butyrate-mediated PPARγ activation, decreasing oral plasma exposure of nateglinide.
Introduction

Nateglinide, a substrate of monocarboxylate transporter 6 (MCT6) (Kohyama et al., 2013), is used widely for treatment of type II diabetes via stimulating transient secretion of insulin in pancreatic β-cells (Halas, 2001). Pharmacokinetic data show that the compound possesses rapid absorption, high bioavailability (~72-90%) and minimal first-pass effect (Karara et al., 1999). In human, nateglinide is predominantly metabolized by hepatic CYP2C9 (~96%) and partly by CYP3A4 (~4%). Its main metabolite is N-[trans-4-(1-hydroxy-1-methylethyl)-cyclohexanecarbonyl]-D-phenylalanine (termed as M1), accounting for about 62-66% of nateglinide dose (Takanohashi et al., 2007). It was found that diabetes did not alter pharmacokinetics of nateglinide following intravenous administration to Goto-Kakizaki rats (Tamura et al., 2010), although some studies reported that diabetic rats induced by streptozocin (STZ) showed significantly lower expressions of hepatic CYP2C11 (Liu et al., 2012) and intestinal CYP3A1/2 but higher expression of hepatic CYP3A1/2 (Hu et al., 2011). CYP2C11 and CYP3A1/2 in rats are corresponding to human orthologues CYP2C9 and CYP3A4 (Martignoni et al., 2006), respectively. Our preliminary experiment found that plasma exposure of nateglinide and its metabolite M1 was decreased significantly following oral administration of nateglinide to diabetic rats induced by combination of high-fat diet and low dose of STZ.

Several drug transporters including oligopeptide transporters (PEPTs), breast cancer resistance protein (BCRP), organic cation transporters (OCTs), organic anion-transporting polypeptides (OATPs) and P-glycoprotein (P-GP) have been identified to account for intestinal absorption of drugs (Estudante et al., 2013). Monocarboxylate transporters (MCTs/SLC16A) have been identified to transport monocarboxylic acids, which are also associated with several diseases, such as atherosclerosis, cancer, mental retardation and cataract formation (Bonen, 2000; Bonen et al., 2006; Jones et al., 2017).
(SLC16A5), a unique member in MCTs family, has been demonstrated to transport nateglinide, probenecid and several diuretics (such as bumetanide, azosemide and furosemide), but not typical substrates for MCTs such as L-lactic acid (Murakami et al., 2005; Kohyama et al., 2013). Furthermore, intestinal protein and mRNA levels of MCT6 are high-expressed (Murakami et al., 2005; Jones et al., 2017). These findings imply that MCT6 is probably responsible for intestinal absorption of nateglinide and that the decreased oral plasma exposure of nateglinide under diabetic states may partly be attributed to alterations in expression and function of intestinal MCT6.

Short chain fatty acids (SCFA) mainly including butyrate, propionate and acetate, are produced by the gut flora in intestine as fermentation products from undigested or unabsorbed food ingredients (Bergman, 1990; Rios-Covian et al., 2016). Accumulating evidences have demonstrated their versatilely healthy effects on human including metabolic fuel for intestinal epithelium (Macfarlane and Macfarlane, 2012; Jung et al., 2015), improvement in intestinal barrier and mucosa (Peng et al., 2009; Kelly et al., 2015), anti-tumorigenesis and anti-inflammatory (Schwab et al., 2006; Louis et al., 2014; Keku et al., 2015). Interestingly, it was reported that butyrate could affect function of intestinal barrier via activating peroxisome proliferator-activated receptor-gamma (PPARγ) (Kinoshita et al., 2002; Peng et al., 2009) and altering expression of transporters (Fukushima et al., 2009) including MCT1 and MCT4 (Ziegler et al., 2016). Several reports showed that PPARγ was also involved in colon cell differentiation and colon cancer (Kliewer et al., 1995; Hamer et al., 2008).

The objective of this study: (1) to explore whether the decrease in oral plasma exposure of nateglinide under diabetic states resulted from impairment of intestinal MCT6; (2) to identify the contributions of MCT6 to transport of nateglinide in Caco-2 cells using both MCT6 inhibitor bumetanide and MCT6 knockdown with siRNA; (3) to document roles of SCFA, especially butyrate, in
alteration of intestinal MCT6 using Caco-2 cells, which was further confirmed using in vivo data; (4) to explore whether the alterations in expression and function of MCT6 by SCFA were involved in PPARγ using both PPARγ antagonist and PPARγ knockdown with siRNA in Caco-2 cells.
Materials and Methods

Reagents

M1 was friendly gifted by Jiang Cheng’s laboratory (College of Pharmacy, China Pharmaceutical University). Nateglinide, bumetanide, phenacetin and verapamil were from Meilun Biological Technology (Dalian, China). Ko143, troglitazone (TGZ) and GW9962 were from MCE (NJ, USA). Glucose-6-phosphate dehydrogenase (G-6PDH), Streptozocin (STZ), glycylsarcosine (Gly-Sar), β-NADP⁺, ketoconazole and D-Glucose-6-phosphate (G-6-P) were from Sigma-Aldrich (MO, USA). Naringin was from TCI (Tokyo, Japan). Tetraethylammonium and L-lactic acid were from Mecklin Biochemical Co., Ltd. (Shanghai, China). BCA kit and RIPA lysis buffer were from Beyotime Biotechnology (Nanjing, China). All other biological reagents or chemicals were of highest grade commercially available.

Animals

Male Sprague-Dawley rats from Super-B&K Laboratory Animal Co., Ltd. (Shanghai, China) were kept in a relative humidity- (50 ± 5%) and temperature-controlled (24 ± 2 °C) environment controlled with 12-h day/night cycles, water and common chow available free. All studies were according to Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and approved by China Pharmaceutical University Animal Ethics Committee. (No. 1621010273).

Induction of diabetic rats

Induction of diabetic rats was according to previous protocol (Shu et al., 2016a). Briefly, male rats, weighing about 100 g, were divided randomly into diabetic (DM) rats, high-fat diet (HFD) rats and
control (CON) rats. The diet for CON rats was common chow, while the diet for both DM and HFD rats was high-fat diet (TROPHIC Animal Feed Co., Ltd, Nantong, China) consisting of 13% lard, 2% sesame oil, 20% sucrose, 3% cholesterol, 5% peanut, 0.1% sodium cholate and 57% normal chow. After 6-week feeding, STZ (35 mg/kg, dissolved in citrate buffer pH 4.5) was intraperitoneally injected to DM rats, while both CON and HFD rats were injected the vehicle. On the 7th day after STZ injection, diabetes development was confirmed by fasting glucose in blood (FBG) using glucose assay kit (Jiancheng Bioengineering Institute, Nanjing, China), and FBG in successful DM rats was higher than 11 mM. Then the rats continued feeding with their own diet for another 3 weeks for following experiments.

Pharmacokinetics of nateglinide in rats

Pharmacokinetics of nateglinide in DM, HFD and CON rats (n=6) following oral administration of nateglinide were investigated. Briefly, the experimental rats, fasted overnight, collected 0.25 mL blood for assessment of biochemical parameters, then orally administrated with nateglinide (10 mg/kg, suspended in 0.25% CMC-Na). At 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 240 and 480 min after administration, about 0.25 mL blood samples from oculi chorioideae vein were collected into heparinized tubes under anesthetized with diethyl ether. After each 4 samplings, the experimental rats received the suitable amount of normal saline via tail vein to compensate blood loss.

On next day (the 22nd day following STZ injection), the animals following fasting overnight were sacrificed under diethyl ether anesthesia. Intestine and liver were obtained for measurement of mRNA or protein and preparation of rat hepatic microsomes, respectively. Levels of total triglyceride (TG), cholesterol (TC) and FBG were detected by assay kits (Jiancheng Bioengineering Institute, Nanjing,
China). Fasting insulin (FINS) was detected by radioimmunoassay insulin kit (North Institute of Biotechnology, Beijing, China). HOMA-IR (Homeostatic model assessment - insulin resistance) was calculated according to equation HOMA-IR=FGB (mM)×FINS (mU/L)/22.5. Small intestinal content was also washed out with 1 mL 0.9% saline for determining SCFA (butyrate, propionate and acetate) following derivatization by HPLC method described previously (Miwa, 2002).

Effect of MCT6 inhibitor bumetanide on pharmacokinetics of nateglinide in rats was investigated. Briefly, 6 normal male rats (about 260 g) orally received nateglinide (10 mg/kg) alone, and another 6 male rats were orally co-administrated with nateglinide (10 mg/kg) and bumetanide (10 mg/kg). Blood samples were obtained as described above.

Effect of butyrate on pharmacokinetics of nateglinide in rats was also investigated according to previous study (Lucas et al., 2018). Briefly, 6 normal male rats drunk water containing butyrate (150 mM) for 4 weeks, and another 6 rats drunk normal water. Then the rats were orally administrated with nateglinide (10 mg/kg). The samples of blood were obtained as aforementioned.

Plasma samples were immediately obtained by centrifugation, and concentrations of nateglinide and M1 in plasma were determined by LC-MS. Pharmacokinetic parameters for individual rats were assessed on Phoenix WinNonlin 7.0 (Pharsight, St. Louis, MO) using non-compartmental analysis.

**Nateglinide metabolism in hepatic microsomes of rats**

Rat hepatic microsomes was prepared according to previous report (Hu et al., 2011). In hepatic microsomes, nateglinide metabolism was documented using M1 formation and nateglinide depletion (Takanohashi et al., 2007). Following pre-incubation of nateglinide (final level 2 µM) and microsomes (1 mg/mL) for 5 min at 37 °C, the reaction was initiated by adding NADPH-regenerating system (1
U/mL G-6PDH, 10 mM G-6-P, 0.5 mM β-NADP⁺ and 5 mM MgCl₂ dissolved in 0.1 M PBS, pH 7.4) and terminated by adding 40 µL HCl (1 M) at 0, 2, 5, 10, 15, 20, 30 and 40 min, respectively. Nateglinide metabolism in microsomes of CON rats was also operated in the presence of CYP3A inhibitor ketoconazole (1 µM). The amount of remaining nateglinide and M1 formation was simultaneously measured. The intrinsic clearance (CL_{int}) of nateglinide depletion was calculated as $CL_{int} = k/C_{mic}$, where $C_{mic}$ was the level of microsomal protein. k was the elimination constant obtained by the curve, logarithm of the remaining amount of nateglinide versus incubation time, via linear regression of the least square.

**Intestinal absorption of nateglinide in rats**

Nateglinide absorption in intestinal of rats was evaluated using SPIP according to the previous method (Zhong et al., 2016). Briefly, following fasting overnight, pentobarbital sodium (45 mg/kg) was intraperitoneally injected to DM, HFD and CON rats (n=6). An isolated jejunum (~10 cm) was fixed with the constant flow pump (flow speed 0.2 mL/min, Q) to input perfusion buffer. Following 5-min pre-perfusion with blank perfusion buffer, the segment of jejunum was perfused with perfusion buffer containing nateglinide (10 µM) for 15 min to achieve stability. In the next two hours, consecutive output solutions were collected through the cannula inserted into the distal segment of isolated jejunum per 15 min. On completion of the perfusion, the rats were sacrificed, then the length and width of the perfused jejunum (A, length×width) were measured. Blank perfusion buffer (pH 6.5) contained NaH₂PO₄ (43 mM), D-glucose (10 mM), mannitol (35 mM), Na₂HPO₄ (28 mM), NaCl (48 mM) and KCl (5.4 mM). All solutions were kept in 37 °C water bath, and animal body temperature was kept in 37 °C by electric blanket. The drug concentrations in input and output solutions ($C_{in}$ and $C_{out}$) were measured by LC-MS. The apparent effective permeability ($P_{eff}$) was obtained using equation $P_{eff} = Q/\ln(C_{out}/C_{in})/A$. And $C_{out}$
was corrected using weight calibration method (Sutton et al., 2001).

Effects of Gly-Sar (PEPT1 inhibitor) and bumetanide (MCT6 inhibitor) on intestinal absorption of nateglinide were investigated in normal rats. Briefly, the isolated jejunum was perfused with perfusion buffer containing nateglinide alone (5 µM) or combined with Gly-Sar (25 mM) or bumetanide (250 µM). Concentrations of inhibitors were according to previous reports (Murakami et al., 2005; Jappar et al., 2010; Posada and Smith, 2013). The $P_{eff}$ values in the presence of transporter inhibitors were calculated.

Another patch of DM rats was established to investigate function of intestinal MCT6. In brief, the intestinal absorption of MCT6 substrate bumetanide and its $P_{eff}$ values were determined via perfusion with perfusion buffer containing bumetanide (5 µM) as described above.

**Cell culture and drug uptake**

Caco-2 cells, Human colorectal adenocarcinoma cell line (Cell Bank of the Chinese Academy of Science, Shanghai, China), were kept in 5% CO$_2$ at 37 °C and cultured in DMEM consisting of 10% FBS (Gibco® ThermoFisher Inc, MA, USA), streptomycin (100 µg/ml), L-glutamine (2 mM), penicillin (100 IU/mL), NaHCO$_3$ (3.7 g/L) and non-essential amino acids. Medium was renewed every 2 days. When 80% confluent, the cells were sub-cultured and seeded in 24-well plates at 1.5×10$^5$ per well. The seeded cells continued culturing for 7 days, when a significant fraction of the cell population exhibited a colonic phenotype, then uptake study was performed according to previous protocol (Kimura et al., 2009; Tsukagoshi et al., 2014; Kimura et al., 2017). Briefly, following removing culture medium, Caco-2 cells were washed with 37 °C Hank’s balanced salt solution (HBSS, 1 mM, pH 6.5) for two times. Uptake was started via addition of 500 µL HBSS (37 °C) containing nateglinide (10 µM) with or without transporter inhibitors [Gly-Sar (25 mM) for PEPT1, verapamil (100 µM) for P-GP, Ko143 (25 µM) for
BCRP, naringin (200 µM) for OATP1A2, tetraethylammonium (5 mM) for OCTs, L-lactic acid (15 mM) for MCT1-4, bumetanide (250 µM) for MCT6] and terminated by ice-cold HBSS washing for three times after 1-h incubation. Concentrations of relevant inhibitors were cited from previous studies (Okamura et al., 2002; Murakami et al., 2005; Ogihara et al., 2009; Posada and Smith, 2013; Jouan et al., 2014; Wen et al., 2015; Zhang et al., 2018). The intracellular concentration of nateglinide was determined by LC-MS.

Effects of SCFA on MCT6 function in Caco-2 cells were further measured using uptake of MCT6 substrate bumetanide. In brief, 5 days after cell seeding, the cells continued culturing for another 2 days with culture medium containing butyrate (1 mM), propionate (1 mM), acetate (10 mM) or their mixture, respectively. Then bumetanide (50 µM) uptake was measured. Levels of SCFA were according to previous reports (Cummings, 1987; Fukushima et al., 2009) and the proportion was based on the data measured in this study. Concentration-dependent effects of butyrate on function of MCT6 were also investigated in Caco-2 cells following 48-h incubation with culture medium containing various levels of butyrate (0, 0.01, 0.1 and 1 mM).

Roles of PPARγ in the altered function of MCT6 were also documented in Caco-2 cells cultured with medium containing butyrate (1 mM), PPARγ agonist troglitazone (5 µM), butyrate (1 mM) + PPARγ antagonist GW9662 (5 µM) or troglitazone (5 µM) + GW9662 (5 µM) for 48 h. Concentrations of agonist and antagonist were cited from previous studies (Wachtershauser et al., 2000; Ulricha et al., 2005; Marion-Letellier et al., 2008).

**MCT6 or PPARγ knockdown with siRNA in Caco-2 cells**

To further estimate the contributions of MCT6 to transport of nateglinide and bumetanide, MCT6
knockdown in Caco-2 cells used lipofectamine® 3000 (Invitrogen, CA, USA) with MCT6 siRNA (120 nM) (Slc16a5: 5'-CCAUCAUCGCUUCAGCAAdTdT-3’ and 5’-UUGCUAGCAUAGGGuUUGCUGdTdT-3’), which were designed by GenePharma Technology (Shanghai, China), according to the manufacture instructions. After 3 days, expression of MCT6 protein was evaluated by western blot and uptake assay of nateglinide (10 µM) and bumetanide (50 µM) was performed. The intracellular concentrations of nateglinide and bumetanide were measured by LC-MS.

To identify whether PPARγ participated in expression of MCT6, PPARγ knockdown in Caco-2 cells was also performed using PPARγ siRNA (120 nM) (PPARγ: 5’-CCAUCAUCGCUUCAGCAAdTdT-3’ and 5’-ACAGCAGCCACUCAACUUGGdTdT-3’)(GenePharma, Shanghai, China) (Vara et al., 2013). After 24 h, the cells continued culturing for another 48 h with culture medium containing butyrate (1 mM) or troglitazone (5 µM). Then western blot was conducted to estimate the expressions of PPARγ and MCT6 protein.

**Quantitative real time-PCR (qPCR)**

Expressions of mRNA including hepatic Cyp2c11v1 (CYP2C11), intestinal Slc15a1 (PEPT1), Slc15a2 (PEPT2), Slco1a5 (OATP1A5), Slc16a1 (MCT1), Slc16a5 (MCT6), Slc16a6 (MCT7) and Slc16a10 (MCT10) in experimental rats were determined by qPCR. Total mRNA was extracted by TriPure® (Aidlab Biotechnology, Inc, Beijing, China) according to manufacture instructions. ReverTra Ace® Master Mix with gDNA Remover (TOYOBO, Osaka, Japan) was used for cDNA synthesis by Eppendorf Mastercycler Nexus (Hamburg, Germany). qPCR primers were designed by Oligo 7.0 (Molecular Biology Insights, CO, USA) or obtained from PrimerBank following NCBI Blast. Sequences
of primers were showed in Supplemental Table 1. qPCR was performed by Roche lightcycler 96 (Penzberg, Germany) using TUNDERBIRD® SYBR Mix (TOYOBO, Osaka, Japan). The protocol was: pre-incubation for 60 s at 95 °C, 3-step amplification (10 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C), melting (10 s at 95 °C, 60 s at 65 °C, 1 s at 97 °C), then cooling 30 s at 37 °C. mRNA levels of target gene were corrected by β-actin according to 2^ΔΔCt method (Livak and Schmittgen, 2001).

**Western Blot**

Protein of tissue and cells was extracted by RIPA lysis buffer containing PMSF (1 mM) and measured by BCA assay kit. Following separating by SDS-polyacrylamide gel electrophoresis, protein (~20 µg) was transferred to PVDF membranes (Millipore, MA, USA) and then blocked at 4 °C overnight in Tris-buffered saline (10 mM) containing 5% non-fat milk and 0.1% Tween 20. Given primary antibodies against PEPT1 (dilution 1:500), PPARγ (dilution 1:200) (Santa Cruz Biotechnology Inc, CA, USA), MCT6 (dilution 1:500) (ThermoFisher Scientific, MA, USA) and β-actin (dilution 1:2500) (Bioworld Technology, MN, USA) were incubated with membranes at 4 °C for 8 h, respectively. Afterwards, a horseradish peroxidase-conjugated secondary antibody (dilution 1:5000) (Bioworld Technology, MN, USA) were incubated with the membranes for 2 h. The immunoreactivity was detected by Tanon 5200 Multi Chemiluminescent System (Shanghai, China) using Super Signal WestFemto Chemiluminescent Substrate (ThermoFisher Scientific, MA, USA). All protein levels were normalized to β-action.

**Drug analysis**
Concentrations of nateglinide and bumetanide were measured by LC-MS. Briefly, 20 µL HCl (1 M), 1 mL ethyl acetate and 10 µL phenacetin (200 ng/mL, internal standard) were added into 100 µL samples. Following mixing rapidly and centrifuging, 800 µL supernatant was volatilized to dryness by nitrogen gas. The sediment was dissolved in 100 µL mixture (60% acetonitrile: 40% water) and centrifuged again. Two µL supernatant was analyzed by Shimadzu LC-MS 2020 System (Kyoto, Japan) using a Shimadzu VP-ODS C18 column (4.6 µm, 250×2.0 mm) (Shimadzu, Tokyo, Japan). The mobile phase was a mixture of acetonitrile (A) and water containing 0.1% formic acid (B) at a constant flow rate (0.2 mL/min). The gradient separation for nateglinide and M1 was: initial wash of 36% A and increased linearly to 68% at 7.5 min, then reduced back to 36% at 15 min until finishing analysis. For bumetanide, the proportion of mobile phase A was kept at 43% using isocratic elution. The analytes [M+H]+ were measured at m/z 318.2 for nateglinide, m/z 365.3 for bumetanide, m/z 334.1 for M1 and m/z 180.1 for phenacetin. For nateglinide, linear range was 0.0031-8 µg/mL in plasma, 0.31-20 µM in microsomes and perfusion buffer, 0.039-2.5 µM in cells. For M1, linear range was 0.0016-4 µg/mL and 0.156-10 µM in plasma and microsomes, respectively. For bumetanide, linear range was 0.156-10 µM and 0.019-1.25 µM in perfusion buffer and cells, respectively. Lower relative standard deviations (less than 10%) was detected in both inter-day and intra-day. The recoveries of all analytes were larger than 95%.
All values were illustrated as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to calculated statistical variations among groups followed by Tukey’s post hoc test, and significant difference was $P < 0.05$. 
Results

Induction of diabetic rats

Physiological and biochemical parameters were determined on the 21st day after injection of STZ (Table 1). Although FINS was similar to CON rats, DM rats showed several typical diabetic characteristics including polyuria, polydipsia, polyphagia and significant increase in liver weight, levels of FGB, TC, TG as well as HOMA-IR, which reflected disease characteristics and natural history of type II diabetes. SCFA levels in small intestinal content of three groups were also measured. Although large individual difference existed, the increased amount of SCFA, especially butyrate, was obviously found in small intestinal content of DM rats, whose order was DM rats>HFD rats>CON rats. Acetate showed the highest amount, and the proportion of acetate: propionate: butyrate in CON rats was measured to be 36:2:1.

Pharmacokinetics of nateglinide in rats

Following oral administration of nateglinide to, plasma concentrations of nateglinide and its metabolite M1 in DM, HFD and CON rats were determined (Fig. 1A and B). It was found that plasma exposure of nateglinide in DM rats was prominently lower than CON rats. Corresponding parameters for pharmacokinetics were also calculated (Table 2). The area under curve (AUC) and peak concentrations (C_{max}) in DM rats were only 28.2\% and 16.9\% of CON rats, respectively, although diabetes was not affect its half-life time (T_{1/2}). Lower plasma concentrations of M1 were also found correspondingly in DM rats, leading to decreases in C_{max} and AUC values of M1 by 80.7\% and 61.8\% of CON rats. HFD feeding also significantly decreased plasma exposure of M1, but only a decreased
trend was found in plasma concentrations of nateglinide.

Co-administration of MCT6 inhibitor bumetanide also remarkably decreased oral plasma concentrations of nateglinide and M1 in normal rats (Fig. 1C and D). The AUC and $C_{\text{max}}$ values of nateglinide were only about 1/3 of nateglinide alone. The $C_{\text{max}}$ and AUC values of M1 were also only about 40% of nateglinide alone (Table 2).

**Metabolism of nateglinide in hepatic microsomes of rats**

In hepatic microsomes, nateglinide metabolism was investigated by M1 formation and nateglinide depletion (Supplemental Figure. 1A and B). It was found that the contribution of M1 formation to nateglinide depletion following 40-min incubation in CON rats was 77.2%, indicating that M1 was the main metabolite of nateglinide. Addition of CYP3A inhibitor ketoconazole (1 µM) significantly decreased M1 formation and $CL_{\text{int}}$ values of nateglinide by about 24% and 40% (Supplemental Figure. 1D and F), respectively, implying that CYP3A was also involved in nateglinide metabolism including M1 formation. Importantly, the $CL_{\text{int}}$ values of nateglinide were comparable in hepatic microsomes of DM, HFD and CON rats (Supplemental Figure. 1A), although M1 formation in DM rats was prominently lower compared with CON rats and the decrease (by about 23%) in M1 formation was also less than that in mRNA expression of CYP2C11 (by about 84%) (Supplemental Figure. 1C).

**Intestinal absorption of nateglinide and expression of intestinal drug transporters related to nateglinide transport in rats**

Intestinal absorption of nateglinide was documented using SPIP in DM, HFD and CON rats. It was indicated that intestinal absorption of nateglinide was remarkably reduced in DM rats (Supplemental
Figure. 2A), whose $P_{\text{eff}}$ values were only about 60% of CON rats (Fig. 2A). Although low intestinal absorption of nateglinide in HFD rats was also found, its level of decrease was less than that in DM rats (Supplemental Figure. 2A). Further study showed that the $P_{\text{eff}}$ values among 120 min were positively correlated with AUC values of nateglinide in DM, HFD and CON rats (Fig. 2B).

Intestinal transporters related to nateglinide transport in DM, HFD and CON rats were detected using qPCR (Fig. 2C). The results indicated that both diabetes and HFD feeding significantly decreased mRNA expression of Slc15a1 (PEPT1) and Slc16a5 (MCT6) in intestine of rats. Diabetes also obviously decreased intestinal mRNA level of Slco1a5 (OATP1A5). Intestinal expressions of corresponding proteins were estimated using Western blot (Fig. 2D and E). In line with the findings in expression of mRNA, both HFD feeding and diabetes significantly decreased intestinal expressions of PEPT1 and MCT6 protein. The extent of decrease in DM rats was larger than that in HFD rats. The measured levels of PEPT1 protein in DM and HFD rats were about 25% and 63% of CON rats, respectively. Similarly, the levels of MCT6 protein in DM and HFD rats decreased by about 81% and 41% of CON rats, respectively. These results inferred that decreases in intestinal absorption of DM and HFD rats resulted from impaired intestinal expression and function of PEPT1 or MCT6.

Roles of MCT6 and PEPT1 in intestinal absorption of nateglinide were documented using MCT6 inhibitor bumetanide and PEPT1 inhibitor Gly-Sar, respectively (Supplemental Figure. 2B and Fig. 2F). It was found that bumetanide, but not Gly-Sar, remarkably inhibited intestinal absorption of nateglinide, indicating that intestinal absorption of nateglinide was mediated by intestinal MCT6 and that the decrease in intestinal absorption of nateglinide resulted from downregulation of intestinal MCT6 function by diabetes. The function of intestinal MCT6 in DM rats was also assessed using another MCT6 substrate bumetanide (Supplemental Figure. 2C and Fig. 2G). In accordance with the findings in
nateglinide, diabetes and HFD feeding also significantly decreased $P_{eff}$ values of bumetanide, further demonstrating impairment of intestinal MCT6 function in DM and HFD rats.

**Roles of SCFA in function and expression of MCT6 in Caco-2 cells**

Effects of several transporter inhibitors including Gly-Sar (for PEPT1), verapamil (for P-GP), Ko143 (for BCRP), naringin (for OATP1A2), tetraethylammonium (for OCTs) and L-lactic acid (for MCT1-4) or bumetanide (for MCT6) on uptake of nateglinide were further investigated in Caco-2 cells (Fig. 3A). It was showed that only bumetanide significantly inhibited uptake of nateglinide, further demonstrating roles of MCT6 might be involved in nateglinide transport.

Contributions of MCT6 to transport of nateglinide were also identified in Caco-2 cells using MCT6 knockdown with siRNA, and bumetanide was served as MCT6 probe. Western blot showed that expression of MCT6 following transfection with siRNA was only about 19% of control cells, demonstrating successful silence of MCT6 (Fig. 3B). It was consistent with findings of bumetanide (33.8% of control cells) that MCT6 knockdown remarkably decreased uptake of nateglinide (42.8% of control cells) (Fig. 3C). The results further indicated that MCT6 was probably the main transporter responsible for nateglinide uptake.

The present study illuminated that diabetes extremely augmented concentrations of SCFA, especially butyrate, in small intestinal content of rats (Table 1). Compared with CON rats, the level of butyrate increased to 13 folds in DM rats. Effects of SCFA including butyrate, propionate, acetate and their mixture on MCT6 function in Caco-2 cells were also documented using uptake of bumetanide (Fig. 3D). The results showed that butyrate and SCFA mixture remarkably decreased uptake of bumetanide in Caco-2 cells, which were only 59.0% and 51.7% of control cells, respectively. Propionate, but not
acetate, only showed slight inhibition on bumetanide uptake. These findings indicated that the inhibition of MCT6 function was mainly attributed to butyrate. Further study showed that butyrate concentration-dependently decreased expression and function of intestinal MCT6 (Fig. 3E and F).

**Involvement of PPARγ in butyrate-mediated MCT6 downregulation in Caco-2 cells**

Involvement of PPARγ in butyrate-mediated MCT6 downregulation in Caco-2 cells was investigated using both PPARγ antagonist GW9662 and PPARγ knockdown with siRNA. The results showed that downregulation of MCT6 expression and function by butyrate in Caco-2 cells was remarkably reversed by GW9662 (Fig. 4A and B). It was also found that troglitazone downregulated expression and function of MCT6, which was also attenuated by GW9662. Moreover, butyrate concentration-dependently induced expression of PPARγ (Fig. 4C). All these results demonstrated that downregulation of MCT6 expression and function by butyrate was involved in PPARγ activation. Roles of PPARγ in butyrate-mediated MCT6 downregulation was further confirmed using PPARγ knockdown with siRNA. Western blot showed that expression of PPARγ following transfection with siRNA was only about 27% of control cells, demonstrating successful silence of PPARγ (Fig. 4D). It was also found that butyrate and troglitazone decreased expression of MCT6 while induced expression of PPARγ in Caco-2 cells transfected with negative control siRNA (Fig. 4E and F). The alterations in expression of MCT6 and PPARγ by butyrate and troglitazone no longer happened in Caco-2 cells transfected with PPARγ siRNA.

**Effect of butyrate on pharmacokinetics of nateglinide in rats**

Pharmacokinetics of nateglinide following oral administration to rats drinking water containing
butyrate for 4 weeks was investigated to further confirm roles of butyrate in function and expression of intestinal MCT6. The results showed that butyrate treatment significantly decreased plasma concentrations of nateglinide and M1 (Fig. 1E and F). The AUC values of nateglinide and M1 in rats drinking butyrate water were only 15.2% and 36.7% of those in normal rats, respectively (Table 2). It was also found that mean residence time (MRT) and time to peak concentration (T_{max}) in rats drinking butyrate water were significantly shortened, but C_{max} was unaltered significantly. The protein levels of MCT6 and PPARγ were detected (Fig. 4H). In accordance with our hypothesis, butyrate treatment downregulated expression of intestinal MCT6, accompanied by increased PPARγ protein level. And expressions of PPARγ were also investigated in DM, HFD and CON rats using western blot (Fig. 4G). Similar alterations of MCT6 and PPARγ were found in intestine of HFD and DM rats. Interestingly, the protein levels of PPARγ was negatively correlated with MCT6 protein levels in intestine of rats (Fig. 4I).
Discussion

The present study mainly reported that diabetes significantly decreased expression and function of intestinal MCT6 in rats, leading to remarkably low oral plasma exposure of nateglinide. The DM rats showed typical diabetic syndromes including polydipsia, polyphagia, polyuria, and significant increase in liver weight, levels of FGB, TC and TG as well as HOMA-IR (Table 1), which reflected disease characteristics and natural history of type II diabetes.

Nateglinide, a substrate of MCT6 (Kohyama et al., 2013), is mainly metabolized to M1 via hepatic CYP2C11 (orthologues of human CYP2C9) in rats. CYP3A1/2 (orthologues of human CYP3A4) also catalyzes M1 formation, although its contribution is minor. We first investigated nateglinide metabolism in hepatic microsomes of rats. It was found that M1 formation from nateglinide following 40-min incubation with hepatic microsomes of rats accounted for about 77.2% of nateglinide depletion (Supplemental Figure. 1A and B), demonstrating that M1 was the main metabolite of nateglinide, which was consistent with nateglinide metabolism in human hepatic microsomes (Takanohashi et al., 2007). And diabetes also significantly impaired M1 formation (Supplemental Figure. 1B). However, CLint values of nateglinide in CON rats and DM rats were comparable, which seemed to explain the findings that diabetes did not alter pharmacokinetics of nateglinide following intravenous administration to Goto-Kakizaki rats (Tamura et al., 2010), but could not explain the decrease in oral plasma exposure of nateglinide in DM rats (Fig. 1A and Table 2). This study also illuminated that hepatic CYP3A was involved in metabolism of nateglinide including M1 formation (Supplemental Figure. 1D and F). Moreover, induction of hepatic CYP3A by diabetes was also reported (Xu et al., 2014; Shu et al., 2016b). These findings indicated that CYP3A induction under diabetic states might compensate alterations in M1 formation and nateglinide depletion mediated by downregulation of CYP2C11 expression, which
seemed to partly explain that diabetes significantly decreased expression of hepatic CYP2C11 without affecting CL$_{int}$ values of nateglinide and that the extent of decrease in M1 formation was less than reduction in hepatic CYP2C11 expression.

Next, we focused on intestinal absorption of nateglinide. In accordance with our expectation, P$_{eff}$ values of nateglinide in intestine of DM rats were only about 60% of CON rats (Fig. 2A). Meanwhile, DM rats showed lower mRNA and protein levels of intestinal PEPT1 and MCT6 (Fig. 2C, D and E), indicating that the decreases of two transporters might be the reasons, at least in part, for impaired intestinal absorption of nateglinide. Further study showed that PEPT1 inhibitor Gly-Sar did not affect intestinal absorption of nateglinide (Fig. 2F), which was consistent with previous report that PEPT1 was not involved in transport of nateglinide (Terada et al., 2000). However, MCT6 inhibitor bumetanide remarkably inhibited absorption of nateglinide, whose P$_{eff}$ values were only about 18% of those without bumetanide, indicating that intestinal absorption of nateglinide was mainly (about 80%) mediated by bumetanide-sensitive transporters and that simple diffusion accounted for less than 20% (Fig. 2F). Bumetanide is used as probe substrate to estimate the function of MCT6 (Murakami et al., 2005), although it is also substrate or inhibitor of other transporters in intestinal tissues, such as organic anion transporter 2 (OAT2), sodium-potassium-2chloride cotransporter 1 (NKCC1), multidrug resistance protein 4 (MRP4) and BCRP (Burckhardt, 2012; Tollner et al., 2015; Romermann et al., 2017; Nigam, 2018). Several reports showed that neither OAT2 nor MRP4 mediated nateglinide transport (Uchida et al., 2007; Kimoto et al., 2018). No evidence demonstrated that NKCC1 and BCRP mediated nateglinide transport. These results indicated that bumetanide decreased intestinal absorption of nateglinide at least in part via inhibiting intestinal MCT6 activity. Contributions of other transporters to intestinal absorption of nateglinide needed further investigation. Additionally, previous reports showed that diabetes
remarkably enhanced intestinal permeability in human (Tian et al., 2016; Cox et al., 2017). Moreover, diabetes also impaired intestinal motility (Marathe et al., 2016), accompanied by slow gastric emptying rate induced by hyperglycemia (Horvath et al., 2015). All these alterations may be reasons leading to decreased intestinal absorption and plasma concentration profile of nateglinide, which seemed to account for the inconsistence between obvious reduction in MCT6 expression and partly decreased P_{eff} values in intestinal of DM rats.

These findings implied that nateglinide absorption was mainly mediated by intestinal MCT6 and that decrease in intestinal absorption of nateglinide by diabetes was mainly attributed to downregulation of intestinal MCT6 function. The downregulation of intestinal MCT6 function by diabetes was further evidenced by decreases in intestinal absorption of bumetanide in DM rats (Fig. 2G). Consistent with pharmacokinetics of nateglinide in DM rats, co-administration of MCT6 inhibitor bumetanide also remarkably inhibited oral plasma exposure of nateglinide in normal rats (Fig. 1C and Table 2). All these results demonstrated that diabetes decreased oral plasma exposure of nateglinide mainly via downregulating expression and function of intestinal MCT6.

Roles of MCT6 in intestinal absorption of nateglinide were further documented using Caco-2 cells. Only bumetanide significantly inhibited uptake of nateglinide, while other transporter inhibitors including Gly-Sar (for PEPT1), verapamil (for P-GP), Ko143 (for BCRP), naringin (for OATP1A2), tetraethylammonium (for OCTs) or L-lactic acid (for MCT1-4) did not show inhibitory effects (Fig. 3A), and silence of MCT6 in Caco-2 cells further demonstrated the important roles of MCT6 in intestinal absorption of nateglinide and bumetanide (Fig. 3B and C). Previous studies reported that K_{m} values of nateglinide uptake in Caco-2 cells (448 and 240 µM) (Okamura et al., 2002; Itagaki et al., 2005) were different from that in Xenopus oocytes injected MCT6-cRNA (45.9 µM) (Kohyama et al. 2013) and
concluded that nateglinide uptake might be not mainly mediated by MCT6. This discrepancy might result from cell source. For example, transport characteristics of Xenopus oocytes were reported to be greatly different from Chinese hamster ovary cells and HEK293 cells (Nieuweboer et al. 2014). Both OATP1B1 and OATP1B3 expressed in Chinese hamster ovary cells or HEK293 cells mediated transport of docetaxel, whereas in Xenopus oocytes, OATP1B3 but not OATP1B1 mediated transport of docetaxel. Moreover, there would be almost twice variability in $K_m$ values of Caco-2 cells from two reports, even if the same cells were used (Okamura et al., 2002; Itagaki et al., 2005). Of course, roles of other intestinal transporters of nateglinide were not excluded.

SCFA are important end products mainly produced by gut flora, which involves intestinal anaerobic fermentation of indigestible peptides and fiber in diet (Cummings, 1987). In the process, fermentation is easily influenced by plentiful physiological and environmental factors including species and interaction of gut flora, disease progress, diet and physical properties of intestinal microenvironment (Jandhyala et al., 2015; O'Toole and Jeffery, 2015; Villanueva-Millan et al., 2015). This study illustrated that HFD feeding and diabetes obviously augmented the amount of SCFA in small intestinal content of rats, especially butyrate (Table 1). It was consistent with previous reports that obesity upregulated SCFA in feces of mice and human (Fernandes et al., 2014; Li et al., 2014), and diabetes also upregulated acetate in feces of kids (Samuelsson and Ludvigsson, 2004). Then, we further investigated effects of SCFA including butyrate, propionate, acetate and their mixture on MCT6 function in Caco-2 cells using uptake of bumetanide (Fig. 3D). The results demonstrated that butyrate and SCFA mixture showed similar inhibition on bumetanide uptake in Caco-2 cells, implying that the inhibition of MCT6 function by SCFA was mainly attributed to butyrate. Furthermore, butyrate concentration-dependently decreased expression and function of MCT6 in Caco-2 cells (Fig. 3E and F).
Previous reports (Wachtershauser et al., 2000; Kinoshita et al., 2002; Marion-Letellier et al., 2008; Tylichova et al., 2017) have implied latent activation of PPARγ by butyrate. We further investigated whether activation of PPARγ was also involved in butyrate-mediated MCT6 downregulation. In accordance to previous reports (Wachtershauser et al., 2000; Marion-Letellier et al., 2008), both butyrate and troglitazone induced protein expression of PPARγ in Caco-2 cells (Fig. 4C and E). Moreover, butyrate and troglitazone downregulated expression and function of MCT6, which were attenuated by PPARγ antagonist GW9662 (Fig. 4A and B). In accordance with our expectation, neither troglitazone nor butyrate could decrease MCT6 expression in Caco-2 cells silenced with PPARγ siRNA (Fig. 4E and F), confirming involvement of PPARγ activation in butyrate-mediated MCT6 downregulation.

Roles of butyrate in intestinal MCT6 expression and function were further confirmed by alteration in oral pharmacokinetics of nateglinide in rats drinking water containing butyrate for 4 weeks. It was consistent with data from DM rats that butyrate treatment significantly decreased oral plasma exposure of nateglinide and M1 (Fig. 1E, F and Table 2). It was also noticed that butyrate treatment remarkably shortened T_max and MRT values of nateglinide in rats, which was possibly due to improvement of intestinal motility by high-concentration butyrate (Canani et al., 2011), in turn, increasing absorptive rate of nateglinide. Rats following butyrate treatment also showed intestinal lower MCT6 and higher PPARγ (Fig. 4H). Expression of intestinal PPARγ protein in DM rats was further detected. Inconsistent with CON rats, higher expression of intestinal PPARγ protein was also showed in DM rats (Fig. 4G). Furthermore, expression of intestinal MCT6 was negatively correlated with intestinal PPARγ protein levels in rats (Fig. 4I).

Although clinical significances of impaired MCT6 by increased butyrate in diabetic rats was still unclear, it seemed to be reasons for the previous studies that oral plasma exposure of MCT6 substrates
(azosemide and furosemide) and diuretic efficacy were attenuated significantly in diabetic rats induced by alloxan (Park et al., 1996; Park et al., 1998). These results indicated that clinical dose of MCT6 substrates should be further considered under diabetic states.

In conclusion, diabetes significantly downregulated expression and function of intestinal MCT6 in rats, decreasing oral plasma exposure of nateglinide. The impairment of MCT6 function and expression by diabetes was partly involved in butyrate-mediated PPARγ activation.
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Interest conflicts

All participants had no conflicts of interest.

Contributions of participants

Research design: Xu, L. Liu, and X.D. Liu.

Experiments conduct: Xu, Xuan, Zhu, Qian, Zhou, Geng, Li, Wu, Zhao, Kong, and Liang.

New reagents: Xu, and Jiang.

Data analysis: Xu, Xuan, and Zhu.

Manuscript writing: Xu, L. Liu, and X.D. Liu.
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Vara D, Morell C, Rodriguez-Henche N, and Diaz-Laviada I (2013) Involvement of PPARgamma in the


Footnotes

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Reprint requests

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Legends for Figures

Fig. 1. Pharmacokinetic profiles of nateglinide (NAT) and its metabolite M1 in rats. Plasma concentrations of nateglinide (A) and M1 (B) following oral administration of nateglinide (10 mg/kg) to CON, HFD and DM rats. Plasma concentrations of nateglinide (C) and M1 (D) following oral administration of nateglinide (10 mg/kg, NOR) alone or co-administration with bumetanide (10 mg/kg, BUM) to normal rats. Plasma concentrations of nateglinide (E) and M1 (F) following oral administration of nateglinide (10 mg/kg) to rats drinking normal water (NOR) and rats drinking water containing butyrate (150 mM, BUT) for 4 weeks. Data are presented as mean ± SD (n=6). *P < 0.05, **P < 0.01 vs CON or NOR; #P < 0.05, ##P < 0.01 vs HFD.

Fig. 2. Intestinal absorption of nateglinide (NAT) and relevant transporters. (A) P_{eff} values in intestine of DM, HFD and CON rats using SPIP with nateglinide (10 µM). (B) Correlation analysis for P_{eff} and AUC values of nateglinide in DM, HFD and CON rats. (C) mRNA levels of transporters related to intestinal absorption in DM, HFD and CON rats. (D and E) Protein levels of PEPT1 and MCT6 in intestine of DM, HFD and CON rats. (F) Effects of PEPT1 inhibitor glycylsarcosine (25 mM, Gly-Sar) or MCT6 inhibitor bumetanide (250 µM, BUM) on P_{eff} values in intestine of normal rats (NOR) using SPIP with nateglinide (5 µM). (G) P_{eff} values in intestine of DM, HFD and CON rats using SPIP with bumetanide (5 µM). Data are presented as mean ± SD (n=6). P_{eff}, apparent effective permeability; SPIP, Single-pass intestinal perfusion. *P < 0.05, **P < 0.01 vs CON or NOR; #P < 0.05, ##P < 0.01 vs HFD.

Fig. 3. Function and expression of MCT6 in Caco-2 cells. (A) Effects of several transporter inhibitors on uptake of nateglinide (10 µM) in Caco-2 cells. (B) Expression of MCT6 protein in Caco-2 cells
transfected with and without MCT6 siRNA. (C) Uptake of nateglinide (10 µM) and bumetanide (50 µM) in Caco-2 cells transfected with negative control siRNA or MCT6 siRNA. (D) Effects of butyrate (1 mM), propionate (1 mM), acetate (10 mM) and their mixture on uptake of bumetanide (50 µM) in Caco-2 cells. (E and F) Concentration-dependent effects of butyrate on uptake of bumetanide (50 µM) and expression of MCT6 protein in Caco-2 cells. Data are presented as mean ± SD (n=6). Symbol: BUM, bumetanide; CON, control cells; Gly-Sar, glycylsarcosine; LAC, L-lactic acid; NAR, naringin; NAT, nateglinide; NC, negative control siRNA; si-MCT6, MCT6 siRNA; TEA, tetraethylammonium; VER, verapamil. *P < 0.05, **P < 0.01 vs CON.

**Fig. 4.** Involvement of PPARγ in butyrate-MCT6 regulation. (A and B) Effects of butyrate (1 mM), troglitazone (5 µM) and GW9662 (5 µM) on uptake of bumetanide (50 µM) and expression of MCT6 protein in Caco-2 cells. (C) Concentration-dependent effects of butyrate on expression of PPARγ protein in Caco-2 cells. (D) Expression of PPARγ protein in Caco-2 cells transfected with and without PPARγ siRNA. (E and F) Effects of butyrate (1 mM) and troglitazone (5 µM) on protein levels of PPARγ and MCT6 in Caco-2 cells transfected with negative control siRNA or PPARγ siRNA. (G) Expression of PPARγ protein in intestine of DM, HFD and CON rats. (H) Protein levels of PPARγ and MCT6 in intestine of normal rats and rats drinking water containing butyrate (150 mM) for 4 weeks. (I) Correlation analysis for protein levels of PPARγ and MCT6 in CON, HFD, DM and BUT rats. Data are presented as mean ± SD (n=6). Symbol: BUT, butyrate; CON, control cells or control diet rats; DM, diabetic rats; GW, GW9662; HFD, high-fat diet rats; NC-siRNA, negative control siRNA; NOR, normal rats; si-PPARγ, PPARγ siRNA; TGZ, troglitazone. *P < 0.05, **P < 0.01 vs CON or NOR; †P < 0.05, ‡P < 0.01 vs HFD; †P < 0.05, ‡‡P < 0.01 vs BUT or NC-BUT; ††P < 0.05, ‡‡‡P < 0.01 vs TGZ or NC-TGZ.
Table 1

Physiological and biochemical parameters of DM, HFD and CON rats.

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<th>Parameters</th>
<th>CON</th>
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<th>DM</th>
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<td>Body Weight (g)</td>
<td>297±13</td>
<td>304±12</td>
<td>250±14**##</td>
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<tr>
<td>Liver Weight (% of Body Weight)</td>
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<td>5.38±0.43**</td>
<td>8.16±0.62**##</td>
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<td>FGB (mM)</td>
<td>5.80±0.53</td>
<td>5.81±0.44</td>
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<td>FINS (mIU/L)</td>
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<td>HOMA-IR</td>
<td>12.1±2.8</td>
<td>12.9±2.7</td>
<td>44.6±6.5***##</td>
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<td>TC (mM)</td>
<td>1.70±0.23</td>
<td>4.51±1.23**</td>
<td>14.1±3.1***##</td>
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<td>TG (mM)</td>
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<td>4.41±1.48**##</td>
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<td>Acetate (mM)</td>
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<td>3.57±1.75*</td>
<td>5.20±1.65**</td>
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<tr>
<td>Propionate (µM)</td>
<td>85.3±50.1</td>
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<td>538±383*</td>
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<tr>
<td>Butyrate (µM)</td>
<td>36.5±12.3</td>
<td>150±93*</td>
<td>477±251***##</td>
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* intestinal content in small intestine was washed using 1 mL normal saline and concentration of the washed solution was measured by HPLC.

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This article has not been copyedited and formatted. The final version may differ from this version.
Data are presented as mean ± SD (n=6). Symbol: CON, normal diet rats; DM, diabetic rats; FBG, fasting glucose in blood; FINS, fasting insulin level; HFD, high-fat diet rats; HOMA-IR, homeostasis model assessment - insulin resistance; TC, total cholesterol level; TG, triglyceride level. *P < 0.05, **P < 0.01 vs CON, #P < 0.05, ##P < 0.01 vs HFD.
Table 2
Pharmacokinetic parameters of nateglinide and M1 following oral administration of nateglinide (10 mg/kg).

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<td>$C_{\text{max}}$ (µg/mL)</td>
<td>4.77±2.46</td>
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<td>3.68±2.34</td>
<td>1.49±0.39*</td>
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<td>$T_{\text{max}}$ (min)</td>
<td>19.3±9.3</td>
<td>16.4±8.0</td>
<td>27.9±13.8</td>
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<td>AUC (min·µg/mL)</td>
<td>244±111</td>
<td>179±56</td>
<td>69.0±41.6**##</td>
<td>304±153</td>
<td>144±39*</td>
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<td>$T_{1/2}$ (min)</td>
<td>64.8±31.6</td>
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<td>MRT (min)</td>
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<tr>
<td>AUC (min·µg/mL)</td>
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<td>105±58**</td>
<td>250±34</td>
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<td>T½ (min)</td>
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<td>127±74</td>
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<td>MRT (min)</td>
<td>136±47</td>
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<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
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<td>T&lt;sub&gt;max&lt;/sub&gt; (min)</td>
<td>19.2±6.7</td>
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<td>AUC (min·µg/mL)</td>
<td>380±122</td>
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<td>MRT (min)</td>
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<td>150±72</td>
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Data are presented as mean ± SD (n=6). Symbol: BUM, normal rats co-administrated with bumetanide (10 mg/kg); BUT, normal rats drinking water containing butyrate (150 mM) for 4 weeks; CON, normal diet rats; DM, diabetic rats; HFD, high-fat diet rats; NOR, normal rats. *P < 0.05, **P < 0.01 vs CON or NOR, †P < 0.05, ‡P < 0.01 vs HFD.
Fig. 1