Dosing time-dependent difference in the suppressive effect of empagliflozin on the development of mechanical pain hypersensitivity in diabetic mice

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EMPA (empagliflozin)
STZ (streptozotocin)
PWT (paw withdrawal threshold)
ABSTRACT
A problem for patients with diabetes is the rise of complications, such as peripheral neuropathy, nephropathy and retinopathy. Among them, peripheral neuropathy, characterized by numbness and/or hypersensitivity to pain in the extremities, is likely to develop in the early stages of diabetes. Empagliflozin (EMPA), a sodium-glucose cotransporter-2 inhibitor, exerts hypoglycemic effects by preventing glucose reabsorption in proximal tubular cells. EMPA can improve cardiovascular and renal outcomes in diabetic patients, but its suppressive effect on the development of diabetic neuropathy remains unclear. In this study, we demonstrated that optimizing the dosing schedule of EMPA suppressed the development of pain hypersensitivity in streptozotocin (STZ)-induced diabetic model mice maintained under standardized light/dark cycle conditions. A single intraperitoneal administration of STZ to mice induced hyperglycemia accompanied by pain hypersensitivity. Although EMPA did not exert anti-hypersensitivity effect on STZ-induced diabetic mice after the establishment of neuropathic pain, the development of pain hypersensitivity in the diabetic mice was significantly suppressed by daily oral administration of EMPA at the beginning of the dark phase. On the other hand, the suppressive effect was not observed when EMPA was administered at the beginning of the light phase. The hypoglycemic effect of EMPA and its stimulatory effect on urinary glucose excretion were also enhanced by the administration of the drug at the beginning of the dark phase. Nocturnal mice consumed their food mainly during the dark phase. Our results support the notion that morning administration of EMPA may be effective in suppressing the development of peripheral neuropathy in diabetic patients.

SIGNIFICANCE STATEMENT: Empagliflozin, a sodium-glucose cotransporter-2 inhibitor suppressed the development of neuropathic pain hypersensitivity in streptozotocin-induced diabetic model mice in a dosing time-dependent manner.
INTRODUCTION

Patients with diabetes often suffer from complications such as peripheral neuropathy, renal dysfunction, and retinopathy. Because these complications significantly reduce patients' quality of life, their prevention and treatment are a critical focus for diabetes management (Galer et al., 2000). Peripheral neuropathy, a common complication of diabetes, develops relatively early in the onset of diabetes and is characterized by sensory nerve symptoms, such as pain and numbness, primarily due to the hyperglycemia-induced accumulation of sorbitol in peripheral nerve cells. Peripheral neuropathy occurs in approximately half of diabetic patients and is associated with pain hypersensitivity in 30%–40% of cases (Abbot et al., 2011). In general, pharmacotherapy is performed for diabetic neuropathy following pain guidelines, and pregabalin and epalrestat are commonly used to treat pain hypersensitivity, but the efficacy of these treatments is unsatisfactory.

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are gaining attention as diabetes treatments because they have a different mechanism of action from conventional drugs that stimulate insulin secretion or potentiate its action (Bailey et al., 1992; Ikenoue et al., 1999; Oku et al., 1999). SGLT2 inhibitors decrease blood glucose levels by suppressing glucose reabsorption at the proximal convoluted tubule of the kidney. In addition, SGLT2 inhibitors have several clinical benefits, including suppression of renal dysfunction associated with diabetes (Wanner et al., 2016), prevention of cardiovascular events (Zinman et al., 2015), and weight loss effects. The inhibitors are typically taken once daily in the morning. Humans generally consume food during the day, so it is thought to be more effective to administer SGLT2 inhibitors before the elevation of blood glucose levels. However, the efficacy and adverse effects of many drugs vary with dosing time and are associated with 24-hour rhythms of various biological processes under the control of the circadian clock (Kato et al., 2020; Omata et al., 2021; Yasukochi et al., 2021). A chronopharmacological strategy can enhance the effects of drugs and lead to the prevention of disease complications. However, there is no clear evidence regarding the most effective dosing regimen for SGLT2 inhibitors in the treatment of diabetes and prevention of its complications.

In the present study, we used a streptozotocin (STZ)-induced diabetic neuropathy mouse model to investigate whether empagliflozin (EMPA), an SGLT2 inhibitor, prevents the development of diabetes-induced neuropathic pain hypersensitivity. Dosing time-dependent differences in drug effects are not only related to diurnal changes in the expression of target molecules but also to drug pharmacokinetics.
Therefore, we investigated the underlying mechanism of the preventive effect of EMPA on the development of diabetic neuropathic pain from the viewpoints of drug sensitivity and pharmacokinetics.

**EXPERIMENTAL PROCEDURES**

**Animals and treatment.** Male ICR mice (5-6 weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in groups (6 to 7 mice per cage) under standardized light/dark cycle conditions (Zeitgeber time 0 [ZT0], lights on; ZT12, lights off) with food and water ad libitum. Room temperature and humidity were controlled at 24 ± 1°C and 60 ± 10%, respectively. To prepare the diabetes-induced neuropathic pain animal model, mice were intraperitoneally (i.p.) administrated 200 mg/kg STZ (FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan). Control mice were injected with an equal volume of saline. We used mice whose blood glucose levels exceeded 300 mg/dL at 1 week after STZ administration. EMPA (Boehringer Ingelheim Pharmaceutical GmbH & Co., Ingelheim, Germany) was dissolved in saline at the indicated concentrations. Drug does were administered in a volume of 50 μL/10 g body weight using a probe for oral administration. All animal experiments were performed following the Guidelines for Animal Experiments of Kyushu University and approved by the Institutional Animal Care and Use Committee of Kyushu University (approval# A23-100-0).

**Measurement of glucose concentrations in plasma and urine.** Blood samples were drawn by orbital sinus collection. Plasma samples were obtained after centrifugation at 3000 rpm for 3 min and stored at −80°C. Urine was collected using a metabolic cage (KN-645, Natsume Co Ltd, Tokyo, Japan) and stored at −20°C. Glucose concentrations in plasma and urine were measured using a glucose assay kit (Fujifilm Wako Pure Chemical) validated for mouse plasma samples, according to the manufacturer’s protocol. To evaluate the hypoglycemic effect of EMPA, the area under the curve (AUC) of plasma glucose concentrations was calculated using the trapezoid rule.

**Assessment of diabetes-induced pain hypersensitivity.** To assess diabetes-induced peripheral neuropathy pain, mice were individually placed in opaque plastic cylinders on a wire mesh and allowed to acclimate to the new environment for
0.5 h. Calibrated von Frey filaments (0.02–2.0 g, North Coast Medical, Morgan hill, CA) were then applied 5 times to the plantar surfaces of the hind paws of the mice. The paw withdrawal threshold (PWT) was assessed using the up-and-down method. Lifting, licking, and biting the hind paw in response to filament stimulation was recorded as a positive response. This pain behavioral test was conducted from ZT8 to ZT11 or from ZT20 to ZT23. Behavioral observations were conducted with the room completely shut off from entry and exit, and the observers were blinded to drug treatment. To evaluate the suppressive effect of EMPA on the development of diabetic neuropathic pain, the AUC of the PWT was calculated using the trapezoid rule.

**Measurement of the consumption of food and water.** Mice were individually placed in metabolic cages (KN-645, Natsume Co Ltd, Tokyo, Japan). The amount of food consumption and water intake was measured in 4 h intervals.

**Measurement of locomotor activity.** Mice were individually housed in breeding cages with food and water ad libitum. The cages were placed in a measurement area, and locomotor activity was measured every 10 min using an infrared sensor. Additionally, locomotor activity was recorded under the light and dark cycles for 24 h on day 21 after STZ treatment.

**Determination of EMPA concentrations in plasma and kidneys.** Blood and kidneys were collected from mice at 1, 2, 4, 6, and 12 h after a single oral administration of EMPA (20 mg/kg). Blood samples were drawn by orbital sinus collection and kidneys were removed after euthanasia by cervical dislocation. Plasma samples were obtained by centrifugation at 3,000 × g and 4°C for 3 min. Then, internal standard was added, 1 µM dapagliflozin (MedChem Express, Monmouth Junction, NJ). Because the EMPA target molecule, SGLT2, is mainly expressed in the proximal renal tubular cells (De Nicola et al., 2014), small sections (100 mg) were prepared from the renal cortex and homogenized in 170 µL of ice-cooled saline containing internal standard (10 µM dapagliflozin). The homogenates were centrifuged at 12,000 × g, 4°C for 5 min, and the supernatants were collected. The plasma and kidney samples (20 µL) were each mixed with 40 µL of trichloroacetic acid and 600 µL of chloroform. After centrifugation (12,000 × g, 4°C) for 10 min, the entire underlayer was collected and evaporated by vacuum centrifugation. These residuals were reconstituted with 20 µL aliquots of the mobile phase. Samples were analyzed using an LC-MS/MS system, ACQUITY (Waters, Tokyo
Chromatographic separation was performed using a CORTECS C18+ column (2.7 μm, 2.1 mm×100 mm; Waters). The mobile phase consisted of 10 mM ammonium formate-methanol (1:3 v/v), and the flow rate was set at 0.3 mL/min. Quantification was performed by multiple reaction monitoring in the positive ion mode, with mass-to-charge ratios (m/z) of 426.2–313 and 468.2–71.1 for dapagliflozin and EMPA, respectively. The EMPA concentrations in the kidney were normalized to protein concentrations measured using the Pierce BCA Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Assessment of SGLT2 protein levels in the kidney. Renal cortices were collected at ZT2 and ZT14. A total of 10 μg of protein lysates was resolved by 8 % or 10 % SDS-PAGE, transferred to a PVDF membrane, and probed with mouse monoclonal antibodies against SGLT2 (1:1000; sc393350, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and Actin (1:10000; sc1616-HRP, Santa Cruz Biotechnology). Specific antigen-antibodies complexes were visualized using horseradish peroxidase-conjugated secondary antibodies (1:10000; ab6820, Abcam, Cambridge, UK) and a chemiluminescence reagent (Nacalai Tesque, Kyoto, Japan).

Statistical analysis. Values were expressed as the mean and S.D. The statistical significance of differences among groups was analyzed by one-way or two-way ANOVA followed by Tukey-Kramer post hoc tests. The Student’s t-test was used for independent comparison between the two groups. The sample size was determined by power analysis using G*Power 3.1 software (Heinrich Heine University, Düsseldorf, Germany). Statistical power was set at 0.8 for all experiments. Equal variances were not formally tested. P < 0.05 was considered significant.

RESULTS
Suppressive effect of EMPA on diabetes-induced neuropathic hypersensitivity. As previously reported (Blay et al., 1985), intraperitoneal administration of 200 mg/kg STZ induced hyperglycemia in mice (Fig. 1A, B). Plasma glucose levels were significantly elevated (above 600 mg/dL) 2 weeks after STZ administration. One day after STZ administration, EMPA was administered orally (once daily) for 21 days at the beginning of the dark phase (ZT12). The duration of EMPA treatment was determined based on the results of a previous study (Akamine et al., 2015). Oral administration of EMPA suppressed STZ-induced hyperglycemia in a dose-dependent manner (Fig. 1B).
Significant suppression of STZ-induced hyperglycemia was observed in mice treated with more than 20 mg/kg EMPA ($P < 0.05$, Fig. 1B right panel). During the development of STZ-induced hyperglycemia, PWT was also assessed before and after the initiation of EMPA administration. No significant difference in PWT was detected among the groups of mice before drug treatment. The PWT was significantly decreased in STZ-treated mice without EMPA treatment (Fig. 1C), indicating that STZ-induced hyperglycemia coincided with the development of diabetic neuropathic pain. Daily oral administration of EMPA dose-dependently suppressed the development of STZ-induced pain hypersensitivity (Fig. 1C). Significant suppression of neuropathic pain hypersensitivity was observed in mice treated with more than 20 mg/kg EMPA ($P < 0.01$). These results suggest that suppression of hyperglycemia by EMPA treatment prevents the development of diabetes-induced pain hypersensitivity.

**Dosing time-dependent changes in the ability of EMPA to suppress the production of pain hypersensitivity in diabetic mice.** For the treatment of diabetes, EMPA is typically taken once daily in the morning. To investigate whether the suppressive effect of EMPA on diabetes-induced neuropathic pain varies depending on the time of administration, mice were orally administered 20 mg/kg EMPA at ZT0 (time at which resting phase begins) or ZT12 (time at which an active phase begins) on the day after STZ treatment (Fig. 2A). Daily oral administration of EMPA at ZT0 had a negligible effect on STZ-induced hyperglycemia, whereas plasma glucose levels in STZ-treated mice were decreased by EMPA administration at ZT12 (Fig. 2B). Consistent with these observations, a significant suppressive effect of EMPA on diabetes-induced pain hypersensitivity was also observed in mice when the drug was administered at ZT12 but not at ZT0 ($P < 0.01$; Fig. 2C). These changes in the anti-hypersensitivity effect of EMPA were independent of differences in the temporal length of time between EMPA administration and the assessment of PWT, because PWT was recovered at 10 and 22 h after dosing when EMPA was administered at ZT12, but PWT remained low at 10 and 22 h after dosing when EMPA was administered at ZT0 (Supplementary Fig. S1). Therefore, the ability of EMPA to suppress the development of diabetes-induced pain hypersensitivity varied depending on the time of administration. Daily administration of EMPA around the beginning of the active phase is effective in decreasing plasma glucose levels and suppressing the production of diabetes-induced neuropathic pain.
Next, we also investigated whether EMPA exhibits an anti-hypersensitivity effect in STZ-induced diabetic mice after the development of neuropathic pain. After confirming the increase in plasma glucose levels and development of pain hypersensitivity, mice were orally administered 20 mg/kg EMPA at ZT0 or ZT12 (Fig. 2D). Daily oral administration of EMPA at ZT0 had a negligible effect on STZ-induced hyperglycemia and pain hypersensitivity (Fig. 2E, F). Conversely, oral administration of EMPA at ZT12 decreased the plasma glucose levels of STZ-treated mice, but the treatment did not show anti-hypersensitivity effects. Therefore, EMPA may not be effective when administered after the development of diabetic peripheral neuropathic pain.

**Dosing time-dependent difference in the ability of EMPA to decrease plasma glucose levels in diabetic mice.** To explore the underlying mechanism of dosing time-dependent changes in the suppressive effect of EMPA on diabetes-induced pain hypersensitivity, we investigated the time course of plasma glucose levels after a single oral administration of 20 mg/kg EMPA at ZT0 and ZT12. The daily amount of water consumption was increased in STZ-induced diabetic mice, but the daily amount of food intake in STZ-treated mice was comparable to those observed in control mice (Fig. 3A left and middle panels). Diurnal variations in water consumption and food intake in STZ-induced diabetic mice were synchronized with their locomotor activity rhythms (Fig. 3A right panel). There were slightly larger differences in the basal glucose levels between the two time points (ZT0 and ZT12). Therefore, the hypoglycemic effects of EMPA were plotted relative to the control groups. A significant decrease in plasma glucose levels was observed up to 8 h after EMPA administration at ZT0, whereas the hypoglycemic effect of EMPA persisted up to 12 h after drug administration at ZT12 ($P < 0.05$; Fig. 3B left panel). The relative AUC value of plasma glucose levels after EMPA administration at ZT12 was significantly lower than that after drug administration at ZT0 ($P < 0.01$; Fig. 3B right panel).

Next, we investigated whether the dosing time-dependent changes in the hypoglycemic effect of EMPA were related to its pharmacokinetics. The plasma and renal concentrations of EMPA in STZ-treated mice reached a maximum 1 to 2 h after oral administration (20 mg/kg). However, there were no significant differences in the time course of EMPA concentrations in the plasma and kidneys between the two dosing times (Fig. 3C). Therefore, we changed our focus from the pharmacokinetics of EMPA to the diurnal variation of its pharmacodynamics.
Dosing time-dependent difference in the ability of EMPA to excrete glucose into the urine of diabetic mice. EMPA promotes the excretion of excess glucose from the blood into the urine by inhibiting its reabsorption through the SGLT2 pathway. Therefore, we investigated whether urinary glucose excretion after EMPA administration was altered according to its dosing time. The protein levels of SGLT2 in the renal cortex of STZ-treated mice were not significantly different between the early light phase (ZT2) and the early dark phase (ZT14) (Fig. 4A). Although glucose was not detectable in the urine of control mice, treatment with 200 mg/kg STZ increased the urinary excretion of glucose. A single oral administration of 20 mg/kg EMPA at ZT0 had a negligible effect on urinary glucose excretion, but the same dose of EMPA at ZT12 significantly increased urinary excretion of glucose ($P < 0.05$; Fig. 4B). Although there was no significant dosing time-dependent difference in the urinary glucose concentrations of STZ-induced diabetic mice after EMPA administration (Fig. 4C), their urine volume was increased after EMPA administration at ZT12 (Fig. 4D). This dosing time-dependent difference in the diuretic effect of EMPA appeared to result in the time-specific enhancement of urinary glucose excretion. Because the hypoglycemic effect of EMPA in STZ-treated mice was also enhanced by administration of the drug at the same time point, this may be a major cause of the dosing time-dependent difference in the suppressive effect of EMPA on the production of diabetic pain hypersensitivity.

DISCUSSION

The diurnal rhythm of biological functions is closely related to the onset and symptoms of various diseases. For example, asthma attacks with dyspnea often occur late at night and early in the morning (Smolensky et al., 2007; Burioka et al., 2010), and allergic diseases such as urticaria and pruritus worsen from evening to night (Ferguson et al., 2021). Pain caused by diabetic peripheral neuropathy has also been reported to show diurnal variations, with exacerbation during the night and early morning (Odrich et al., 2005). The diurnal rhythm of biological functions and disease symptoms affects the pharmacological actions of many drugs, and their efficacy and toxicity vary depending on the time of day when they are administered (Filipski et al., 2014; Kanemitsu et al., 2017; Matsunaga et al., 2018; Yasukochi et al., 2021; Tsuruta et al., 2022). Blood glucose concentrations show diurnal variations depending on feeding time, with diurnal humans showing high levels during the active daytime period, whereas nocturnal experimental animals such as mice and rats show high levels during the dark phase.
For the treatment of diabetic patients, EMPA is commonly taken orally once daily after breakfast, but the scientific rationale for this dosing regimen has not been clarified.

Although it has been demonstrated that STZ-induced diabetic model mice have high blood glucose levels throughout the day and do not show significant diurnal rhythms (Akamine et al, 2018), EMPA exhibited a hypoglycemic effect on STZ-induced diabetic model mice when the drug was administered in the beginning of the active phase (ZT12). In addition, administration of EMPA at the same time of day significantly suppressed the development of diabetes-induced peripheral neuropathic pain. In diabetic patients, the polyol pathway is upregulated as a compensatory mechanism for decreased glucose uptake into cells, resulting in the accumulation of sorbitol and other substances in neurons and the development of peripheral neuropathy (Gabbay et al., 1966). In the mouse model of STZ-induced diabetes, the development of pain hypersensitivity is observed in association with sorbitol accumulation in peripheral nerves (Akamine et al., 2018). Therefore, administration of EMPA to mice at the beginning of their active period suppresses the increase in blood glucose levels and the accumulation of sorbitol, thereby preventing the development of pain hypersensitivity associated with peripheral neuropathy.

In this study, we used STZ-induced diabetic male mice, but this animal model mimics type 1 diabetes, which is characterized by a deficiency in insulin secretion (Chen et al., 2009). Therefore, we also investigated the dosing time-dependency of the pharmacological action of EMPA using mice with hyperglycemia induced by taking a high-fat diet (HFD), representing a typical type 2 diabetic animal model. Plasma glucose levels in HFD-induced diabetic mice gradually increased after the initiation of HFD feeding and reached a plateau around 12 weeks. However, the timing of the onset of pain hypersensitivity in HFD-induced diabetic mice showed large individual variations, and pain intensity was modest compared with that of STZ-induced diabetic mice (data not shown). The individual variations and modest pain intensity may hinder the accurate evaluation of the anti-hypersensitivity effect of EMPA. Similar drawbacks were also observed in STZ-induced diabetic female mice (data not shown). Further studies are required to investigate the dosing time-dependent difference in the suppressive effect of EMPA on the development of neuropathic pain in type 2 diabetic models and STZ-induced diabetic female animals.

A single dose of EMPA also showed a significant hypoglycemic effect in STZ-induced diabetic mice, but the hypoglycemic effect was also enhanced by the administration of EMPA at ZT12. In intestinal epithelial cells, efflux transporters, such as
P-glycoprotein and breast cancer resistance protein, recognize EMPA as a substrate and suppress its intestinal absorption (Gu et al., 2020). The intestinal expression of these transporters shows a diurnal oscillation in mice and causes dosing time-dependent changes in the absorption and bioavailability of orally administered drugs (Murakami et al., 2008; Hamdan et al., 2012). In healthy humans, it has been reported that the pharmacokinetic parameters of EMPA do not exhibit differences at the level of bioequivalence when the drug is administered in the morning and evening (El-Dash et al., 2021). In the present study, we also demonstrated that there are no significant dosing time-dependent differences in EMPA concentrations in the blood and kidneys of STZ-induced diabetic mice after administration of the drug at ZT0 and ZT12. Therefore, the dosing time-dependent differences in the hypoglycemic effects of EMPA and its suppressive effect on peripheral neuropathic pain are unlikely related to diurnal variations in its pharmacokinetics.

On the other hand, urinary glucose excretion in STZ-induced diabetic mice was increased by a single oral administration of EMPA at the beginning of the active phase (ZT12). Circulating EMPA is filtered through glomeruli in the kidney and prevents glucose reabsorption by inhibiting the transport activity of SGLT2, which is expressed on the luminal side of proximal tubules (Chasis et al., 1933; Rossetti et al., 1987; Kanai et al., 1994; Oku et al., 1999). There were no significant time-dependent differences in the expression levels of SGLT2 protein in mouse kidneys. Renal clearance of inulin has been shown to increase in nocturnal mice during the dark phase, suggesting that the glomerular filtration rate (GFR) exhibits a diurnal variation (Oda et al., 2014). Although it was difficult to measure the amount and affinity of EMPA bound to SGLT2 on the proximal renal tubular cells, the amount of EMPA reaching the site of action may have been increased by administering the drug at ZT12, considering that the GFR of nocturnal mice increases during the dark phase. However, even if large amounts of glucose reabsorption were suppressed by the administration of EMPA at ZT12, the urinary glucose concentrations may have been diluted by the diuretic effects of EMPA because SGLT2 inhibitors also have osmotic diuresis and natriuresis actions (Tanaka et al., 2017). There was no significant dosing time-dependent difference in the urinary glucose concentration of STZ-induced diabetic mice after EMPA administration, but their urine volume was increased after EMPA administration at ZT12. This dosing time-dependent difference in the diuretic effect of EMPA appeared to be associated with its effects on urinary glucose excretion.
Other hypoglycemic drugs, such as dapagliflozin, an SGLT2 inhibitor, and sidagliptin, a DDP-4 inhibitor, have been reported to significantly decrease plasma glucose concentrations in mice when administered at ZT2, the early light phase (Yoshioka et al., 2019a; Yoshioka et al., 2019b). Diurnal variations in the secretion of GLP-1 and GIP are also observed in humans (Lindgren et al., 2011). Therefore, hypoglycemic drugs may exert similar suppressive effects on the development neuropathic pain in both experimental animals and humans, although it is necessary to evaluate the optimal dosing time of EMPA in the patients with diabetes.

In this study, we used the STZ-induced diabetic mouse model to demonstrate that optimizing the dosing schedule of EMPA suppresses the development of neuropathic pain hypersensitivity. In general, temporal blood glucose levels in diabetic patients increase during the day depending on the feeding pattern (Hajime et al., 2018). Therefore, SGLT2 inhibitors, including EMPA, are administered in the morning because glucose filtration from the glomeruli is also assumed to increase during the day. No significant diurnal variation in blood glucose levels was observed in STZ-induced diabetic mice (Akamine et al., 2018), but the hypoglycemic effect of EMPA was enhanced when the drug was administered at the beginning of the active phase, suppressing the production of pain hypersensitivity. Our results support the practice of administering EMPA in the morning, to effectively suppress the development of peripheral neuropathy in diabetic patients.
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DATA AVAILABILITY STATEMENT
All the data supporting the findings of this study are contained within the paper.

AUTHORSHIP CONTRIBUTIONS
Participated in research design: Sato, Koyanagi.
Conducted experiments: Sato, Yasukochi, Iwanaka, Koyanagi, Yamauchi.
Contributed new reagents or analytic tools: Yasukochi, Yamauchi, Tsuruta.
Performed data analysis: Sato, Iwanaka, Koyanagi.
Wrote or contributed to the writing of the manuscript: Sato, Koyanagi, Ohdo.
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FOOTNOTES
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No author has an actual or perceived conflict of interest with the contents of this article.
FIGURE LEGENDS

Figure 1 EMPA suppresses the development of pain hypersensitivity in STZ-induced diabetic mice. (A) Schematic diagram of dosing regimen of EMPA in STZ-induced diabetic model animals. Mice were intraperitoneally administered 200 mg/kg STZ and then orally administered with a single daily dose of EMPA at ZT12. (B) Dose-dependent suppression of hyperglycemia in STZ-treated mice by EMPA. Right panel shows the AUC value of glucose levels in plasma of STZ-treated mice after EMPA administration. Values are shown as means with S.D. (n=6). In the left panel, **P<0.01; * P<0.05, compared with STZ+Saline group at corresponding time points (F_{12,100}=3.836, P<0.001, two-way ANOVA with the Tukey Kramer post-hoc test). In the right panel, **P<0.01; * P<0.05 compared between the two groups (F_{4,25}=18.705, P<0.001, one-way ANOVA with the Tukey Kramer post-hoc test). (C) Dose-dependent suppression of the development of pain hypersensitivity in STZ-treated mice by EMPA. The paw withdrawal threshold (PWT) of mice was assessed by the von Frey up down method. Right panel shows the AUC value of PWT of STZ-treated mice after EMPA administration. Values are shown as means with S.D. (n=6). In the left panel, **P<0.01; * P<0.05, compared with STZ+saline group at corresponding time points (F_{28,200}=2.205, P<0.001, two-way ANOVA with the Tukey Kramer post-hoc test). In the right panel, **P<0.01, compared between the two groups (F_{4,25}=16.807, P<0.001, one-way ANOVA with the Tukey Kramer post-hoc test).

Figure 2 Dosing time-dependent changes in the suppression effect of EMPA on the development of pain hypersensitivity in STZ-induced diabetic mice. (A) Schematic diagram of dosing regimen of EMPA in STZ-induced diabetic mice before the development of pain hypersensitivity. Mice were intraperitoneally administered 200 mg/kg STZ and then orally administered with a single daily dose of EMPA at ZT0 or ZT12. (B) Dosing time-dependency of suppressive effect of EMPA (20 mg/kg, p.o.) on the development of in STZ-induced diabetic mice. Values are shown as means with S.D. (n=5-6). There was a significant dosing time-dependent difference in hypoglycemic effect of EMPA (F_{3,76}=4.210, P<0.01, two-way ANOVA with the Tukey Kramer post-hoc test). (C) Dosing time-dependency of suppressive effect of EMPA on the development of pain hypersensitivity in STZ-induced diabetic mice. The PWT of mice was assessed by the von Frey up down method from ZT8 to ZT10. Right panel shows the AUC value of PWT of STZ-treated mice after EMPA administration. Values are shown as means with S.D. (n=5-6). In the right panel, *P<0.01, compared between the two groups (F_{3,152}=16.830, P<0.001, two-way ANOVA with the Tukey Kramer post-hoc test). *P<0.05, compared between the two groups (F_{3,19}=4.985, P<0.05, one-way ANOVA with the Tukey Kramer post-hoc test). (D) Schematic diagram of dosing regimen of EMPA in STZ-induced diabetic mice after the development of pain hypersensitivity. Mice were intraperitoneally administered 200 mg/kg STZ. After confirming the development of pain hypersensitivity, a single daily dose of EMPA was orally administered at ZT0 or ZT12.
(E) Dosing time-dependency of hypoglycemic effect of EMPA (20 mg/kg, p.o.) in STZ-induced diabetic mice. Values are shown as means with S.D. (n=5-6). (F) Influence of EMPA dosing time on the pain hypersensitivity effect in STZ-induced diabetic mice. The PWT of mice was assessed by the von Frey up down method from ZT8 to ZT10. Right panel shows the AUC value of PWT of STZ-treated mice after the initiation of EMPA administration. Values are shown as means with S.D. (n=5-6). **P<0.01, compared between the two groups (Student’s T-test).

Figure 3 Dosing time-dependent changes in the hypoglycemic effect of EMPA in STZ-induced diabetic mice. (A) Temporal profile of amount of water consumption, food intake, and locomotor activity of healthy and STZ-induced diabetic mice. Values are shown as means with S.D. (n=5-6). (B) The time course of plasma glucose levels in STZ-induced diabetic mice after a single oral administration of EMPA (20 mg/kg) at ZT0 or ZT12. Right panel shows the AUC value of glucose levels in plasma of STZ-treated diabetic mice after EMPA administration. Values are shown as means with S.D. (n=6-7). In the left panels, **P<0.01; *P<0.05, compared with saline treated group at corresponding time points. (F_{12,105}=7.773, P < 0.001, two-way ANOVA with the Tukey Kramer post-hoc test). In the right panel, **P<0.01, compared between the groups (F_{3,21}=22.985, P < 0.001, one-way ANOVA with the Tukey Kramer post-hoc test). (C) The time course of EMPA concentrations in plasma (left) and kidney (right) of STZ-induced diabetic mice after a single oral administration of EMPA (20 mg/kg) at ZT0 or ZT12. Values are shown as means with S.D. (n=5).

Figure 4 Dosing time-dependent changes in the promotion effect of EMPA on urinary excretion of glucose in STZ-induced diabetic mice. (A) Temporal profiles of protein levels for SGLT2 in the kidney of STZ-induced diabetic mice. Values are shown as means with S.D. (n=4). (B) The time course of urinary glucose excretion in STZ-induced diabetic mice after a single oral administration of EMPA (20 mg/kg) at ZT0 or ZT12. Values are shown as means with S.D. (n=5). *P<0.05; compared between the two groups (Student’s t-test). (C) The time course of urinary glucose concentrations of STZ-induced diabetic mice after a single oral administration of EMPA (20 mg/kg) at ZT0 or ZT12. Values are shown as means with S.D. (n=5). (D) The time course of urine volume of STZ-induced diabetic mice after a single oral administration of EMPA (20 mg/kg) at ZT0 or ZT12. Values are shown as means with S.D. (n=5).
**Figure 2**

**A**

- STZ (200 mg/kg, i.p.)
- Daily administration of saline or EMPA (20 mg/kg, p.o.) at ZT0 or ZT12
- Blood sampling (ZT0 and ZT12)
- PWT assessment

**B**

- Plasma glucose levels (mg/dL)
- Saline (ZT0) vs. EMPA (ZT0)

**C**

- PWT (g)
- Days after the initiation of drug administration

**D**

- STZ (200 mg/kg, i.p.)
- Development of diabetic pain hypersensitivity
- Daily administration of saline or EMPA (20 mg/kg, p.o.) at ZT0 or ZT12
- Blood sampling (ZT0 and ZT12)
- PWT assessment

**E**

- Plasma glucose levels (mg/dL)
- Saline (ZT12) vs. EMPA (ZT12)

**F**

- PWT (g)
- Days after the initiation of drug administration
Figure 3
Figure 4
Dosing time-dependent difference in the suppressive effect of empagliflozin on the development of mechanical pain hypersensitivity in diabetic mice
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Supplementary Figure S1 Influence of differences in the temporal length of time between empagliflozin (EMPA) administration and the assessment of paw withdrawal threshold. The PWT was assessed at 10 and 22 h after administration of EMPA at ZT0 or ZT12. The PWT was recovered at 10 and 22 h after dosing when EMPA was administered at ZT12, but the PWT remained low at 10 and 22 h after dosing when EMPA was administered at ZT0. The PWT of EMPA administrated group tended to be significantly different from that of saline group ($P = 0.069$, two-way ANOVA).