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Effect of ipragliflozin, an SGLT2 inhibitor, on cardiac histopathological changes in a non-diabetic rat model of cardiomyopathy

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ABSTRACT

Aims: We investigated the effect of the selective sodium-dependent glucose cotransporter 2 (SGLT2) inhibitor ipragliflozin on cardiac dysfunction and histopathology in a non-diabetic rat model of cardiomyopathy.
Main methods: Ipragliflozin was mixed with chow (0.01%, w/w) and administered to male DahlS.*Z-Lepr*^{fa}/*Lepr*^{fa} (DS/obese) rats for 8 weeks. Male DahlS.Z-*Lepr*$^{+}$/*Lepr*$^{+}$ (DS/lean) rats of the same age were used as controls. Systolic blood pressure (SBP) and heart rate (HR) were measured every 4 weeks. After 8 weeks of treatment, echocardiography and histopathological examinations were performed. Further, the effect of ipragliflozin on blood and urine parameters were investigated.
Key findings: In the DS/obese rats, ipragliflozin delayed the age-related increase in SBP without affecting HR, reduced left ventricular (LV) mass and intraventricular septal thickness in echocardiography, and ameliorated hypertrophy of cardiomyocytes and LV fibrosis in histopathological examination. Although ipragliflozin significantly increased both urine volume and urinary glucose excretion in DS/obese rats, it did not alter plasma glucose levels.
Significance: Ipragliflozin prevented LV hypertrophy and fibrosis in non-diabetic DS/obese rats without affecting plasma glucose levels. These findings suggest that SGLT2 inhibitors have a cardio-protective effect in non-diabetic patients with cardiomyopathy.

1. Introduction

Sodium-dependent glucose cotransporter 2 (SGLT2) inhibitors have been developed to manage glycemic control in patients with type 2 diabetes mellitus (T2DM) [1]. Recently, the EMPA-REG OUTCOME trial demonstrated that the SGLT2 inhibitor empagliflozin in combination with standard therapy reduced the rate of death from cardiovascular causes, all-cause death and heart failure (HF) hospitalization in T2DM patients with established cardiovascular diseases (CVD) [2]. Treatment with the other SGLT2 inhibitors canagliflozin and dapagliflozin also resulted in a lower rate of death or hospitalization for HF in patients with T2DM compared with those who received placebo [3,4]. Given these findings, the cardiovascular benefits observed in these studies can be assumed to be a class effect of SGLT2 inhibitors. Interestingly, however, analyses of the EMPA-REG OUTCOME trial revealed that there was no significant relationship between glycemic control and HF outcomes [5,6], suggesting that the cardiovascular benefits of SGLT2 inhibitors are mediated by mechanisms other than glycemic control. Moreover, the cardiovascular benefits of SGLT2 inhibitors were exhibited even in non-diabetic patients with established CVD. While there are a number of possible mechanisms by which SGLT2 inhibitors may exhibit cardiovascular benefits beyond glycemic control, including body weight reduction, reduction in systemic blood pressure, and potential direct cardiac effects [7–10], the exact mechanisms remain unclear. Studies in non-diabetic animals can help elucidate the mechanism of action of SGLT2 inhibitors and the possible benefits of SGLT2 inhibition in non-diabetic patients with established CVD, because SGLT2 inhibitors rarely decrease plasma glucose levels in such animals. To our knowledge, however, studies of the effect of SGLT2 inhibition on cardiac dysfunction using non-diabetic animals are limited [11].

DahlS.*Z-Lepr*^{fa}/*Lepr*^{fa} (DS/obese) rats were established by crossing Dahl salt-sensitive rats with Zucker rats harboring a missense mutation in the leptin receptor gene (*Lepr*) [12], and are characterized by normoglycemia, salt-sensitive hypertension and spontaneous development of cardiomyopathy [12,13]. Here, we investigated the effect of ipragliflozin, a selective SGLT2 inhibitor that was reported to have beneficial effects in both a rodent model of diabetes [14–18] and rodent disease models without diabetes [19–21], on the cardiac dysfunction and histopathological changes in the left ventricle in non-diabetic DS/obese rats.

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2. Materials and methods

2.1. Compounds

Ipragliflozin L-proline (PubChem CID: 57339444) was synthesized at Astellas Pharma Inc. (Tsukuba, Japan). The compound was administered as a 0.01% ipragliflozin rat chow diet.

2.2. Animals and experimental protocol

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Tsukuba Research Center of Astellas Pharma Inc., which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All animal experiments were conducted at CMIC Pharma Science Co., Ltd. (Yamanashi, Japan), which is also accredited by AAALAC International. Five-week-old male DahlS.*Z-Lepr*$^{+}$/*Lepr*$^{+}$ rats (DS/lean rats) and DahlS.Z-*Lepr*^{fa}/*Lepr*^{fa} rats (DS/obese rats) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rats were housed in plastic cages floored with a substrate of autoclaved wood chips in a temperature- and humidity-controlled room with a 12-h light and dark cycle (lights on 7:00–19:00). Water and standard laboratory diet CE-2 (CLEA Japan, Inc., Tokyo, Japan) were available ad libitum. The animals were acclimatized to the laboratory conditions for 6 days. Animals with no abnormalities in clinical signs were included in the group allocation. At 6 weeks of age, male DS/obese rats were divided into pathological control (DS/obese Control) (n = 14) and ipragliflozin-treated (DS/obese Ipragliflozin) (n = 14) groups, such that mean SBP, body weight, plasma glucose and insulin levels, and their variance were as equal as possible between the groups. Male DS/lean rats (n = 10) were used as a normal control group. Ipragliflozin was mixed with chow (0.01%, w/w) and given to the rats ad libitum. The dose of ipragliflozin was based on that of a previous study, which was found to promote urinary glucose excretion in rats [14,19,22]. Twenty-four-hour food consumption was measured at 6 weeks of age and once every 4 weeks thereafter.

2.3. Blood chemical analysis

Blood samples were collected at 6 and 11 weeks of age from the jugular vein in the morning under non-fasting conditions, and also at 15 weeks of age from the abdominal vena cava after measuring echocardiography under isoflurane anesthesia. Plasma levels of glucose, triglyceride (TG), total cholesterol (T-Cho), non-esterified fatty acid (NEFA), and serum levels of electrolyte (Na, Cl, K) were measured using an automatic clinical chemistry analyzer (JCA-BM6010, Nihon Denshi, Co., Ltd.). Plasma levels of insulin were measured using a rat insulin ELISA kit (Lbis® Insulin-Rat-T, Shibayagi Co., Ltd., Gunma, Japan) at designated time points. Plasma levels of leptin, angiotensin II (Ang II), aldosterone, and B-type natriuretic peptide (BNP), and the serum level of renin were measured using a Mouse/Rat Leptin ELISA Kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan), Rat Ang II ELISA Kit (Life Span BioSciences, Inc., WA, USA), Aldosterone ELISA kit (Abcam plc., Cambridge, UK), Rat BNP Assay Kit 1-Plate Kit (Meso Scale Diagnostics, LLC., MD, USA), and Rat Renin (REN) ELISA (Kamiya Biomedical Company, WA, USA), respectively.

2.4. Urinalysis

Twenty-four-hour urine samples were collected at 6, 10 and 14 weeks of age under non-fasting conditions. In each sample, glucose and electrolyte levels were measured using an automatic clinical chemistry analyzer (JCA-BM6010, Nihon Denshi, Co., Ltd.). Twenty-four-hour food consumption, water intake, urine volume and urinary glucose excretion levels were also measured.

2.5. Blood pressure and heart rate

Measurement of SBP and HR was performed by a tail-cuff method using a noninvasive automatic blood pressure measurement apparatus (BP-98A or BP-98A-L, Softron Co., Ltd., Tokyo, Japan). Animals were pre-warmed in a thermostatic box (about 32 °C), then placed in a thermostatic unit (about 38 °C) before measurement of SBP and HR.

2.6. Echocardiography

Echocardiography was performed using a modified version of the methods described by Reffelmann et al. [23] and Murase et al. [24]. At the end of the administration period at 15 weeks of age, each animal was anesthetized by inhalation with isoflurane, and placed on a heating pad to maintain body temperature. Rats were placed in the left lateral position, and the LV short-axis cross-sectional image (papillary muscle level) was visualized by M-mode using the linear probe (UST-547, Hitachi-Aloka medical Co., Ltd., Tokyo, Japan) of an ultrasonic diagnostic apparatus (SSD-α5, Hitachi-Aloka medical Co., Ltd.). LV end-diastolic dimension (LVDd), end-systolic dimension (LVDs), thickness of the interventricular septum (IVSTd) and LV posterior wall thickness (LVPWTd) were measured, and relative wall thickness (RWT) and LV mass were calculated as follows: RWT = (LVSTd + LVPWTd)/LVDd; LV mass = $1.04 \times ((\mathrm{IVSTd} + \mathrm{LVDd} + \mathrm{LPWTd})^3 - \mathrm{LVDd}^3) \times 0.8) + 0.14$. LV ejection fraction (EF), fractional shortening (FS), cardiac output (CO) and stroke volume (SV) were also calculated. In addition, for assessment of LV diastolic function, the cardiac apical five-chamber tomographic image was visualized and the blood flow velocity pattern of the LV inflow passage and LV outflow passage were measured by the pulse Doppler method. E/A and *E*-wave deceleration time (DecT) were measured in animals in which the E-wave and A-wave were distinct and separated in the echocardiogram.

2.7. Histopathological examination

Immediately after measuring the echocardiogram, the LV of each rat was dissected and immersed in 10% neutral-buffered formalin solution for fixation. The LV was then trimmed, placed in a cassette and embedded in an automatic fixed embedding device (ETP-150 CV and VIP-J0, Sakura Finetek Japan Co, Ltd.), and thin sections were made for microscopic examination. LV sections were stained with hematoxylin and eosin (HE) for measurement of myocardial cross-sectional area, with azocarimine G and aniline blue (Azan-Mallory) for measurement of perivascular/interstitial fibrosis, and with an anti-CD68 Antibody mouse monoclonal/Anti-mouse ENVISION kit for measurement of macrophages. Kidney sections were stained with periodic acid Schiff (PAS) to score glomerulosclerosis. LV myocardial cross-sectional area, cardiac fibrosis, and the number of CD68-positive cells were measured using an image analysis software (WinRoof 2013, Mitani Corporation, Tokyo, Japan) as follows. LV myocardial cross-sectional area: 3 fields were randomly selected, and then the number of cells was counted using a 40-fold objective lens. The area of the observed field was divided by the number of cells to calculate the average of LV myocardial cross-sectional area. LV cardiac fibrosis: 5 fields were randomly selected, and the fibrotic area was then measured using a 40-fold objective lens and summed. The sum was divided by the number of fields to calculate the average of fibrosis area. To count the number of CD68-positive cells, 5 fields were randomly selected, and the number of CD68-positive cells in those 5 fields was counted using a 40-fold objective lens and summed. The summed number was divided by the number of fields to calculate the average of CD68-positive cells. To score glomerulosclerosis, 50 glomerular bodies in PAS staining slides were observed for each rat. Glomerulosclerosis in each rat was scored in the manner determined by the judgment criteria of Uehara et al. [25], with the score graded according to the approximated proportion of glomeruli affected as follows: 0 (intact glomerulus), 1 (≤1/4), 2 (1/4–1/2), 3 (1/2–3/4) or 4 (> 3/4). Glomerulosclerosis score was calculated as Glomerulosclerosis

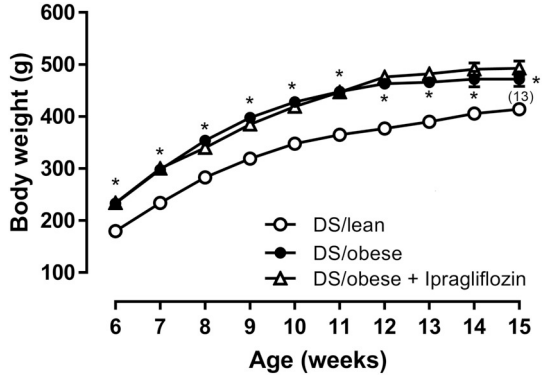

Fig. 1. Body weight changes in DS/lean and DS/obese rats.
Treatment with ipragliflozin was started at 7 weeks of age. Data are presented as mean ± SEM. $^{*}p < 0.05$ compared to DS/lean group. Number of rats per group at the start of administration: DS/lean control group, 10; DS/obese control group, 14; ipragliflozin-treated group, 14. At 15 weeks of age, the number of rats in the DS/obese group was 13.

score = ([0 × a] + [1 × b] + [2 × c] + [3 × d] + [4 × e])/50, where a is the number of glomerular bodies of 'grade 0', b is the numbers of those of 'grade 1', c is the number of those of 'grade 2', d is the number of those of 'graded 3', and e is the number of those of 'graded 4'.

2.8. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). DS/lean and DS/obese control groups, and the DS/obese control and ipragliflozin-treated group were compared using an *F* test for homogeneity of variance followed by Student's *t*-test or Aspin-Welch's t-test. For histopathological findings, differences between the DS/lean and DS/obese control groups, and DS/obese and ipragliflozin-treated group were assessed using the Wilcoxon rank sum test. $p < 0.05$ was considered significant. Statistical analyses were conducted using SAS for Windows, version 9.3. (SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Body weight

Fig. 1 shows body weight changes in each group. At 6 weeks of age, mean body weight of the DS/lean group and DS/obese groups was 180 g and 233 to 234 g, respectively. Mean body weight of the DS/obese control group was higher than that of the DS/lean group at all measurement time points. In the ipragliflozin group, no significant difference in body weight was observed between the ipragliflozin-treated group and DS/obese control group.

3.2. Urine volume and urinary glucose excretion

Fig. 2A and B show mean daily urine volume and urinary glucose

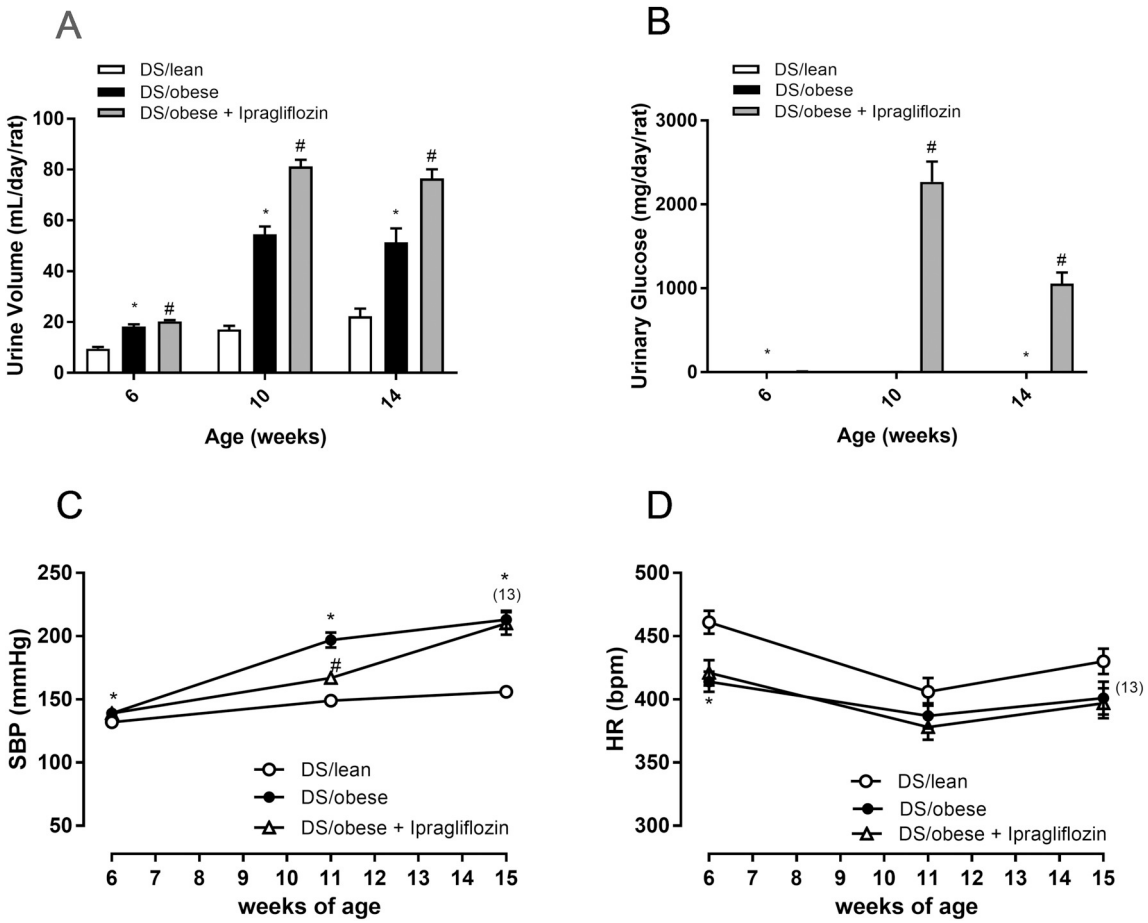

Fig. 2. Urine volume (A), urinary glucose excretion (B), SBP (C) and HR (D) of DS/lean and DS/obese rats.
Treatment with ipragliflozin was started at 7 weeks of age. Data are presented as mean ± SEM, $^{*}p < 0.05$ compared to DS/lean group, $^{\#}p < 0.05$ compared to DS/obese control group. Number of rats per group at the start of administration: DS/lean, 10; DS/obese control, 14; ipragliflozin, 14. Animal numbers for measurement of SBP and HR at 15 weeks of age in the DS/obese control were 13, respectively.

Table 1
Echocardiography parameters of DS/lean and DS/obese rats on echocardiography after 8 weeks of treatment.

	DS/lean	DS/obese	
	Control $n = 10$	Control $n = 13$	Ipragliflozin $n = 13$
Left ventricle wall and lumen			
LVDd (mm)	7.8 ± 0.2	7.7 ± 0.1	7.6 ± 0.1
LVDs (mm)	4.4 ± 0.2	3.7 ± 0.2*	3.4 ± 0.2
IVSTd (mm)	1.9 ± 0.1	2.3 ± 0.0*	2.1 ± 0.0#
LVPWTd (mm)	1.9 ± 0.1	2.2 ± 0.0*	2.1 ± 0.1
RWT	0.490 ± 0.024	0.581 ± 0.017*	0.559 ± 0.015
LV mass (mg)	915.2 ± 47.1	1128.7 ± 33.7*	1007.1 ± 29.5#
Systolic function			
EF (%)	79.6 ± 2.6	87.5 ± 1.4*	88.9 ± 1.0
FS(%)	44.0 ± 2.2	52.9 ± 1.8*	54.8 ± 1.7
CO (L/min)	0.30 ± 0.02	0.31 ± 0.02	0.28 ± 0.01
SV (mL)	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0
Diastolic function			
E/A	1.71 ± 0.10 (8)	1.58 ± 0.05 (11)	1.59 ± 0.07 (10)
DecT (msec)	42 ± 2 (9)	50 ± 2*	46 ± 2

( ): Number of animals.
LVDd and LVDs indicate left ventricle dimensions at end-diastole and end-systole, respectively; IVSTd, interventricular septal thickness at end-diastole; LVPWTd, left ventricle posterior wall thickness; RWT, relative wall thickness; EF, ejection fraction; FS, fractional shortening; CO, cardiac output; SV, stroke volume; E/A, index of the diastolic function of the left ventricle; DecT, *E*-wave deceleration time; LV mass = $1.04 \times ((\text{IVSTd} + \text{LVDd} + \text{LPWTd})^3 - \text{LVDd}^3) \times 0.8) + 0.14$.
RWT = (LVSTd + LVPWTd) / LVDd. E/A and DecT were measured in animals in which the E-wave and A-wave were distinct and separated in the echocardiogram. Data are presented as mean ± SEM.
* $p < 0.05$ compared to DS/lean control group.
# $p < 0.05$ compared to DS/obese control group.

excretion levels in each group at 6, 10 and 14 weeks of age, respectively. Mean urine volume of the DS/obese control group was higher than that of the DS/lean control group at all age points. Although no urinary glucose excretion was observed in DS/lean control or DS/obese control groups, treatment with ipragliflozin increased both urine volume and urinary glucose excretion.

3.3. Blood pressure and heart rate

Fig. 2C and D show SBP and HR, respectively. At 6 weeks of age, mean SBP of the DS/obese control group was slightly but significantly higher than that of the DS/lean group. After that, SBP of the DS/obese control group gradually increased. In the ipragliflozin-treated group, SBP was lower than that of the DS/obese control group at 11 weeks of age, but no difference was shown between the groups at 15 weeks of age. HR of the DS/obese control group was lower than that of the DS/lean group at all age points. There was no obvious difference between the ipragliflozin-treated group and DS/obese control group.

3.4. Echocardiography

Table 1 shows the results of echocardiography conducted at 15 weeks of age, after the 8-week treatment period. Regarding measurement of the LV wall and lumen, LVDd was comparable and LVDs was decreased in DS/obese rats compared with DS/lean rats. Ipragliflozin did not alter these parameters. IVSTd, LVPWTd, RWT and LV mass were increased in DS/obese rats compared with DS/lean rats. In the ipragliflozin-treated group, significant decreases were observed in LV mass and IVSTd as compared with the DS/obese control group. With regard to systolic function parameters, EF and FS were increased, whereas CO and SV were comparable in DS/obese rats as compared with those in DS/lean rats. Ipragliflozin did not alter these parameters. In diastolic function parameters, E/A tended to be lower and DecT, an index of LV relaxation, was significantly higher in the DS/obese group as compared with the DS/lean group. DecT of the ipragliflozin-treated group was lower than that of the DS/obese group, albeit without statistical significance ($p = 0.1499$).

3.5. Histopathological examination

Fig. 3 shows HE staining for the myocardial cross-sectional area in LV. Microscopic analysis revealed that the cross-sectional area of cardiac myocytes was increased in the DS/obese control group compared

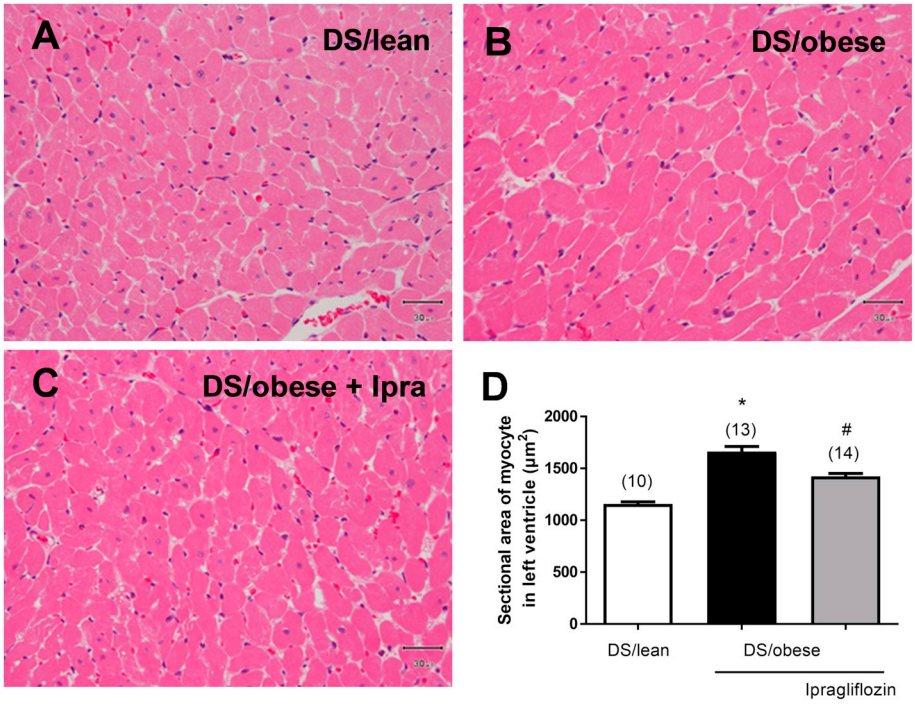

Fig. 3. Cross-sectional area of cardiomyocytes in the LV of DS/lean and DS/obese rats.
A–C: Typical photographs of the sectional area of cardiomyocytes in the DS/lean, DS/obese control, and ipragliflozin-treated groups, respectively. Thin sectional area of cardiomyocytes of LV was stained with hematoxylin and eosin. Scale bar equals 30 μm.
D: Cross sectional area of cardiomyocytes in the LV. Data are presented as mean ± SEM, $^*p < 0.05$ compared to DS/lean group, $^\#p < 0.05$ compared to DS/obese control group. Numbers in parentheses are the number of animals per group.

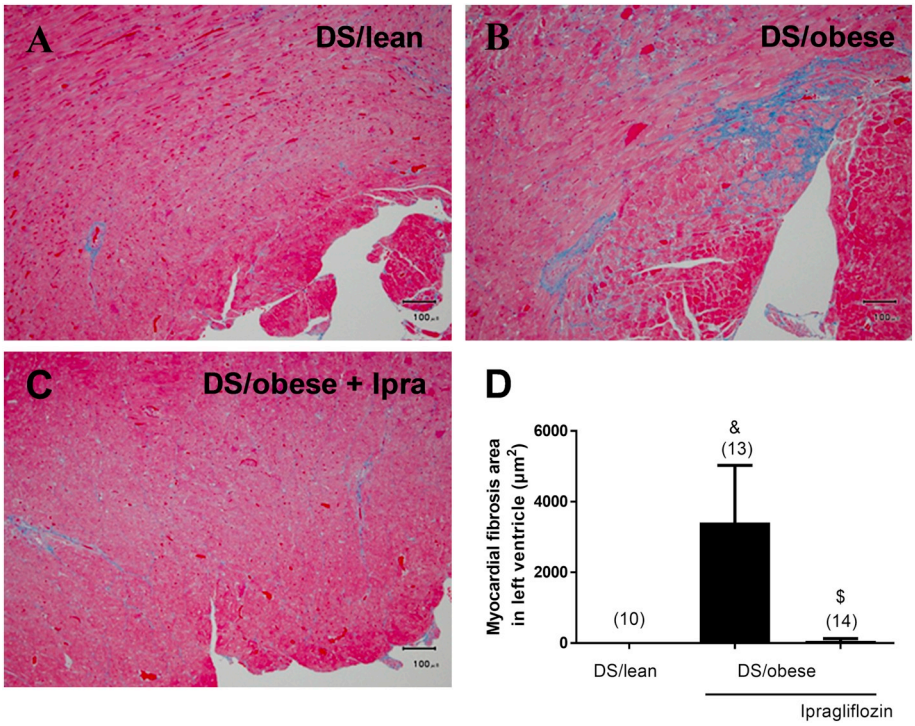

Fig. 4. Myocardial fibrosis in the LV of DS/lean and DS/obese rats.
A–C: Typical photographs of the myocardial fibrosis in the LV of the DS/lean, DS/obese control, and ipragliflozin-treated groups, respectively. Thin sectional area of LV specimens was stained with Azocarimine G and Aniline blue (Azan-Mallory). Scale bar equals 100 μm. D: Myocardial fibrosis in the LV. Data are presented as mean ± SEM, $^{\&}p < 0.05$ compared to DS/lean group, $^{\$}p < 0.05$ compared to DS/obese control group. Numbers in parentheses are the number of animals per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the DS/lean group. In the ipragliflozin-treated group, cross-sectional area was significantly decreased compared with the DS/obese control group. Fig. 4 shows Azan-Mallory staining for fibrosis area in LV. Fibrosis area in the LV myocardium was increased in the DS/obese control group compared with the DS/lean group. In the ipragliflozin-treated group, fibrosis area was significantly decreased compared with the DS/obese control group. Fig. 5 shows immunostaining for the monocyte-macrophage marker CD68-positive cells in LV. No CD68-positive cells were seen in LV of the DS/lean group; in contrast, such cells were evident in LV of DS/obese control group. The mean number of CD68-positive cells in the ipragliflozin-treated group was slightly lower but not significantly different from those in the DS/obese control group. Fig. 6 shows PAS staining for glomerulosclerosis in the kidney. Glomerulosclerosis score was significantly increased in the DS/obese control group compared with the DS/lean group. In the ipragliflozin group, a significant decrease was observed in glomerulosclerosis score as compared with DS/obese control group.

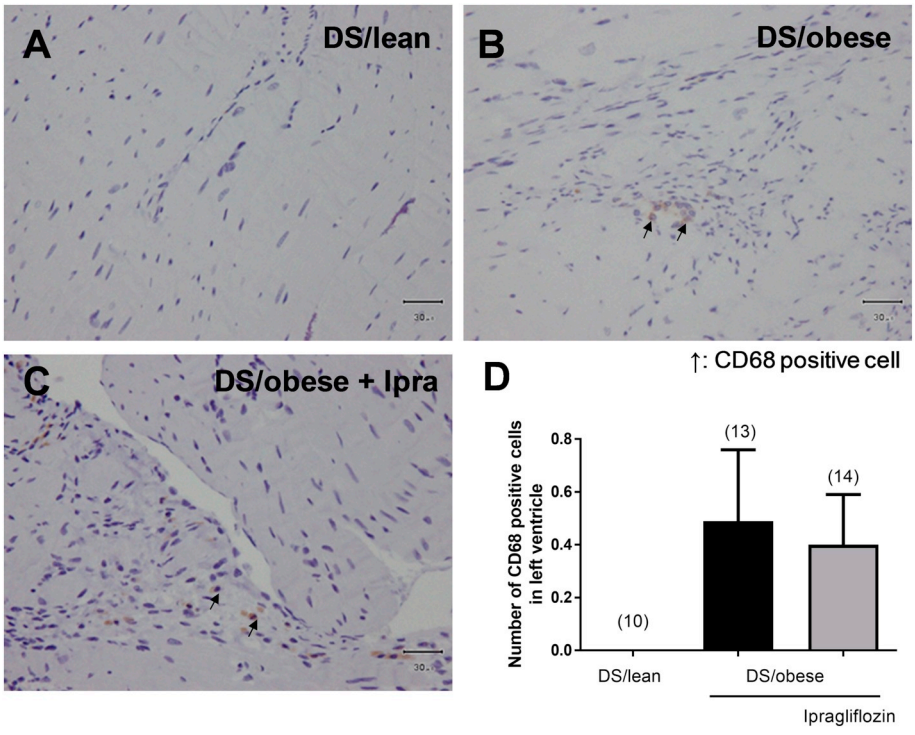

Fig. 5. Number of CD68-positive cells in the LV of DS/obese rats.
A-C: Typical photographs of inflammation in the LV of the DS/lean, DS/obese control, and ipragliflozin-treated groups, respectively. Thin sectional area of LV specimens was stained with anti-CD68 antibody, and a mouse monoclonal and anti-mouse ENVISON Kit. Scale bar equals 30 μm. D: The number of CD68-positive cells in the LV. Data are presented as mean ± SEM, with no significant differences among the groups. Numbers in parentheses are the number of animals per group.

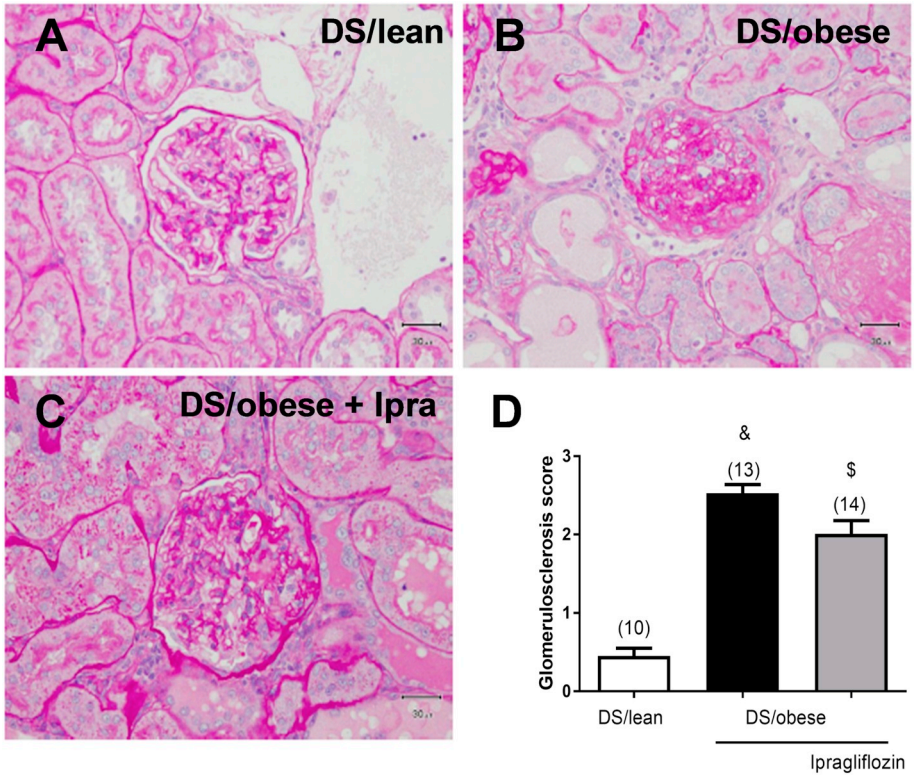

Fig. 6. Glomerulosclerosis and glomerulosclerosis score in the kidneys of DS/lean and DS/obese rats. A–C: Typical photographs of glomerulosclerosis in the DS/lean, DS/obese control, and ipragliflozin-treated groups, respectively. Thin kidney specimens were stained with PAS. Scale bar equals 30 μm. D: Glomerulosclerosis score was graded according to the approximated proportion of glomeruli affected, as follows: 0 (intact glomerulus), 1 (≤1/4), 2 (1/4–1/2), 3 (1/2–3/4) or 4 (> 3/4). Data are presented as mean ± SEM, $^{\&}p < 0.05$ compared to DS/lean group, $^{\$}p < 0.05$ compared to DS/obese control group. Numbers in parentheses are the number of animals per group.

3.6. Blood chemical analysis

Table 2 shows the results of blood chemistry at ages 6 and 11 weeks. Mean plasma glucose levels were almost identical among the groups at both age points. Plasma insulin, TG, and T-Cho levels in the DS/obese control group were obviously higher than those of the DS/lean control group. Treatment with ipragliflozin did not affect these parameters. Plasma NEFA levels in the DS/obese control group at age 6 weeks was lower than those in the DS/lean control group. In the ipragliflozin-treated group, a significant decrease was observed in plasma NEFA at 11 weeks of age as compared with the DS/obese control group. Table 3 shows the results of serum electrolytes at ages 6 and 11 weeks. Mean serum Na, Cl and K levels in the DS/lean control group remained stable between ages, whereas levels in the DS/obese control group decreased with age. Treatment with ipragliflozin did not affect serum electrolyte levels. Table 4 shows levels of blood pressure-related hormones at age 15 weeks. In the DS/obese control group, significant increases were observed in serum renin, plasma BNP and leptin levels, but both plasma Ang II and aldosterone levels were almost identical as compared with DS/lean group. Treatment with ipragliflozin caused a decrease in plasma Ang II and BNP levels, and tended to cause a decrease in plasma leptin levels ($p = 0.0503$) compared with the DS/obese control group.

Table 2
Blood chemistry in DS/lean and DS/obese rats at 6 and 11 weeks of age.

		DS/lean	DS/obese	
		Control	Control	Ipragliflozin
	Age (week)	$n = 10$	$n = 14$	$n = 14$
Glucose (mg/dL)	6	136 ± 3	137 ± 4	137 ± 5
	11	111 ± 2	113 ± 3	109 ± 5
Insulin (ng/mL)	6	0.68 ± 0.19	14.61 ± 2.51*	14.61 ± 1.94
	11	1.17 ± 0.12	12.30 ± 5.83	18.95 ± 10.44
TG (mg/dL)	6	175 ± 20	392 ± 33*	448 ± 57
	11	498 ± 48	8851 ± 1181*	9708 ± 809
T-Cho (mg/dL)	6	120 ± 4	198 ± 8*	218 ± 13
	11	123 ± 10	909 ± 66*	912 ± 73
NEFA (mEq/L)	6	0.57 ± 0.04	0.31 ± 0.02*	0.32 ± 0.02
	11	0.77 ± 0.08	0.60 ± 0.03	0.44 ± 0.03#

Treatment with ipragliflozin was started at 7 weeks of age.
Data are presented as mean ± SEM
* $p < 0.05$ compared to DS/lean control group.
# $p < 0.05$ compared to DS/obese control group.

4. Discussion

It has been postulated that the cardio-protective benefits of SGLT2 inhibitors are mediated by mechanisms other than glycemic control. Against this background, we investigated the effect of the SGLT2 selective inhibitor ipragliflozin on cardiac dysfunction and histopathology in LV in a non-diabetic rat model of cardiomyopathy, DS/obese rats. DS/obese rats show normoglycemia, hypertension, cardiac diastolic dysfunction, and cardiac remodeling, including LV hypertrophy, LV wall thickness and fibrosis in the myocardium, and rising left ventricular end-diastolic pressure (LVEDP) [12,13,26,27]. In

Table 3
Serum electrolytes in DS/lean and DS/obese rats at 6 and 11 weeks of age.

		DS/lean	DS/obese	
		Control	Control	Ipragliflozin
	Age (week)	$n = 10$	$n = 14$	$n = 14$
Na (mEq/L)	6	142 ± 0	143 ± 0	143 ± 0
	11	143 ± 0	130 ± 2*	131 ± 1
Cl (mEq/L)	6	100 ± 0	97 ± 1*	97 ± 0
	11	100 ± 0	85 ± 2*	84 ± 1
K (mEq/L)	6	5.7 ± 0.1	5.9 ± 0.1*	5.8 ± 0.1
	11	5.6 ± 0.1	4.6 ± 0.2*	4.6 ± 0.1

Treatment with ipragliflozin was started at 7 weeks of age.
Data are presented as mean ± SEM
* $p < 0.05$ compared to DS/lean control group.

Table 4
Blood pressure-related hormones in DS/lean and DS/obese rats after 8 weeks of treatment.

	DS/lean	DS/obese	
	Control $n = 9$	Control $n = 13$	Ipragliflozin $n = 13$
Renin (pg/mL)	73.4 ± 5.2	371 ± 59*	300 ± 48
Angiotensin II (pg/mL)	22.1 ± 1.9	18.5 ± 2.5[a]	7.94 ± 2.60#
Aldosterone (pg/mL)	788 ± 73	872 ± 148[a]	1190 ± 280
BNP (pg/mL)	55.2 ± 8.9	96.9 ± 11.8*	51.6 ± 4.7#
Leptin (ng/mL)	1.61 ± 0.18	91.2 ± 4.5*[a]	77.1 ± 5.0[b]

Data are presented as mean ± SEM.
* $p < 0.05$ compared to DS/lean control group.
# $p < 0.05$ compared to DS/obese control group.
[a] $n = 11$ because of two missing values.
[b] $p = 0.0503$ compared to DS/obese control group.

addition, increases in plasma leptin levels and upregulation of *BNP* gene in the LV were noted, as previously reported [12,13]. Treatment with ipragliflozin clearly ameliorated LV hypertrophy, and LV wall thickness and fibrosis in association with increased urine volume and urinary glucose excretion levels, indicating that SGLT2 in the rats was significantly inhibited. However, treatment did not affect plasma glucose and lipid levels, body weight, or heart rate. The ipragliflozin-treated group did show a delay in the age-related increase in SBP, a decrease in plasma BNP levels and a lowered plasma leptin levels.

In the present study, the SGLT2 inhibitor ipragliflozin demonstrated a cardio-protective effect without affecting glycemic levels in DS/obese rats. These findings are similar to those observed in the EMPA-REG OUTCOME trial, and suggest that the cardio-protective effects of SGLT2 inhibitors might be expected even in CVD patients without diabetes. In addition, DS/obese rats might be an appropriate model in which to investigate the cardio-protective mechanism of SGLT2 inhibitors other than glycemic control.

There are several possible mechanisms to explain the cardio-protective effects deduced from the present study. A delay in the age-related increase in SBP in the ipragliflozin-treated group is highly likely to explain the cardio-protective action. Lowered blood pressure levels, likely due to osmotic diuresis by ipragliflozin, should reduce cardiac pressure load. Osmotic diuresis should reduce cardiac volume load as well. Alleviation of this cardiac burden might have contributed to the cardio-protective effect. The decrease in plasma BNP levels in the ipragliflozin-treated group supports this reduction in cardiac burden given that is known that the hormone BNP is secreted primarily by cardiac ventricular myocytes in response to increased ventricular wall stress induced by volume expansion and pressure overload [28]. Although treatment with ipragliflozin decreased plasma angiotensin II levels, the significance of this effect remains unclear as it was not accompanied by changes in renin and aldosterone levels or SBP. Alterations in sympathetic nervous system activity following SGLT2 inhibition might be another possible mechanism of action for the cardio-protective effect. However, no significant alterations in HR was observed in the present study. Another SGLT2 inhibitor, luseogliflozin, did not change HR in animal models of T2DM [29]. Moreover, a number of clinical trials using SGLT2 inhibitors have shown no meaningful changes in HR in T2DM patients [30]. These observations indicate that SGLT2 inhibitors do not affect HR, suggesting that inhibition of SGLT2 inhibition may have little impact on sympathetic nervous system activity. In the present study, DS/obese control rats showed significant hyperleptinemia. It is known that increased circulating levels of leptin, a hormone which is a marker of leptin resistance, is common in obesity and is independently associated with cardiovascular disease in humans [31]. Leptin is a hormone predominantly secreted from white adipose tissue, and circulating levels are positively correlated with fat mass [32]. Treatment with ipragliflozin decreased plasma leptin levels but the change did not reach statistical significance ($p < 0.0503$). We previously reported that ipragliflozin reduced body fat mass by increasing fatty acid oxidation in obese rats [19] and promoted the preferential loss of fat mass in non-obese diabetic rats [33]. Although we did not measure fat mass in the present study, it is plausible that treatment with ipragliflozin decreased circulating leptin levels through reduced fat mass.

In the present study, we did not evaluate the effect of ipragliflozin on vascular resistance or vascular function. However, ipragliflozin might have reduced vascular resistance, which might in turn have contributed to cardio-protection. In fact, Salim et al. reported that ipragliflozin ameliorated endothelial dysfunction in streptozotocin-induced diabetic mice [34]. Two other SGLT2 inhibitors, dapagliflozin, and empagliflozin, also ameliorated endothelial dysfunction in diabetic animal models [35–37]. These investigators noted that SGLT 2 inhibitors reduced glucotoxicity and oxidative stress, which may represent an important mechanism underlying the cardiovascular benefits of SGLT2 inhibitor treatment. SGLT2 inhibitors may reduce the steep increase in blood glucose levels after meals (glucose spike), and might therefore reduce post-prandial oxidative stress even in non-diabetic animals. However, validating this hypothesis will require further study.

Renal diseases are common in T2DM patients with established CVD patients and frequently coexist [38,39]. House et al. reported that cardiac disease is often associated with worsening renal function and vice versa [40]. Thus, prevention of renal damage or preservation of renal function might result in cardio-protection. It is known that glycemic control prevents the deterioration of microvascular complications such as diabetic nephropathy. Recently, it was proposed that SGLT2 inhibitors have reno-protective action through modulation of the tubuloglomerular feedback (TGF) system [41]. Inhibition of SGLT2 reduces proximal reabsorption of glucose and sodium, which leads to significant increases in early distal sodium and chloride levels and consequently reduces single nephron glomerular filtration rate [42] and thereby decreases intraglomerular pressure [41,43]. Such a mechanism would work not only in hyperglycemia but also in normoglycemia. In the present study, a low glomerulosclerosis score was observed in the ipragliflozin-treated group, which is likely via the TGF mechanism. Such reno-protective action observed in the ipragliflozin-treated group might contribute to the cardio-protective effect.

In the present study, we demonstrated that treatment with ipragliflozin clearly ameliorated LV hypertrophy, and LV wall thickness and fibrosis in a non-diabetic rat model of cardiomyopathy without affecting plasma glucose and lipid levels or heart rate. These findings suggest that SGLT2 inhibition prevents the deterioration of cardiomyopathy even in patients without T2DM. However, several limitations of the study warrant mention. First, the myocardial function of rats shows marked physiological distinctions compared to the human heart. For example, the rat myocardium exhibits a very short action potential [44], and the resting heart rate is five times that of humans [45]. Thus, translational aspects of a rat model of cardiomyopathy must be interpreted with caution. Second, the mechanism of the cardio-protective effect of SGLT2 inhibition has not been fully elucidated. One likely contributor to this protective effect is a delay in the age-related increase in SBP; however, our present findings suggested several other potential mechanisms of action. Further studies are needed to determine the mechanisms underlying the cardio-protective effect of SGLT2 inhibitors and its translatability to humans.

5. Conclusion

Ipragliflozin ameliorated LV hypertrophy, and LV wall thickness and fibrosis without altering plasma glucose levels in non-diabetic cardiomyopathy model rats. The present data suggests that SGLT2 inhibitors may prevent the deterioration of cardiomyopathy with or without diabetes.

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Declaration of competing interest

T. Takasu and S. Takakura are employees of Astellas Pharma Inc. The study was funded by Astellas Pharma Inc., Japan.

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