

Luseogliflozin inhibits high glucose-induced TGF- β 2 expression in mouse cardiomyocytes by suppressing NHE-1 activity

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Abstract

Objective: Sodium-glucose cotransporter-2 (SGLT2) inhibitors exhibit cardioprotective properties in patients with diabetes. However, SGLT2 is not expressed in the heart, and the underlying molecular mechanisms are not fully understood. We investigated whether the SGLT2 inhibitor luseogliflozin exerts beneficial effects on high glucose-exposed cardiomyocytes via the suppression of sodium-hydrogen exchanger-1 (NHE-1) activity.

Methods: Mouse cardiomyocytes were incubated under normal or high glucose conditions with vehicle, luseogliflozin, or the NHE-1 inhibitor cariporide. NHE-1 activity and gene expression were evaluated by the SNARF assay and real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, respectively. Six-week-old male db/db mice were treated with vehicle or luseogliflozin for 6 weeks, and the hearts were collected for histological, RT-PCR, and western blot analyses.

Results: High glucose increased NHE-1 activity and transforming growth factor (*Tgf*)- β 2 mRNA levels in cardiomyocytes, both of which were inhibited by luseogliflozin or cariporide, whereas

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their combination showed no additive suppression of *Tgf-β2* mRNA levels. Luseogliflozin attenuated cardiac hypertrophy and fibrosis in db/db mice in association with decreased mRNA and protein levels of TGF-β2.

Conclusions: Luseogliflozin may suppress cardiac hypertrophy in diabetes by reducing *Tgf-β2* expression in cardiomyocytes via the suppression of NHE-1 activity.

Keywords

Cariporide, diabetic cardiomyopathy, fibrosis, luseogliflozin, sodium-hydrogen exchanger-1, sodium-glucose cotransporter-2 inhibitor, transforming growth factor-β2

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Introduction

Accumulating evidence has shown that diabetes is an independent risk factor and poor prognostic marker of heart failure.^{1,2} Cardiac hypertrophy and fibrosis are associated with an increased risk of heart failure in patients with diabetes.³ However, the underlying molecular mechanisms are not fully understood. Therefore, identifying a potential therapeutic target for cardiac hypertrophy and fibrosis is urgently needed in patients with diabetes.

Sodium-glucose cotransporter-2 (SGLT2) inhibitors are a new class of oral antidiabetic agents that improve hyperglycemia in patients with diabetes by inhibiting urinary glucose reabsorption at the S1 segment of renal proximal tubules.^{4,5} Although SGLT2 is not expressed in the heart,⁴ several animal studies have shown that SGLT2 inhibitors can prevent cardiac hypertrophy and fibrosis in rodent models of diabetes.⁶⁻⁸ Furthermore, recent cardiovascular outcome trials have consistently shown that compared with placebo, treatment with SGLT2 inhibitors significantly reduces the risk of heart failure in patients with type 2 diabetes.⁹ However, the molecular mechanism for this remains to be elucidated.

Sodium-hydrogen exchanger (NHE) is a plasma membrane transporter involved in

the regulation of intracellular pH via proton extrusion driven by the transmembrane Na⁺ gradient.¹⁰ Among the isoforms, NHE-1 is predominantly expressed in the heart.¹⁰ Although NHE-1 plays an essential role in the maintenance of cellular homeostasis under physiological conditions, NHE-1 activation has been reported to be involved in cardiac hypertrophy and fibrosis in the heart of a type 2 diabetic rat model.¹¹ Recently, SGLT2 inhibitors were shown to inhibit NHE-1 activity by directly binding to NHE-1 protein located on the plasma membrane in rodent cardiomyocytes.^{12,13} Accordingly, we speculate that the suppression of NHE-1 by SGLT2 inhibitors may contribute to their cardioprotective properties in diabetes.

We previously reported that high glucose and diabetic conditions increase the cardiomyocyte and cardiac tissue expression levels of transforming growth factor (TGF)-β2, respectively, contributing to cardiac hypertrophy and fibrosis in type 2 diabetic mice.¹⁴ However, it remains unclear whether SGLT2 inhibitors attenuate cardiac hypertrophy and fibrosis in diabetic animals by reducing TGF-β2 expression in the heart via the suppression of NHE-1 activity. Therefore, in the present study, we investigated the effects of luseogliflozin, a selective SGLT2 inhibitor, on NHE-1

activity and TGF- β 2 expression in high glucose-exposed cultured mouse cardiomyocytes and examined the influence of luseogliflozin on cardiac TGF- β 2 expression, hypertrophy, and fibrosis in db/db mice, an animal model of type 2 diabetes with obesity.¹⁵

Materials and methods

Chemical agents

The NHE-1 inhibitor cariporide and nuclear factor of activated T cells (NFAT) inhibitor 11R-VIVIT were purchased from Selleck Biotech (Bunkyo, Tokyo, Japan) and Merck Millipore Japan (Meguro, Tokyo, Japan), respectively. Luseogliflozin was provided by Taisho Pharmaceutical (Toshima, Tokyo, Japan).

Cell culture experiments

Cardiomyocytes isolated from neonatal ICR mice were obtained from Cosmo Bio (Koto, Tokyo, Japan). Cardiomyocytes were seeded onto fibronectin-coated 48-well plates and cultured in Dulbecco's Modified Eagle Medium (Gibco, Waltham, MA, USA) containing 10% fetal bovine serum and 5.5 mM glucose.¹⁴ For real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis, cells at 60% to 70% confluence were pre-treated with vehicle, cariporide (10 μ M), 11R-VIVIT (1 μ M), luseogliflozin (1 μ M), or their combination for 1 hour and further cultured under normal (5.5 mM) or high (30 mM) glucose conditions for 48 hours.^{16–19} Experiments were conducted with at least three independent cell cultures.

RT-PCR

Total RNA was extracted from tissues and cells and then used to synthesize cDNA for RT-PCR assays as previously described.¹⁴ Quantitative real-time RT-PCR was

performed using the TaqMan gene expression assay and sequence detection system (StepOne Plus; Life Technologies Japan, Minato, Tokyo, Japan).¹⁴ The pre-designed TaqMan probe sets used were as follows: *Sglt2*, Mm00453831_m1; *Nhe-1*, Mm00444270_m1; *Tgf- β 1*, Mm01178820_m1; *Tgf- β 2*, Mm00436955_m1; myosin heavy chain beta (*β -Mhc*), Mm00600555_m1; and connective tissue growth factor (*Ctgf*), Mm1192932_g1. The 18S ribosomal RNA probe (*18s rna*, Mm03928990_g1) was used as an internal control.

Intracellular pH measurement

NHE-1 activity was assessed by intracellular pH recovery after ammonium chloride prepulse treatment in accordance with previously described methods with some modifications.^{13,20} Briefly, cardiomyocytes at 60% to 70% confluence were cultured under normal (5.5 mM) or high glucose (30 mM) conditions for 48 hours prior to the assay. Dulbecco's phosphate-buffered saline (Thermo Fisher Scientific Japan [Gibco], Minato, Tokyo, Japan; catalog ID 14040-133; media formulation: 0.9 mM CaCl₂, 0.5 mM MgCl₂·6H₂O, 2.7 mM KCl, 1.5 mM KH₂PO₄, 137.9 mM NaCl, 8.1 mM Na₂HPO₄·7H₂O, 8 mM HEPES, 5.5 mM [normal] or 30 mM [high] glucose) was used as an assay buffer. 5-(and-6)-Carboxy SNARF-1 acetoxymethyl ester (SNARF-1, Thermo Fisher Scientific Japan) dissolved in the assay buffer at a final concentration of 10 μ M was used as a loading buffer. NH₄Cl dissolved in assay buffer was used as the stimulation buffer.

First, cell culture medium was replaced with the loading buffer without electrical pacing and incubated for 30 minutes. After five washes with the fresh assay buffer, cells were incubated with the stimulation buffer containing vehicle (0.1% dimethylsulfoxide [DMSO]), cariporide

(10 μ M), or luseogliflozin (10 μ M) for 10 minutes. Then, the stimulation buffer was replaced with the fresh assay buffer containing vehicle (0.1% DMSO), cariporide (10 μ M), or luseogliflozin (10 μ M), and the ratio of two fluorescence intensities (emission 580 nM, excitation 514 nM; emission 640 nM, excitation 514 nM) was measured from multiple cells located in the central area of each well in 60-second intervals using a microplate reader (TECAN Infinite Pro200; TECAN Japan, Kawasaki, Kanagawa, Japan). At the end of each experiment, the fluorescence intensity was calibrated using an Intracellular pH Calibration Buffer Kit (Thermo Fisher Scientific Japan) in accordance with the manufacturer's instruction. Cells were maintained at 37°C in a CO₂ incubator during SNARF-1 loading and NH₄Cl stimulation and room air during the measurement of fluorescence intensity. Δ intracellular pH was calculated between each time point, and Δ intracellular pH/minute was expressed by the average of Δ intracellular pH over the first 6 minutes after stimulation with NH₄Cl.

Animal study

The reporting of this study conforms to ARRIVE 2.0 guidelines.²¹ The protocol of the animal experiment was approved by the Animal Care Committee of Showa University School of Medicine before the study (approval number: 09059; date of approval: 28 July 2020). All experiments were conducted under adherence to the Guide for the Care and Use of Laboratory Animals (8th Edition).²² Invasive procedures were conducted under general anesthesia using isoflurane (1.5% to 2.0%, depending on the toe pinch reflex).²³ We minimized the number of animals based on an estimation from our previous studies.²⁴ We carefully observed the health conditions of mice (at least 6 days a week) to

decrease their suffering. Leptin receptor-deficient db/db mice fed a high-fat diet are a widely used mouse model of type 2 diabetes with obesity.¹⁵ Five-week-old male db/db mice (BKS.Cg-*Dock7^m*+/*+**Lepr^{db}*/Jcl, *n* = 19) purchased from CLEA Japan (Meguro, Tokyo, Japan) were housed in a single cage in the animal care facility of Showa University and maintained on standard rodent chow with free access to water.

After 1 week of acclimatization, mice were switched to a 5.0 g/day high-fat diet (45% of total calories, D12451, Research Diet, New Brunswick, NJ, USA) and then randomly assigned to the vehicle (*n* = 9) or luseogliflozin group (*n* = 10) according to their cage numbers (odd number: luseogliflozin, even number: vehicle). Luseogliflozin was given to mice via food, and its dosage (20 mg/kg/day) was determined based on a previous study.²⁵ After 6 weeks of intervention, all mice were sacrificed by isoflurane overdose. The heart, liver, kidney, and epididymal fat pad were carefully isolated and weighed to calculate the index for each organ as follows: organ weight (mg)/left tibial length (mm). The apices of the isolated hearts were excised and snap-frozen for RT-PCR and western blot analyses, and the remaining parts of the hearts were immersed in 4% paraformaldehyde for histological analysis. No unexpected adverse event was observed during the experimental period.

Measurement of plasma levels and blood pressure

Blood samples were collected after 6 hours of fasting at the end of experiments.^{14,24} Blood levels of glycated hemoglobin (HbA1c) were measured by an immunoassay (Roche Diagnostics, Minato, Tokyo). Plasma levels of glucose, lipids, and insulin were determined with enzyme electrode (Sanwa Kagaku, Nagoya, Aichi, Japan), colorimetric (Fuji Film WAKO, Osaka,

Osaka, Japan), and enzyme-linked immunosorbent (ELISA) assays (Ultra-sensitive Mouse Insulin ELISA Kit; Product ID: M1104; Morinaga Institute of Biological Science, Yokohama, Kanagawa, Japan), respectively. Systolic blood pressure and pulse rates were measured 1 week before the end of experiments using a non-invasive tail-cuff method as previously described.^{14,24}

Histological assessment of hearts

Cardiac hypertrophy and fibrosis were evaluated as previously described.¹⁴ Briefly, the hearts were embedded in paraffin blocks, and cross-sections at the papillary muscle level were stained with hematoxylin & eosin for the assessment of cardiomyocyte size and Masson's trichrome for left ventricular interstitial fibrosis area measurements. The images were analyzed by a researcher blinded to group assignments using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Protein expression levels were determined by western blot analysis as previously described.²⁶ Briefly, 10 μ g of proteins extracted from cardiac tissues were electrophoresed in polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After 1-hour incubation with a blocking reagent, the membranes were incubated with primary antibodies overnight at 4°C, followed by secondary antibody for 1 hour at room temperature. The following antibodies were used for western blot analyses: TGF- β 2 (Abcam Japan, Chuo, Tokyo, Japan; Product ID: ab36495, RRID: AB_778343; mouse monoclonal antibody; dilution: 1:2000; molecular weight [MW]: 25 kDa [bioactive dimer]), NHE-1 (Santa Cruz Biotechnology, Dallas, TX, USA; Product ID: sc-136239,

RRID: AB_2191254; mouse monoclonal antibody; dilution: 1:500; MW: 110 kDa), β -actin (Santa Cruz Biotechnology; Product ID: sc-47778; RRID: AB_2714189; mouse monoclonal antibody; dilution: 1:10,000; MW: 43 kDa), and anti-mouse IgG from sheep (GE Healthcare Japan, Hino, Tokyo, Japan; Product ID: NA931; RRID: AB_772212; dilution: 1:20,000). The bands on immunoblots were detected using the Amersham ECL Prime Kit (GE Health Care Japan), digitized using a WSE-6100 LuminoGraph (ATTO, Taito, Tokyo, Japan), and quantified using CS Analyzer 4 software (ATTO).²⁶

Statistics

Data are expressed as the mean \pm standard deviation (SD). Statistical comparisons were performed using JMP software (version 13; SAS Institute Inc., Cary, NC, USA). Comparisons were tested by an unpaired two-sided t-test for two groups and one-way ANOVA followed by Tukey's test for three or more groups. The number of mice was decided as follows: comparison type, unpaired two-sided t-test; α error, 5%; β error, 20%; estimated mean difference, 15%; and estimated SD of each group, 25%. Correlation was tested by Pearson's correlation coefficient. The significance level was defined as $p < 0.05$.

Results

Luseogliflozin inhibited NHE-1 activity and Tgf- β 2 gene expression in mouse cardiomyocytes under high glucose conditions

First, we evaluated the effects of luseogliflozin on cultured mouse cardiomyocytes (4–10 samples per group were obtained from at least three independent experiments). In contrast to the levels in positive control mouse kidneys, *Sglt2* gene

expression was barely detected in cardiomyocytes (Figure 1a). *Nhe-1* mRNA levels in cardiomyocytes were comparable to those in mouse kidneys (Figure 1a). Although high glucose did not affect

Nhe-1 mRNA levels in cardiomyocytes (Figure 1b), it significantly increased NHE-1 activity, as assessed by intracellular pH recovery after ammonium chloride prepulse treatment ($p < 0.01$) (Figure 1c, 1d).

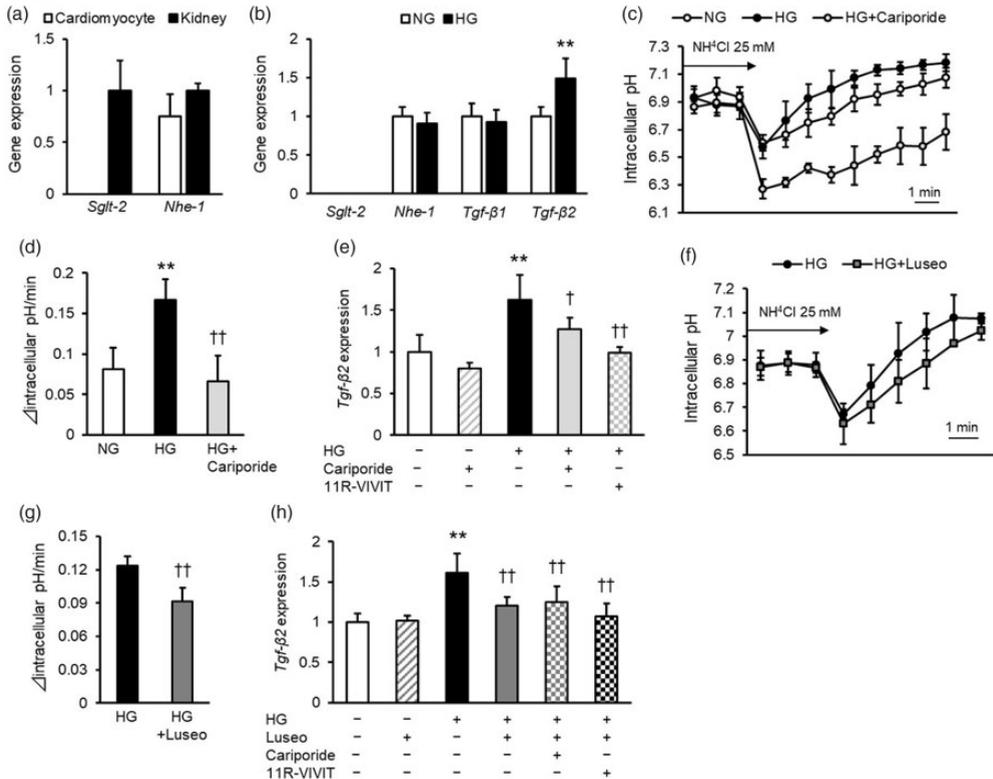


Figure 1. Effects of cariporide and luseogliflozin on NHE-1 activity and *Tgf-β2* gene expression levels in mouse cardiomyocytes. (a) mRNA levels of *Sglt2* and *Nhe-1* in mouse cardiomyocytes. Data of the kidneys collected from db/db mice were used as a positive control for SGLT2. (b) mRNA levels of *Sglt2*, *Nhe-1*, *Tgf-β1*, and *Tgf-β2* in mouse cardiomyocytes under normal and high glucose conditions for 48 hours. (c) Effects of high glucose and cariporide on intracellular pH recovery after ammonium chloride (NH_4Cl) prepulse treatment. (d) Quantified data of intracellular pH recovery after ammonium chloride prepulse treatment. Δ intracellular pH was calculated between each time point, and Δ intracellular pH/minute was expressed by the average of Δ intracellular pH over the first 6 minutes after stimulation with NH_4Cl . (e) Effect of cariporide and 11R-VIVIT on *Tgf-β2* mRNA levels in mouse cardiomyocytes under high glucose conditions. (f) Effects of luseogliflozin on intracellular pH recovery after ammonium chloride prepulse treatment. (g) Quantified data of intracellular pH recovery after ammonium chloride prepulse treatment. (h) Effect of luseogliflozin on *Tgf-β2* mRNA levels in mouse cardiomyocytes under high glucose conditions. NHE-1, sodium-hydrogen exchanger-1; TGF, transforming growth factor; SGLT2, sodium-glucose cotransporter-2; NG, normal glucose; HG, high glucose; Luseo, luseogliflozin. Four to ten samples per group were obtained from at least three independent experiments. * and **, $p < 0.05$ and $p < 0.01$ vs. normal glucose, respectively. † and ††, $p < 0.05$ and $p < 0.01$ vs. high glucose, respectively.

Furthermore, high glucose significantly increased the mRNA levels of *Tgf-β2* in cardiomyocytes ($p < 0.01$) but did not affect *Tgf-β1* gene expression levels (Figure 1b). Cariporide, an inhibitor of NHE-1, inhibited the effects of high glucose on NHE-1 activity ($p < 0.01$) and *Tgf-β2* mRNA levels ($p < 0.05$) in cardiomyocytes (Figures 1c–1e). Moreover, 11R-VIVIT, an inhibitor of NFAT (essential transcriptional factor downstream of NHE-1²⁷) blocked the high glucose-induced upregulation of *Tgf-β2* mRNA levels in cardiomyocytes ($p < 0.01$) (Figure 1e).

The selective SGLT2 inhibitor luseogliflozin significantly decreased NHE-1 activity in high glucose-exposed cardiomyocytes ($p < 0.01$) (Figure 1f, 1g). In addition, luseogliflozin inhibited the high glucose-induced increase in *Tgf-β2* mRNA levels ($p < 0.01$), whereas cariporide or 11R-VIVIT did not affect *Tgf-β2* gene expression levels in cardiomyocytes exposed to high glucose and luseogliflozin (Figure 1h).

Luseogliflozin prevented cardiac hypertrophy and fibrosis in db/db mice in association with reduced cardiac gene expression levels of Tgf-β2

We next evaluated the effect of luseogliflozin on cardiac hypertrophy and fibrosis in high-fat-fed db/db mice, an animal model of type 2 diabetes with obesity. The metabolic parameters of animals are shown in Figure 2a and Table 1. Body weight was significantly decreased by luseogliflozin treatment at 7 days but increased after 28 days ($p < 0.01$) (Figure 2a). Liver and kidney weight indexes, HbA1c levels, and plasma glucose levels were significantly decreased by treatment with luseogliflozin (all $p < 0.01$) (Table 1).

As shown in Figure 2b and 2c, *Sglt2* mRNA was not detected in the hearts of db/db mice, whereas *Nhe-1* mRNA was

detected at similar levels in the hearts and kidneys. Luseogliflozin treatment did not affect the mRNA or protein levels of NHE-1 in the hearts (Figure 2c and 2d). Luseogliflozin treatment significantly reduced cardiomyocyte size and cardiac interstitial fibrosis area compared with the vehicle ($p < 0.01$) (Figure 2e and 2f). Furthermore, luseogliflozin treatment significantly decreased *Tgf-β2* mRNA levels and bioactive dimetric TGF-β2 protein levels in the hearts of db/db mice ($p < 0.01$ and $p < 0.05$, respectively) (Figure 2g and 2h). These changes were accompanied by decreased gene expression levels of *β-Mhc*, a marker of cardiac hypertrophy²⁸ ($p < 0.01$) (Figure 2g), and *Ctgf*, a fibrogenic growth factor downstream of TGF-β2 signaling²⁹ ($p < 0.05$) (Figure 2g). Finally, there was a positive correlation between *Tgf-β2* and *β-MHC* gene expression levels ($r = 0.87$, $p < 0.01$) (Figure 2i).

Discussion

In this study, we found for the first time that high glucose increased NHE-1 activity and *Tgf-β2* mRNA levels in cultured mouse cardiomyocytes, both of which were inhibited by the selective SGLT2 inhibitor luseogliflozin and NHE-1 inhibitor cariporide. Although the NFAT inhibitor 11R-VIVIT, which suppressed the downstream signaling of NHE-1, also blocked the effects of high glucose on *Tgf-β2* gene expression, the combination of cariporide and 11R-VIVIT did not have additive effects on *Tgf-β2* gene suppression in high glucose-exposed luseogliflozin-treated cardiomyocytes. Furthermore, luseogliflozin treatment attenuated cardiac hypertrophy and fibrosis in db/db mice in association with decreased gene expression levels of *Tgf-β2* and its downstream molecules *β-Mhc* and *Ctgf*.^{28,29} Therefore, our present observations suggest that luseogliflozin may exert anti-hypertrophic and anti-fibrotic effects

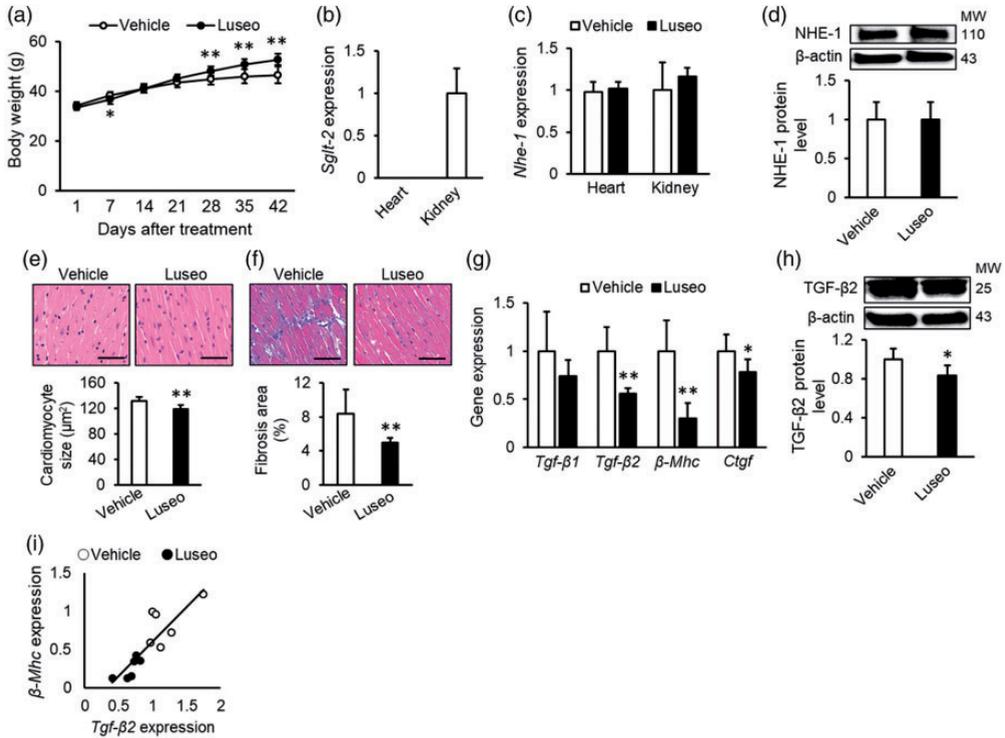


Figure 2. Effects of luseogliflozin treatment on cardiac hypertrophy and fibrosis in diabetic mice with obesity. High-fat-fed db/db mice were treated with vehicle or luseogliflozin for 6 weeks. (a) Body weight changes after treatment. (b) Cardiac mRNA levels of *Sglt2*. Data of the kidney were used as a positive control for SGLT2. (c) Cardiac mRNA levels of *Nhe-1*. (d) Cardiac protein levels of NHE-1. Upper panels show the representative immunoblot images. (e) Cardiomyocyte sizes. (f) Interstitial fibrosis area. Upper panels show the representative microscopic images of left ventricle stained with hematoxylin and eosin (E) and Masson's trichrome (f). Scale bars, 1 mm. (g) Cardiac mRNA levels of *Tgf-β1*, *Tgf-β2*, *β-Mhc*, and *Ctgf*. (h) Cardiac protein levels of bioactive dimetric TGF-β2. Upper panels show the representative immunoblot images. (i) Correlation between gene expression levels of *Tgf-β2* and *β-Mhc*. (a), (e), (f): Vehicle, $n = 9$, Luseogliflozin, $n = 10$; (b), (c): $n = 4$ per group; (d): $n = 5$ per group; (g), (i): $n = 6$ per group; (h): Vehicle, $n = 5$, Luseogliflozin, $n = 6$. Gene and protein expression levels of target molecules were normalized to those of the internal controls *18s rna* and β -actin, respectively; the data were shown as relative levels to the vehicle. NHE-1, sodium-hydrogen exchanger-1; TGF, transforming growth factor; SGLT2, sodium-glucose cotransporter-2; β -Mhc, myosin heavy chain beta; *Ctgf*, connective tissue growth factor; MW, molecular weight; Luseo, luseogliflozin. * $p < 0.05$ vs. vehicle, ** $p < 0.01$ vs. vehicle.

in the diabetic hearts of db/db mice by reducing cardiac *Tgf-β2* gene expression levels, partly via the suppression of NHE-1 activity. This effect may be one of the potential mechanisms underlying the cardioprotective property of SGLT2 inhibitors in patients with diabetes.

Heart failure is a leading cause of death in patients with diabetes.¹⁻³ Cardiac hypertrophy and fibrosis are the characteristic structural changes observed in the hearts of patients with diabetes and are thus predictors of heart failure in these patients.³⁰ However, the molecular mechanisms

Table 1. Anthropometric and biochemical parameters of diabetic mice treated with vehicle or luseogliflozin.

	Vehicle	Luseogliflozin
Number	9	10
Food intake (g/day)	5.0	5.0
Heart weight index	8.9 ± 0.5	8.6 ± 0.6
Kidney weight index	18.7 ± 1.4	14.7 ± 1.6**
Liver weight index	142.6 ± 11.4	108.3 ± 15.9**
Visceral fat weight index	85.7 ± 5.1	88.7 ± 5.5
Pulse rate (/minute)	597 ± 63	638 ± 38
Systolic blood pressure (mmHg)	127 ± 23	127 ± 23
HbA1c (%)	12.4 ± 0.3	7.8 ± 0.6**
Plasma glucose (mg/dL)	564 ± 49	255 ± 46**
Plasma insulin (ng/mL)	0.3 ± 0.2	0.7 ± 0.6
Plasma total cholesterol (mg/dL)	234 ± 32	230 ± 42
Plasma HDL cholesterol (mg/dL)	139 ± 27	140 ± 29
Plasma triglycerides (mg/dL)	108 ± 38	108 ± 58

Mean ± standard deviation. HDL, high-density lipoprotein; HbA1c, glycated hemoglobin. * $p < 0.05$, ** $p < 0.01$ vs. Vehicle.

underlying cardiac hypertrophy and fibrosis in diabetes are not fully understood. TGF- β is a multifunctional growth factor that has been shown to play a crucial role in the pathogenesis of cardiac hypertrophy and fibrosis by inducing cellular hypertrophy in cardiomyocytes and extracellular matrix production in cardiac fibroblasts.²⁹ Indeed, we previously reported that cardiac gene expression levels of *Tgf- β 2* are increased and associated with cardiac hypertrophy and fibrosis in diabetic animals.¹⁴ In this study, we found that luseogliflozin treatment prevented cardiac hypertrophy and fibrosis with a concomitant reduction in TGF- β 2 gene and protein expression levels in the hearts of diabetic mice with obesity. We also found that luseogliflozin reduced cardiac gene expression levels of *β -Mhc*, a marker of cardiac hypertrophy,²⁸ which were positively correlated with those of *Tgf- β 2*. In addition, luseogliflozin decreased the cardiac gene expression levels of *Ctgf*, a mediator of TGF- β fibrogenic signaling.²⁹ These observations suggest that the suppression of cardiac

TGF- β 2 expression may partly account for the anti-hypertrophic and anti-fibrotic effects of luseogliflozin.

In the present study, we found that luseogliflozin attenuated the high glucose-induced increase in NHE-1 activity and *Tgf- β 2* gene expression in cardiomyocytes, and the NHE-1 inhibitor cariporide mimicked the effects of luseogliflozin. Because cariporide did not exert additive effects on luseogliflozin-induced downregulation of *Tgf- β 2* gene expression in high-glucose-exposed cardiomyocytes, luseogliflozin may inhibit *Tgf- β 2* gene expression in cardiomyocytes via the suppression of NHE-1 activity. Pharmacological inhibition of NHE-1 has been shown to prevent hypoxia-induced TGF- β 1 expression and subsequently attenuate cardiac fibrosis in rats,³¹ thus supporting our speculation. SGLT2 inhibitors, such as empagliflozin, dapagliflozin, and canagliflozin, are reported to directly bind to NHE-1 protein and inhibit its biological activity.¹³ Luseogliflozin may exert the same inhibition against NHE-1 activity. Therefore,

the inhibition of cardiac NHE-1 activity by SGLT2 inhibitors may be a novel molecular strategy in the prevention of cardiac hypertrophy and fibrosis in diabetes.

NFAT is a transcriptional factor activated by the calcium-sensitive molecule calmodulin.³² NHE-1 overexpression causes Na⁺-induced Ca²⁺ overload via the compensative activation of Na⁺-Ca²⁺ channels and subsequently activates NFAT in cardiomyocytes.³³ Cardiomyocyte-specific overexpression of NFAT in mice induces cardiac hypertrophy and fibrosis,³² whereas treatment with an NFAT inhibitor prevents pressure overload-induced cardiac hypertrophy and fibrosis.³⁴ Furthermore, NFAT inhibition has been reported to reduce *Tgf-β* gene expression in platelet-derived growth factor-exposed vascular smooth muscle cells.³⁵ Taken together, these findings suggest that the NHE-1/NFAT pathway may play a crucial role in the pathogenesis of cardiac hypertrophy. In this study, we found that 11R-VIVIT suppressed the high glucose-induced *Tgf-β2* gene expression in cardiomyocytes but did not affect the gene expression levels of *Tgf-β2* in high glucose-exposed luseogliflozin-treated cells. Therefore, our findings suggest that luseogliflozin may attenuate high glucose-induced *Tgf-β2* gene expression by suppressing NFAT via the inhibition of NHE-1, as shown in the graphical abstract in Supplemental Figure 1.

There are several limitations to this study. First, we did not evaluate TGF-β2 protein levels in high glucose-exposed cultured cardiomyocytes. Therefore, although luseogliflozin treatment decreased the mRNA and protein levels of TGF-β2 in the hearts of db/db mice, it remains unclear whether luseogliflozin suppresses the translation of *Tgf-β2* mRNA in cardiomyocytes exposed to high glucose. Second, consistent with the previous observations showing that SGLT2 inhibitors, such as luseogliflozin, increase body weight in db/db mice, we

found that the final body weights were significantly higher in luseogliflozin-treated mice than in vehicle-treated mice.^{36–39} Although the effects of luseogliflozin on body weight in mice differ from those in humans, the mechanism underlying this difference remains unknown. Third, although luseogliflozin did not affect blood pressure or lipid parameters, it significantly improved hyperglycemia in diabetic mice. However, we did not determine if the glucose-lowering effects of luseogliflozin partly accounted for the suppression of cardiac hypertrophy and fibrosis in our animal model, although a meta-analysis revealed that intensive glycemic control exhibited no impact on the risk of heart failure in patients with type 2 diabetes.⁴⁰ Fourth, we could not evaluate cardiac function because of a technical difficulty. Therefore, whether treatment with luseogliflozin improves cardiac function and ultimately reduces the risk of heart failure in db/db mice remains unknown. Finally, we demonstrated direct effects of luseogliflozin on mouse cardiomyocytes in this study. However, our findings need to be further validated by future studies using human cardiomyocytes or heart tissues, which may provide more clinically relevant insight into the molecular mechanisms underlying the cardioprotective property of SGLT2 inhibitors.

Conclusions

Our present findings suggest that luseogliflozin directly inhibits the harmful effect of high glucose on cardiomyocytes by reducing TGF-β2 expression via the suppression of NHE-1 activity, which may be one of potential mechanisms underlying the cardioprotective property of SGLT2 inhibitors in diabetes.

Author contributions

NO performed experiments, analyzed data, interpreted the results of experiments, and

drafted and revised manuscript. YM conceived and designed the research, performed experiments, analyzed data, interpreted the results of experiments, prepared figures, drafted manuscript, and edited and revised manuscript. TS performed experiments, interpreted the results of experiments, and edited and revised manuscript. MT, MH, HY, YS, RK, MO, TF, and SY interpreted the results of experiments and edited and revised manuscript. All authors have approved the final version of the manuscript. YM is the guarantor of this work and is responsible for its integrity. Data are available upon reasonable request.

Declaration of conflicting interest

The authors declare that they have no competing interests.

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