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ORIGINAL ARTICLE

Luseogliflozin and caloric intake restriction increase superoxide dismutase 2 expression, promote antioxidative effects, and attenuate aortic endothelial dysfunction in diet-induced obese mice

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ABSTRACT

Aims/Introduction: The mechanisms underlying the effect of sodium-glucose cotransporter 2 (SGLT2) inhibitors on aortic endothelial dysfunction in diet-induced obesity are not clearly understood. This study investigated whether SGLT2 inhibition by luseogliflozin improved free fatty acid (FFA)-induced endothelial dysfunction in high-fat diet (HFD)-induced obese mice.

Materials and Methods: Mice were fed a control diet or high-fat diet for 8 weeks, and then each diet with or without luseogliflozin was provided for an additional 8 weeks under free or paired feeding. Afterward, the thoracic aortas were removed and utilized for the experiments.

Results: Luseogliflozin treatment decreased body weight, fasting blood glucose, insulin, and total cholesterol in HFD-fed mice only under paired feeding but not under free feeding. Endothelial-dependent vasodilation under FFA exposure conditions was significantly lower in HFD-fed mice than in control diet-fed mice, and luseogliflozin treatment ameliorated FFA-induced endothelial dysfunction. Reactive oxygen species (ROS) production induced by FFA was significantly increased in HFD-induced obese mice. Luseogliflozin treatment increased the expression of superoxide dismutase 2 (SOD2), an antioxidative molecule, and reduced FFA-induced ROS production in the thoracic aorta. Superoxide dismutase reversed FFA-induced endothelial dysfunction in HFD-fed mice.

Conclusions: It was shown that caloric restriction is important for the effect of luseogliflozin on metabolic parameters and endothelial dysfunction. Furthermore, SGLT2 inhibition by luseogliflozin possibly ameliorates FFA-induced endothelial dysfunction by increasing SOD2 expression and decreasing reactive oxygen species production in the thoracic aorta.

INTRODUCTION

The prevalence of obesity has nearly tripled since 1975[1]. Obesity and visceral adipose fat accumulation have been reported to be associated with impaired glucose tolerance (IGT), insulin resistance, and diabetes mellitus, which are related to cardiovascular disease (CVD) risk[2–4]. CVD is a leading cause of morbidity and mortality worldwide[5], and it has been reported that the risk of death from ischemic heart disease among diabetic patients is several times higher than that among nondiabetic patients[6, 7]. Therefore, it is important to reduce obesity, prevent the progression to diabetes, and reduce the risk of cardiovascular disease.

Aortic endothelial dysfunction develops as an early lesion of atherosclerosis and plays a central role in the progression of

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atherosclerosis and cardiovascular disease[8]. Aortic endothelial dysfunction is observed in impaired glucose tolerance[9]; furthermore, endothelial dysfunction has been reported to occur in obesity even without diabetes[10]. Therefore, it seems important to intervene at an earlier stage to prevent atherosclerosis and cardiovascular disease progression. However, treatment has not been established for aortic endothelial dysfunction, especially in the early stage of diabetes.

Sodium glucose cotransporter 2 (SGLT2) inhibitors are drugs that lower blood glucose by inhibiting glucose reabsorption in the kidney. Recently, large-scale clinical trials have reported that SGLT2 inhibitors reduce blood glucose levels, body weight, blood pressure, and the risks of cardiovascular disease and renal dysfunction[11–13]. In addition, SGLT2 inhibitors have been reported to have various effects, such as lowering uric acid[14] and improving fatty liver[15], and multifaceted effects in addition to lowering glycemia are expected.

It has been reported that SGLT2 inhibitors reduce oxidative stress and inflammation in the aorta and ameliorate the aortic endothelial dysfunction accompanying diabetes mellitus in an animal model[16–18]. However, most animal models are more representative of type 1 diabetes as they are established using streptozotocin, or are characterized by prominent hyperglycemia, and there are few reports of the effects of SGLT2 inhibitors on aortic endothelial dysfunction with diet-induced obesity and early-stage of diabetes. Therefore, in the present study, we examined the effect and mechanism of SGLT2 inhibitors on aortic endothelial dysfunction using luseogliflozin in an animal model of diet-induced obesity and early-stage diabetes mellitus.

MATERIALS AND METHODS

Animals, diets, and luseogliflozin treatment

Male, 4-week-old C57BL/6J mice were purchased from Kyudo Co., Ltd (Saga, Japan), Luseogliflozin was supplied by Taisho Pharmaceutical Co., Ltd (Tokyo, Japan). A powdered control diet (MF; 61.5% of calories from carbohydrate, 12.8% from fat, 25.7% from protein) and powdered high-fat diet (HFD-60; 19.6% of calories from carbohydrate, 62.2% from fat, 18.2% from protein) were purchased from Oriental Yeast (Tokyo, Japan). After 1 week of acclimatization, the mice were fed a control diet or a high-fat diet for 16 weeks. In week 9 of feeding, we divided the mice into four groups according to diet and drug administration: (1) control diet (Con) group, (2) Con + luseogliflozin (Lus) group, (3) high-fat diet (HFD) group, and (4) HFD + Lus group. Luseogliflozin was mixed into the diets at a concentration of 0.01%. Feeding was performed by free or paired feeding. Paired feeding was performed as follows: each animal treated with luseogliflozin was restricted to the food intake of an assigned untreated counterpart measured 24 h earlier. Mice were maintained under a 12 h light/12 h dark cycle with free access to water. All experimental procedures were reviewed and approved by the Laboratory Animal Committees of Kagoshima University Graduate School and were performed in accordance with the guidelines for the care and use of laboratory animals.

Measurement of metabolic parameters and hepatic cholesterol and triglycerides

At the time of killing, blood samples from the inferior vena cava, liver, and epididymal adipose tissue (EAT) were collected. Serum triglycerides, nonesterified fatty acids (NEFAs), and total cholesterol were measured using LabAssay kits (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan). Serum leukocyte cell-derived chemotaxin 2 (LECT2), a liver-derived hepatokine, was measured using a CircuLex Mouse LECT2 ELISA kit (Medical & Biological Laboratories Co., Ltd, Tokyo, Japan). Serum interleukin-6 (IL-6) and monocyte chemotactic protein 1/C-C motif chemokine 2 (MCP-1/CCL2) were measured using mouse IL-6 and MCP-1 ELISA kits (Proteintech Japan, Inc., Tokyo, Japan) and plasma insulin was measured using a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc., Kanagawa, Japan). Lipid in the liver was extracted using the Bligh and Dyer method, and cholesterol and triglyceride in the liver were measured using LabAssay kits (Fujifilm Wako Pure Chemical Corporation). If the results were below the lower limit of sensitivity of the assays, the lower limit was used as the result. In this report, we defined hyperglycemia, hyperinsulinemia, insulin resistance, and lipid abnormalities induced by obesity as metabolic disorders.

Glucose tolerance test and insulin tolerance test

A glucose tolerance test was performed in the morning after an overnight fast. D-Glucose (2 mg/g body weight) was injected intraperitoneally. Capillary blood samples were collected using the tail cut method, and blood glucose was measured with Stat Strip XP3 (NIPRO Corporation, Osaka, Japan) before glucose injection and at 30, 60, 90, and 120 min after glucose injection.

An insulin tolerance test was performed in the early afternoon in the random fed state. Insulin (Humulin R, Eli Lilly Japan K.K., Hyogo, Japan) was injected intraperitoneally at 0.8 mU/g body weight, and blood glucose was measured before insulin injection and at 30 and 60 min after insulin injection.

Vascular reactivity assay

The analysis of vascular reactivity was performed as described previously[19]. After the mice had been fasted overnight, fasting blood glucose and blood 3-hydroxybutyric acid (3-OHBA) were measured using Stat Strip XP3. Then, after the mice were anesthetized, the thoracic aortas were isolated and cut into 1–2 mm rings. The aortic rings were mounted in a Multiwire Myograph Systems 620M (Danish Myo Technology A/S, Hinnerup, Denmark) filled with physiological salt solution (PSS; 130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO4, 1.17 mM $MgSO_4 \cdot 7H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM glucose, 0.026 mM EDTA, and 1.6 mM $CaCl_2$) aerated with 95% O_2 and 5% CO_2 at 37°C. The mounted aortic rings were equilibrated for 1 h. For the first 40 min of the equilibration period, the aortic rings

were equilibrated in one of the following three conditions: (1) normal (PSS only) condition, (2) PSS containing a 50-fold dilution of 20% Intralipos (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) and lipoprotein lipase (Fujifilm WAKO) at 30 U/mL (Lipos + LPL) and (3) PSS containing diluted Intralipos, lipoprotein lipase and superoxide dismutase (Sigma–Aldrich, Tokyo, Japan) at 100 U/mL (Lipos + LPL + SOD). Intralipos and LPL were used to expose aortic rings to free fatty acid (FFA)[20]. After equilibration, the aortic rings were constricted with 1×10^{-7} M phenylephrine. When plateaus were attained, the aortic rings were exposed to increasing concentrations of acetylcholine (ACh) and sodium nitroprusside (SNP). At the end of the experiments, 1×10^{-4} M papaverine (PPV) was added. The isometric tension was recorded on a polygraph. The responses of the aortic rings to ACh and SNP were expressed as a percentage of the responses to papaverine.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously[16, 21, 22]. Total RNA was extracted from mouse thoracic aortas by using TRIzol Reagent (Life Technologies Japan Ltd, Tokyo, Japan). Reverse transcription was performed by using High Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific K.K., Tokyo, Japan). Quantitative real-time PCR was performed on a StepOnePlus Real-Time PCR system with the gene-specific primers listed in Table S1 and Power-Track SYBR Green Master Mix (Thermo Fisher Scientific K.K.). Relative gene expression was calculated using the $\Delta\Delta C_t$ method. The expression of target genes was normalized to that of β-actin.

Dihydroethidium fluorescence

The oxidative fluorescent dye dihydroethidium (DHE) was used to evaluate the production of ROS in the aorta[19, 23]. After exposure to Lipos + LPL for 40 min, unfixed segments of the thoracic aorta were frozen in OCT compound, and transverse sections (8 μm) were generated with a cryostat and placed on glass slides. Sections were incubated in a light-protected chamber at room temperature for 20 min with 5 μmol/L dihydroethidium (Thermo Fisher Scientific). Images were obtained with an optical microscope (EVOS FL Auto 2; Thermo Fisher Scientific). The DHE fluorescence intensity was measured after correcting the background intensity by using Image J. Because the amount of blood vessel in each image may be different, the area of auto fluorescence was counted to represent the sample size with Image J and the DHE signal/autofluorescence signal ratio was calculated for normalization[23].

Statistical analysis

All numerical values are expressed as the mean ± standard error of the mean. Comparisons between multiple groups were performed to assess the effect of high-fat diet feeding and luseogliflozin treatment with two-way analysis of variance (ANOVA). When interactions were significant with two-way ANOVA, the comparisons were performed with one-way ANOVA. The comparisons of vascular responses were performed with two-factor repeated-measures ANOVA. A value of $P < 0.05$ was considered indicative of significance.

RESULTS

Luseogliflozin did not affect diet-induced obesity or obesity-induced metabolic disorders under free feeding

Body weight, fasting blood glucose, and plasma insulin levels were higher under high-fat diet feeding. The luseogliflozin treatment did not affect these metabolic disorders under free feeding (Table 1).

Luseogliflozin ameliorated HFD-induced obesity and metabolic disorders in paired feeding

The high-fat diet group had significantly higher body weight, fasting blood glucose, plasma insulin levels, and total cholesterol levels than the Con group, and luseogliflozin significantly reduced these parameters under paired feeding. There was no difference in blood 3-OHBA, triglyceride, nonesterified fatty acid, IL-6, and MCP-1 levels (Table 2).

Luseogliflozin improved HFD-induced fatty liver under paired feeding

Luseogliflozin significantly reduced liver weight, the liver weight/body weight ratio, and the cholesterol and triglyceride

Table 1 | Body weight and metabolic parameters in control or diet induced obese mice in free feeding treated with or without luseogliflozin

					Two-way ANOVA		
	Con	Con + Lus	HFD	HFD + Lus	*P* HFD	*P* Lus	*P* Int
BW (g)	29.07 ± 1.4	30.53 ± 1.4	47.73 ± 1.8	47.73 ± 1.3	<0.001	0.632	0.632
FBG (mg/dL)	89.17 ± 3.5	100 ± 9.2	154.5 ± 8.5†††	133.3 ± 1.8†††	<0.001	0.442	0.025
Insulin (ng/mL)	0.25 ± 0.092	0.24 ± 0.094	1.26 ± 0.304	1.88 ± 0.307	<0.001	0.186	0.180
3-OHBA (mol/L)	1.77 ± 0.16	1.60 ± 0.17	1.57 ± 0.19	2.0 ± 0.25	0.614	0.502	0.140

The data are presented as the mean ± standard error of the mean. Group comparisons were performed using two-way ANOVA. When interactions were significant with two-way ANOVA, group comparisons were performed with one-way ANOVA. $^{\dagger\dagger\dagger}P < 0.001$ compared with Con group using one-way ANOVA. 3-OHBA, 3-hydroxybutyric acid; BW, body weight; Con, control diet; FBG, fasting blood glucose; HFD, high fat diet; Int, interaction; Lus, luseogliflozin; *P* HFD, *P*-value of HFD factor in two-way ANOVA; *P* Lus, *P*-value of Lus factor in two-way ANOVA.

Table 2 | Body weight, metabolic parameters, and serum inflammatory cytokine levels in control or diet-induced-obese mice in paired feeding treated with or without luseogliflozin

					Two-way ANOVA		
	Con	Con + Lus	HFD	HFD + Lus	*P* HFD	*P* Lus	*P* Int
BW (g)	30.48 ± 0.9	26.0 ± 0.6†	47.64 ± 1.0†††	38.06 ± 1.5†††,¶¶¶	<0.001	<0.001	0.020
FBG (mg/dL)	108.7 ± 4.6	102.2 ± 6.3	174.6 ± 10.1†††	121.5 ± 6.6¶¶¶	<0.001	0.442	0.025
Insulin (ng/mL)	0.024 ± 0.044	0.048 ± 6.4	0.9 ± 0.15†††	0.084 ± 0.017¶¶¶	<0.001	<0.001	<0.001
3-OHBA (mol/L)	1.44 ± 0.14	1.21 ± 0.12	1.1 ± 0.16	1.35 ± 0.21	0.54	0.96	0.140
TG (mg/dL)	53.1 ± 6.1	48.8 ± 6.4	55.5 ± 5.6	52.6 ± 5.2	0.60	0.546	0.902
NEFA (mEq/L)	0.55 ± 0.030	0.59 ± 0.033	0.49 ± 0.037	0.54 ± 0.038	0.11	0.17	0.98
T-chol (mg/dL)	55.4 ± 3.1	54.0 ± 3.0	132.86 ± 8.6†††	99.6 ± 5.5†††,¶¶¶	<0.001	0.020	0.030
IL-6 (pg/mL)	13.37 ± 0.8	12.2 ± 3.16	14.82 ± 1.51	14.04 ± 3.2	0.51	0.69	0.94
MCP-1 (pg/mL)	17.81 ± 2.09	15.59 ± 1.5	36.35 ± 8.74	24.67 ± 4.13	0.39	0.14	0.069

The data are presented as the mean ± standard error of the mean. Group comparisons were performed using two-way ANOVA. When interactions were significant with two-way ANOVA, group comparisons were performed with one-way ANOVA. $^{\dagger}P < 0.005$, $^{\dagger\dagger\dagger}P < 0.001$ compared with Con group, $^{\P\P\P}P < 0.001$ compared with HFD group using one-way ANOVA. 3-OHBA, 3-hydroxybutyric acid; BW, body weight; Con, control diet; FBG, fasting blood glucose; HFD, high fat diet; IL-6, interleukin-6; Int, interaction; Lus, luseogliflozin; MCP-1, monocyte chemotactic protein 1; NEFA, nonesterified fatty acid; *P* HFD, *P*-value of HFD factor in two-way ANOVA; *P* Lus, *P*-value of Lus factor in two-way ANOVA; T-chol, total cholesterol; TG, triglyceride.

Table 3 | Weights of the liver and epididymal adipose tissue and serum LECT2 in control or diet induced obese mice in paired feeding treated with or without luseogliflozin

					Two-way ANOVA		
	Con	Con + Lus	HFD	HFD + Lus	*P* HFD	*P* Lus	*P* Int
Liver (g)	1.2 ± 0.026	1.2 ± 0.034	2.3 ± 0.16†††	1.2 ± 0.036¶¶¶	<0.001	<0.001	<0.001
Liver to BW (%)	4.1 ± 0.074	4.6 ± 0.10	5.0 ± 0.44†	3.1 ± 0.067†,¶¶¶	0.318	0.005	<0.001
Liver chol (mg/g liver)	0.46 ± 0.079	0.32 ± 0.086	2.23 ± 0.23†††	0.52 ± 0.22¶¶¶	<0.001	<0.001	<0.001
Liver TG (mg/g liver)	20.3 ± 3.42	17.7 ± 6.71	115 ± 11.4†††	29.8 ± 7.34¶¶¶	<0.001	<0.001	<0.001
LECT2 (ng/mL)	20.3 ± 2.6	18.1 ± 1.6	58.7 ± 5.3†††	35.9 ± 2.6†,¶¶¶	<0.001	0.001	0.005
EAT (g)	0.94 ± 0.11	0.36 ± 0.043	2.1 ± 0.76†††	2.1 ± 0.17†††	<0.001	0.014	0.014
EAT to BW (%)	3.0 ± 0.28	1.4 ± 0.15	4.5 ± 0.23††	5.5 ± 0.29†††,¶	<0.001	0.230	<0.001

The data are presented as the mean ± standard error of the mean. Group comparisons were performed using two-way ANOVA. When interactions were significant with two-way ANOVA, group comparisons were performed with one-way ANOVA. $^{\dagger}P < 0.005$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ compared with Con group, $^{\P}P < 0.05$, $^{\P\P\P}P < 0.001$ compared with HFD group using one-way ANOVA. BW, body weight; chol, cholesterol; Con, control diet; EAT, epididymal adipose tissue; HFD, high fat diet; Int, interaction; LECT2, leukocyte cell-derived chemotaxin 2; Lus, luseogliflozin; *P* HFD, *P*-value of HFD factor in two-way ANOVA; *P* Lus, *P*-value of Lus factor in two-way ANOVA; TG, triglyceride.

levels in the liver. LECT2 is a liver-derived hepatokine which increases in association with hepatic fat accumulation[24]. The HFD group had significantly higher LECT2 levels than the Con group, and luseogliflozin significantly reduced the LECT2 levels. There was no difference in the epididymal adipose tissue (EAT) weight between the HFD group and the HFD + Lus group. The HFD + Lus group had a higher EAT weight/body weight ratio than the Con group and HFD group (Table 3).

Luseogliflozin ameliorated glucose intolerance and insulin resistance

The area under the curve of blood glucose in the glucose tolerance test (GTT) was significantly greater under high-fat diet feeding, and luseogliflozin treatment significantly reduced it (Figure 1a,b). In the insulin tolerance test (ITT), the reduction of blood glucose levels at 30 min after insulin administration was significantly greater under luseogliflozin treatment (Figure 1c,d). There was no difference in the blood glucose change at 60 min in the ITT (Figure 1e).

Luseogliflozin ameliorated FFA-induced aortic endothelial dysfunction

The endothelium-dependent vasodilation in response to acetylcholine was not different between the group fed the control diet and the group fed the high-fat diet in the control (PSS only) condition (Figure 2a). On the other hand, the HFD group had a significantly lower response to ACh than the Con group in the increasing concentration of FFA condition induced by Lipos + LPL and luseogliflozin treatment significantly

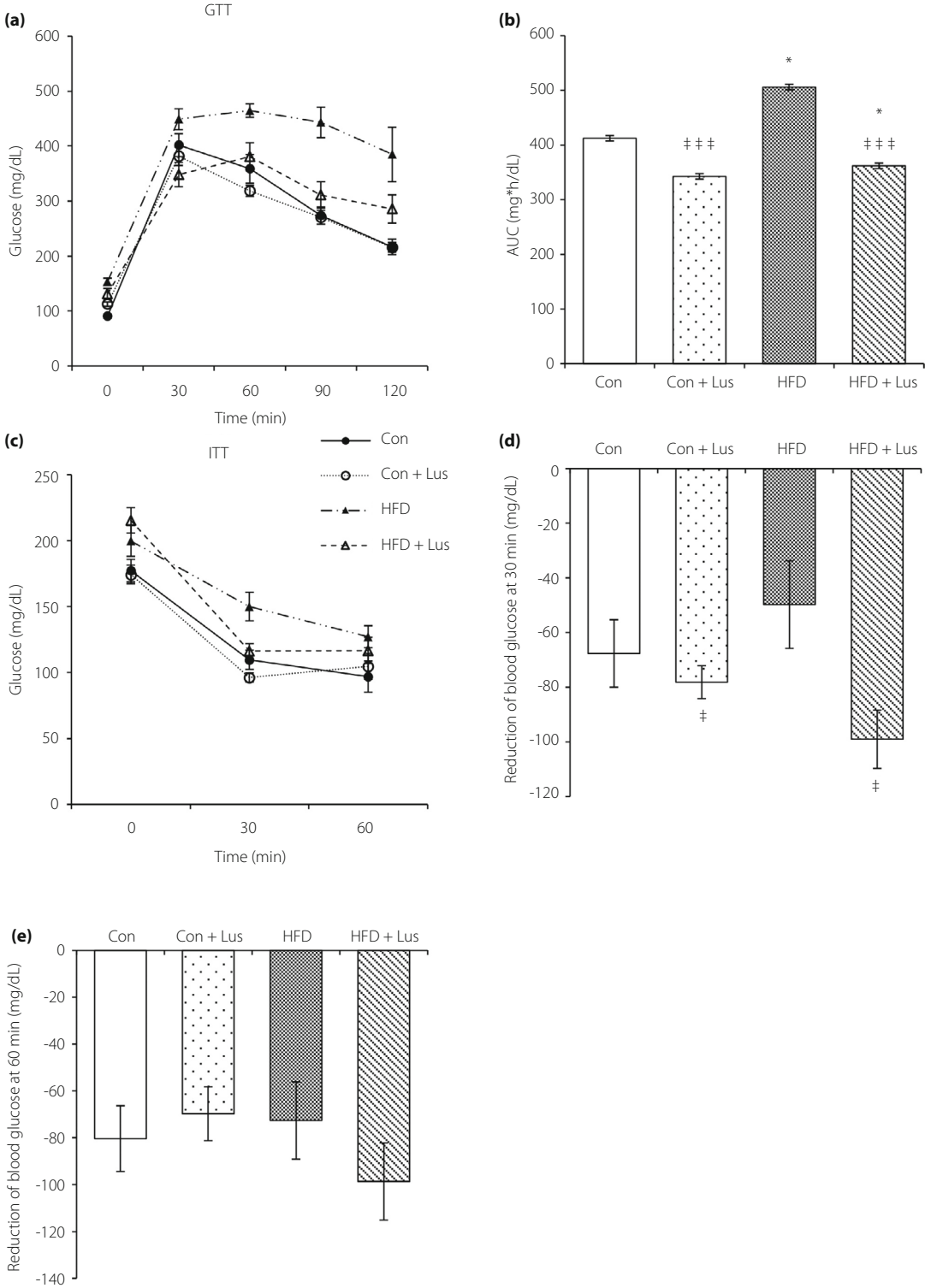

Figure 1 | Changes in blood glucose level in glucose tolerance test or ITT in control or diet induced obese mice treated with or without luseogliflozin. (a) Changes in blood glucose level of the GTT. (b) AUC of blood glucose level during GTT. (c) Changes in blood glucose level of the ITT. (d) Reduction of blood glucose at 30 min in the ITT. (e) Reduction of blood glucose at 60 min in the ITT. The data are presented as the mean ± standard error of the mean. *$P < 0.05$ for the comparison of HFD feeding factor with two-way ANOVA. ‡$P < 0.05$ and ‡‡‡$P < 0.001$ for the comparison of luseogliflozin treatment factor with two-way ANOVA. AUC, area under the curve; Con, control diet; GTT, glucose tolerance test; HFD, high-fat diet; ITT, insulin tolerance test; Lus, luseogliflozin.

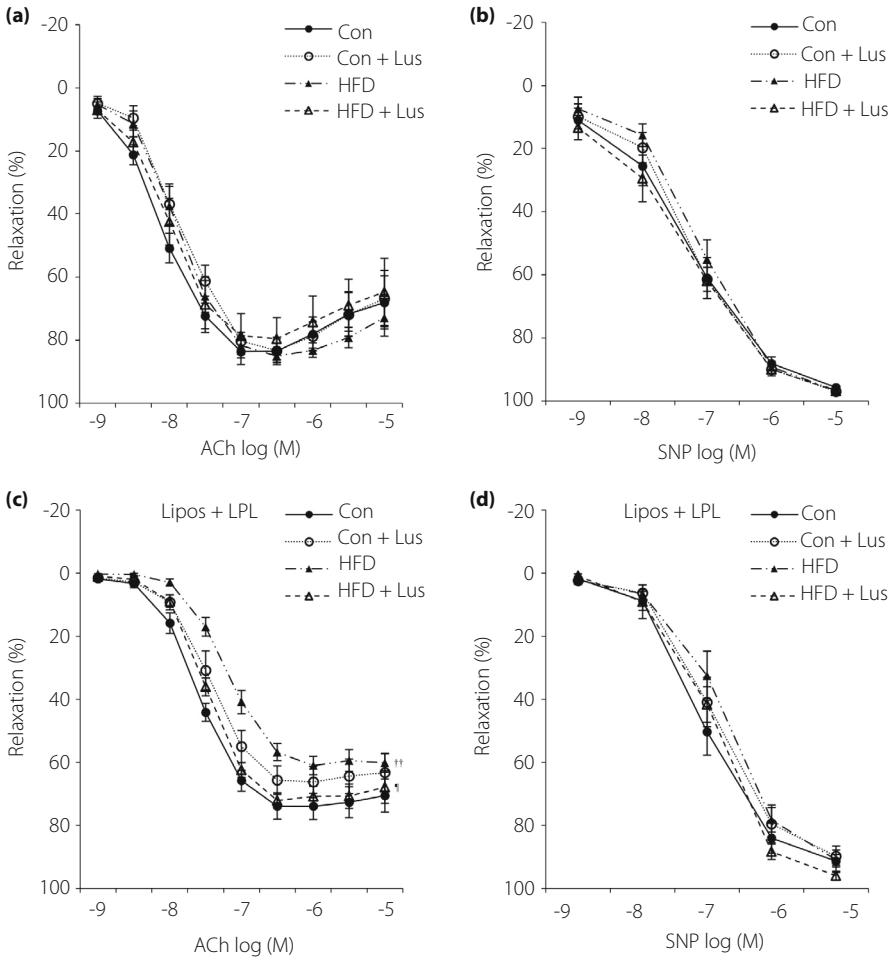

Figure 2 | Vascular response to acetylcholine or sodium nitroprusside under the normal condition or Lipos + LPL condition in control or diet induced obese mice treated with or without luseogliflozin. (a) The vascular response to acetylcholine under the normal condition. (b) The vascular response to sodium nitroprusside under the normal condition. (c) The vascular response to acetylcholine under the Lipos + LPL condition. (d) The vascular response to sodium nitroprusside under the Lipos + LPL condition. The data are presented as the mean ± standard error of the mean. †† $P < 0.01$ compared with the Con group; ¶ $P < 0.05$ compared with the HFD group using two-factor repeated-measures ANOVA. ACh, acetylcholine; Con, control diet; HFD, high-fat diet; Lipos, Intralipos; LPL, lipoprotein lipase; Lus, luseogliflozin; SNP, sodium nitroprusside.

ameliorated the FFA-induced endothelial dysfunction (Figure 2c). The endothelium-independent vasodilation in response to sodium nitroprusside was not different between the group fed the control diet and the group fed the high-fat diet under either condition (Figure 2b,d).

Luseogliflozin suppressed FFA-induced ROS production

The dihydroethidium intensity was significantly higher in the thoracic aortas from the HFD group than in those from the Con group. The luseogliflozin treatment significantly suppressed the ROS production induced by FFA (Figure 3).

Luseogliflozin significantly increased antioxidative molecule levels

The mRNA expression levels of SOD1 and NOX4 in the aorta were not affected by either high-fat diet feeding or luseogliflozin treatment (Figure 4a,c). On the other hand, luseogliflozin treatment significantly increased the mRNA expression of SOD2 in the aorta (Figure 4b). The mRNA expression of MCP-1 was greater under HFD feeding (Figure 4d). The mRNA expression levels of ICAM-1 and VCAM-1 in the aorta were not significantly affected by HFD feeding or luseogliflozin treatment (Figure 4e,f).

SOD prevented fatty acid-induced endothelial dysfunction

To assess whether superoxide dismutase could prevent fatty acid-induced endothelial dysfunction, we added SOD to Lipos + LPL and tested vascular function. Superoxide dismutase reversed FFA-induced endothelial dysfunction in the high-fat diet group (Figure 5a). The endothelium-independent vasodilation in response to sodium nitroprusside was not different among the four groups (Figure 5b).

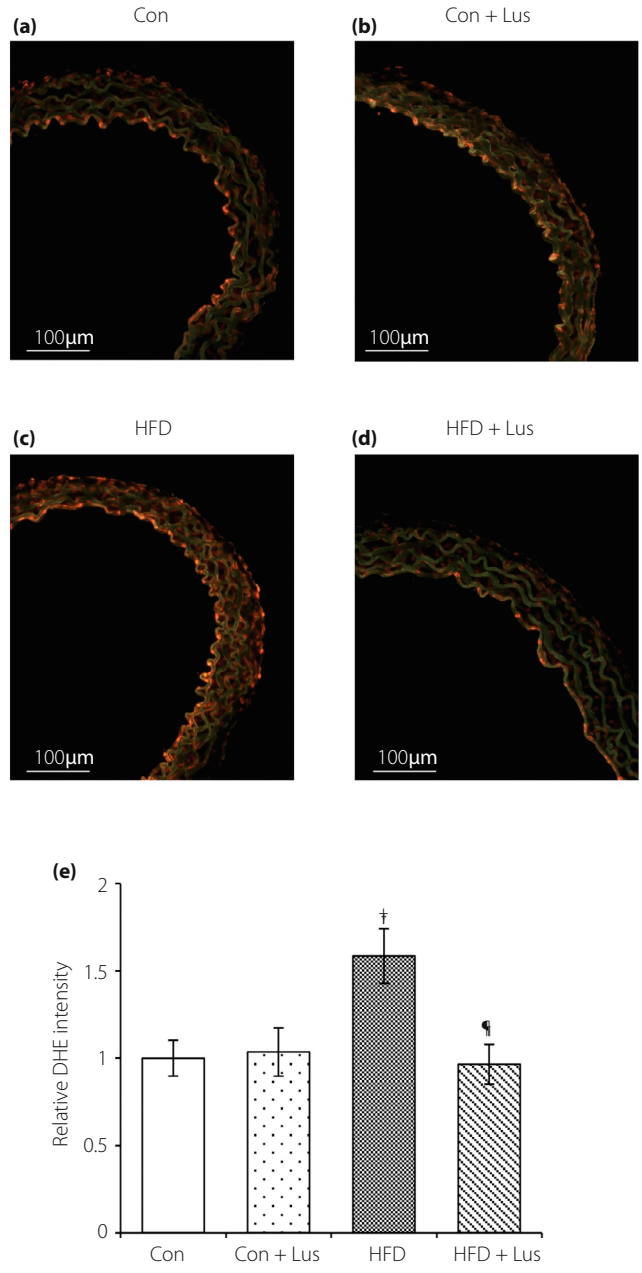

Figure 3 | Free fatty acid induced ROS production in the thoracic aorta from control or diet induced obese mice treated with or without luseogliflozin. (a–d) Representative photomicrographs of aortas stained with dihydroethidium (DHE). (a) Con group. (b) Con + Lus group. (c) HFD group. (d) HFD + Lus group. (e) Relative DHE intensity. The data are presented as the mean ± standard error of the mean. $^{\dagger}P < 0.05$ compared with the Con group; $^{\P}P < 0.05$ compared with the HFD group using one-way ANOVA. Con, control diet; HFD, high-fat diet; Lus, luseogliflozin.

DISCUSSION

It was demonstrated that luseogliflozin treatment and caloric intake restriction could decrease body weight, insulin resistance, fatty liver, and FFA-induced aortic endothelial dysfunction. Furthermore, luseogliflozin treatment induced the expression of the antioxidative molecule SOD2 and reduced FFA-induced ROS production in the thoracic aorta. These results suggest that luseogliflozin treatment and caloric intake restriction ameliorate obesity-induced metabolic disorders and may promote vascular antioxidant capacity and protect aortic endothelial function.

In the present study, luseogliflozin affected body weight and metabolic disorders under paired feeding but not under free feeding. Several studies have shown that SGLT2 inhibitors increase food intake[25], and this attenuates body weight reduction and hypoglycemic effects[26, 27]. It has been also reported that the respiratory exchange ratio (RER) did not change with SGLT2 inhibitor treatment without food intake restriction, on the other hand, SGLT2 inhibitor treatment with food intake restriction decreased the RER, suggesting that sources of energy shifted from glucose to fat. Furthermore, it has been reported that SGLT2 inhibitor treatment decreased body weight and the hepatic gluconeogenic response, and increased energy consumption under controlled feeding in obese mice but free feeding diminished these effects of the SGLT2 inhibitor[26]. Consequently, caloric intake restriction is important for the effects of SGLT2 inhibitors. SGLT2 inhibitors increase urinary glucose excretion and calorie loss and consequently decrease body weight and blood glucose levels. It has been speculated that the increased food intake compensates for the calorie loss caused by SGLT2 inhibitors. Although we could not have measured food intake under free feeding, we hypothesized that luseogliflozin may not have ameliorated diet-induced obesity and hyperglycemia under free feeding by increasing food intake. This is the reason that paired feeding was performed. Thus, caloric intake restriction in addition to luseogliflozin treatment attenuated not only body weight gain but also hyperglycemia, hyperinsulinemia, and visceral adipose obesity, which can cause vascular endothelial dysfunction and atherosclerosis progression.

In this study, the high-fat diet group exhibited a significant increase in ROS production, attenuated endothelial-dependent vasodilation under increasing concentrations of FFA and SOD ameliorated the FFA induced endothelial dysfunction. On the other hand, a previous study that examined endothelial function with a similar experimental system showed increasing oxidative stress and aortic endothelial dysfunction under hyperglycemic conditions in diet-induced obesity and insulin resistance model rats[19]. In the study, an antioxidant protected the aortic endothelial function as in our study. Given this evidence, the results of the present study are not FFA-specific and indicate that oxidative stress is an important factor in endothelial dysfunction.

In the present study, FFA caused endothelial dysfunction in the high-fat diet group and SOD ameliorated the FFA-induced endothelial dysfunction in vascular reactivity experiments. Because FFA enters endothelial cells via transporters, increases oxidative stress, and induces endothelial dysfunction[28],

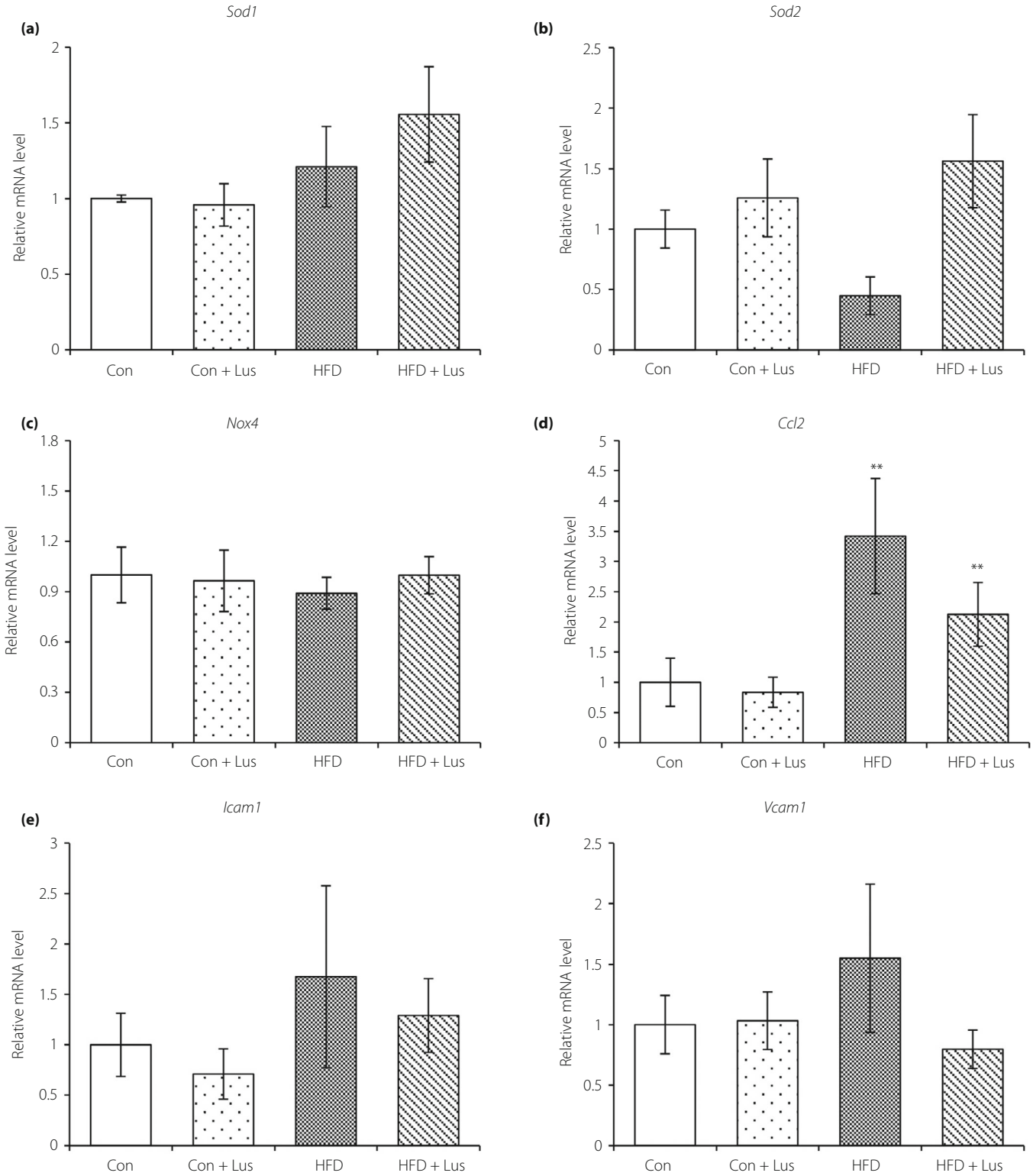

Figure 4 | Relative levels of cytokines and antioxidant molecules in the thoracic aorta from control or diet induced obese mice treated with or without luseogliflozin. (a–f) Real-time PCR analysis of the expression of (a) *Sod1*, (b) *Sod2*, (c) *Nox4*, (d) *Ccl2*, (e) *Icam1*, and (f) *Vcam1*. The data are presented as the mean ± standard error of the mean. $^{**}P < 0.01$ and $^{‡}P < 0.05$ for the comparison between HFD feeding or luseogliflozin treatment factors with two-way ANOVA. *Ccl2*, C-C motif chemokine 2; Con, control diet; HFD, high-fat diet; *Icam1*, intercellular adhesion molecule 1; Lus, luseogliflozin; *Nox4*, NADPH oxidase 4; *Sod1*, superoxide dismutase 1; *Sod2*, superoxide dismutase 2; *Vcam1*, vascular cell adhesion molecule 1.

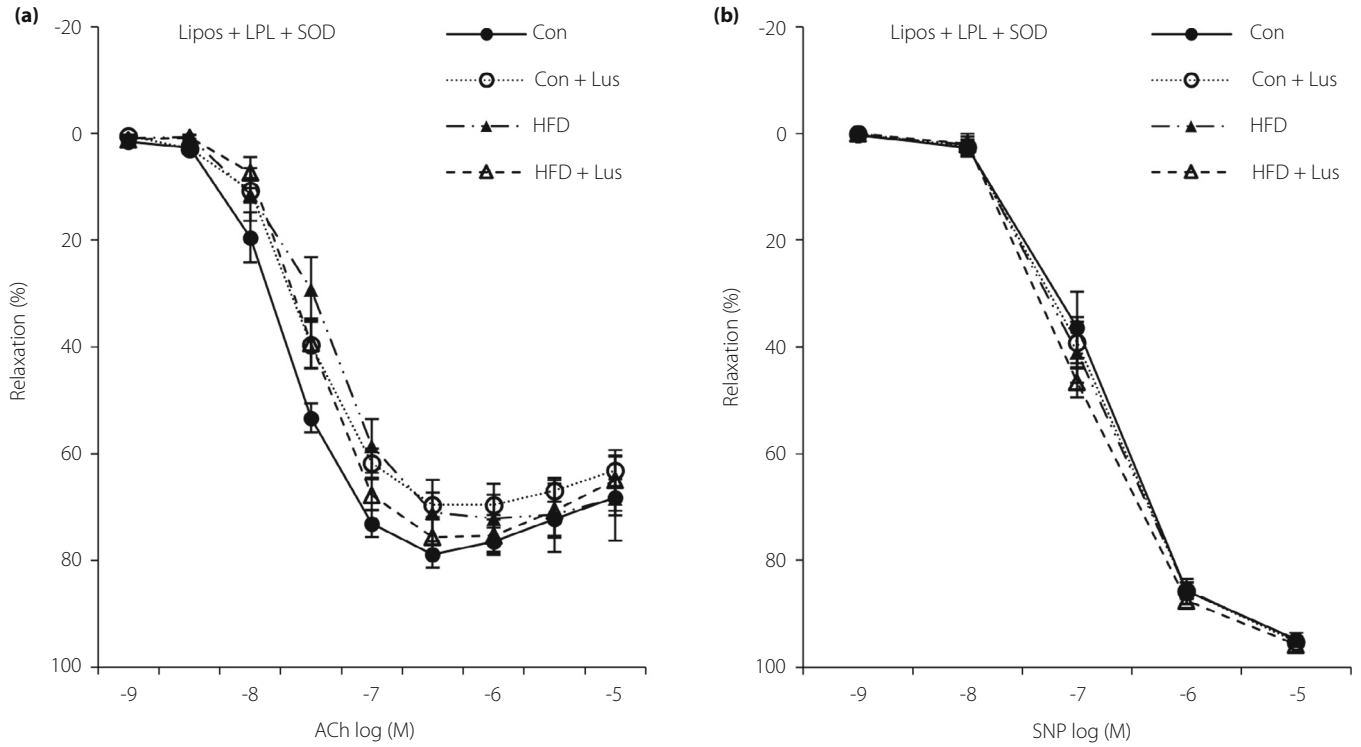

Figure 5 | Vascular response to acetylcholine or sodium nitroprusside under the Lipos + LPL + SOD condition in control or diet induced obese mice treated with or without luseogliflozin. (a) The vascular response to acetylcholine under the Lipos + LPL + SOD condition. (b) The vascular response to sodium nitroprusside under the Lipos + LPL + SOD condition. The data are presented as the mean ± standard error of the mean. ACh, acetylcholine; Con, control diet; HFD, high-fat diet; Lipos, Intralipos; LPL, lipoprotein lipase; Lus, luseogliflozin; SNP, sodium nitroprusside; SOD, superoxide dismutase.

intercellular superoxide anion is important for FFA induced endothelial dysfunction and the SOD effects observed in this study.

A previous study showed that urinary SOD activity is significantly higher in type 2 diabetes patients treated with SGLT2 inhibitors than in patients not treated with SGLT2 inhibitors[29]. Another study showed that dapagliflozin increased the expression of SOD in renal tissue in an animal model of type 2 diabetes[30]. In this study, luseogliflozin treatment significantly increased the expression of SOD2 in the thoracic aorta. The expression and activity of SOD are changed by several factors including body weight. It has been reported that high fat diet decreases SOD2 expression[31]. Another study has reported that SOD activity is decreased in individuals with obesity[32]. In the present study, luseogliflozin treatment reduced body weight significantly in both Con and HFD groups, therefore, body weight reduction by luseogliflozin may be associated with the increased expression of the SOD2 gene. NF-E2-related factor 2 (Nrf2), a major transcription regulator that activates antioxidants, including SOD2, and anti-inflammatory and cell protective gene expression, may be another factor that increases the expression of SOD2 in the thoracic aorta with luseogliflozin administration. It has been reported that activating Nrf2 by bardoxolone methyl increased SOD2 gene expression[19]. Furthermore, SGLT2 inhibitors could increase Nrf2 expression in both diabetic and non-diabetic animal models[33, 34]. Although we cannot say for certain because we did not measure the expression of Nrf2, based on the above reports, it is speculated that luseogliflozin treatment possibly promote Nrf2 expression, thus increasing the expression of SOD2 and reducing FFA-induced ROS production in the present study. In this study, increasing the mRNA expression of SOD2 in thoracic aorta was statistically significant under luseogliflozin treatment in two-way ANOVA. Based on this result, it is possible that luseogliflozin has not only an indirect effect but also a direct effect on thoracic aorta.

People with cardiovascular disease and nonalcoholic fatty liver disease (NAFLD) have overlapping risk factors, such as obesity and insulin resistance. A previous study showed that increases in inflammatory cytokines and plasminogen activator inhibitor-1 (PAI-1) are associated with inflammation and thrombus formation and are linked to vascular endothelial dysfunction and atherosclerosis in coronary and cerebral arteries in patients with NAFLD[35]. In the present study, the high-fat diet group had significantly increased liver weight and triglyceride and cholesterol levels in the liver, and luseogliflozin treatment reduced these parameters. In stepwise multiple regression analysis, liver weight was negatively correlated with endothelial-dependent vascular relaxation (Table S2). Furthermore, in

simple regression analysis, liver weight was significantly and positively correlated with body weight and levels of fasting blood glucose, fasting insulin, liver triglyceride, and liver cholesterol levels (Figure S3). These results indicate that luseogliflozin treatment ameliorated metabolic disorders by reducing fatty liver and improved endothelial dysfunction in obese mice. LECT2, a liver-derived hepatokine, is related to obesity and NAFLD[36, 37]. It has been reported that LECT2 induces proinflammatory cytokines and is significantly correlated with aortic atherosclerosis[38, 39]. In the present study, plasma LECT2 levels were significantly increased, and luseogliflozin treatment reduced them. Luseogliflozin treatment may have decreased plasma LECT2 levels by preventing diet-induced obesity and fatty liver, and this reduction in LECT2 levels may be correlated with the protective effect on endothelial function.

In contrast to a previous study, diet-induced obesity alone did not cause endothelial dysfunction in the present study. There are several reports that showed preserved aortic endothelial function in obese animal models[40, 41]. It has been postulated that obesity increases cardiac output and consequently increases aortic blood flow and shear stress, which could contribute to the preservation of aortic endothelial function[41]. This may be one reason why diet-induced obesity alone did not cause endothelial dysfunction in the current study. Another reason for the preservation of endothelial function in obese mice may be the removal of perivascular adipose tissue (PVAT) when we evaluated aortic endothelial function. PVAT releases vasoactive substances, such as nitric oxide (NO), and has a protective effect on vascular function under physiological conditions; on the other hand, PVAT becomes dysfunctional and releases proinflammatory factors, cytokines and chemokines to the aorta, inducing endothelial dysfunction in obesity[42, 43]. Therefore, we may not have been able to demonstrate aortic endothelial dysfunction in obese mice by removing dysfunctional PVAT.

In conclusion, we showed that caloric intake restriction and luseogliflozin administration ameliorated obesity-induced metabolic disorders and aortic endothelial dysfunction in diet-induced obese mice. We also showed that luseogliflozin treatment significantly increased SOD2 expression in the thoracic aorta, promoted antioxidative effects, and ameliorated FFA-induced aortic endothelial dysfunction.

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DISCLOSURE

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Approval of the research protocol: All experimental procedures were reviewed and approved by the Laboratory Animal Committees of Kagoshima University Graduate School and were performed in accordance with the guidelines for the care and use of laboratory animals.
Informed consent: N/A.
Registry and registration no. of the study/trial: The study was approved on October 12, 2020 (Approval No. MD20072), and June 22, 2022 (Approval No. MD22036).
Animal studies: All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S3 | Simple regression analysis of liver weight with (a) BW (body weight), (b) FBG (fasting blood glucose), (c) insulin, (d) T-chol (total cholesterol), (e) liver chol (liver cholesterol), (f) liver TG (liver triglyceride), (g) LECT2 (Leukocyte cell-derived chemotaxin 2).

Table S1 | Primer sequences used for the quantitative real-time PCR analysis.

Table S2 | Stepwise multiple regression analysis showing the independent association of liver weight with maximum response by acetylcholine.