ORIGINAL ARTICLE

The sodium-glucose co-transporter 2 inhibitor tofogliflozin suppresses atherosclerosis through glucose lowering in ApoE-deficient mice with streptozotocin-induced diabetes

Masahiko Iwamoto[1,2] | Tetsuya Kubota[1,2,3,4,5,6] (iD) | Yoshitaka Sakurai[1] | Nobuhiro Wada[1,3] | Seiji Shioda[7] | Toshimasa Yamauchi[1] | Takashi Kadowaki[1,8] | Naoto Kubota[1,9]

[1]Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

[2]Division of Diabetes and Metabolism, The Institute of Medical Science, Asahi Life Foundation, Tokyo, Japan

[3]Department of Clinical Nutrition, National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN), Tokyo, Japan

[4]Laboratory for Intestinal Ecosystem, RIKEN Center for Integrative Medical Sciences (IMS), Kanagawa, Japan

[5]Intestinal Microbiota Project, Kanagawa Institute of Industrial Science and Technology Ebina, Kanagawa, Japan

[6]Division of Cardiovascular Medicine, Toho University Ohashi Medical Center, Tokyo, Japan

[7]Global Research Center for Innovative Life Science, Peptide Drug Innovation, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, Tokyo, Japan

[8]Toranomon Hospital, Tokyo, Japan

[9]Department of Clinical Nutrition Therapy, The University of Tokyo, Tokyo, Japan

Correspondence
Naoto Kubota or Takashi Kadowaki, Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.
Email: nkubota-tky@umin.ac.jp, kadowaki-3im@h.u-tokyo.ac.jp

Funding information
Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Scientific Research, Grant/Award Number: 18H02860; Kowa Company; Ministry of Education, Culture, Sports, Science, and Technology of Japan, Grant/Award Number: 15H04847

Abstract

Epidemiological and animal studies have revealed that sodium-glucose cotransporter 2 (SGLT2) inhibitors suppress cardiovascular events in subjects with type 2 diabetes and atherosclerosis in animal models of diabetes. However, it still remains unclear if the anti-atherosclerotic effect of SGLT2 inhibitors is entirely dependent on their glucose-lowering effect. Tofogliflozin, a highly specific SGLT2 inhibitor, was administrated to apolipoprotein-E-deficient (ApoEKO) with streptozotocin (STZ)-induced diabetes and nondiabetic ApoEKO mice. After 6 weeks, samples were collected to investigate the histological changes and peritoneal macrophage inflammatory cytokine levels. Tofogliflozin suppressed atherosclerosis in the diabetic ApoEKO mice. The atherosclerosis lesion areas and accumulation of macrophages in these areas were reduced by tofogliflozin treatment. The expression levels of interleukin (IL)-1β and IL-6 in the peritoneal macrophages were significantly suppressed in the tofogliflozin-treated diabetic ApoEKO mice. Tofogliflozin treatment failed to inhibit atherosclerosis

in the nondiabetic ApoEKO mice. No significant difference in the anti-atherosclerotic effects of insulin and tofogliflozin was observed between diabetic ApoEKO mice with equivalent degrees of glycemic control achieved with the two treatments. Insulin treatment significantly reduced the IL-1β and IL-6 expression levels in the peritoneal macrophages of the diabetic ApoEKO mice. Significant decrease of the LPS-stimulated IL-1β concentrations was also observed in the conditioned medium of the peritoneal macrophages collected from insulin- and tofogliflozin-treated diabetic ApoEKO mice. These results suggest that tofogliflozin suppresses atherosclerosis by improving glucose intolerance associated with inhibition of inflammation. Tofogliflozin suppresses atherosclerosis in ApoEKO mice with STZ-induced diabetes via its glucose-lowering effect.

KEYWORDS
atherosclerosis, diabetes, macrophage, SGLT-2 inhibitor, Tofogliflozin

1 | INTRODUCTION

Diabetic patients are reported to be at a two- to fourfold higher risk of the development of coronary artery disease as compared to nondiabetic patients.[1,2] Atherosclerotic cardiovascular disease (ASCVD) is now reported to be the major cause of death in diabetic patients.[3,4] The increase in the risk of ASCVD in patients with type 2 diabetes has been reported to show a strong association with poor control of hyperglycemia,[5-7] suggesting that control of hyperglycemia is essential for preventing ASCVD in patients with type 2 diabetes mellitus.

Sodium-glucose cotransporter 2 (SGLT2) inhibitors, a recently approved class of antidiabetic drugs, reduce the blood glucose levels by inhibiting glucose reabsorption in the proximal tubules of the kidney and increasing the urinary glucose excretion.[8,9] EMPA-REG OUTCOME (The Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Patients-Removing Excess Glucose), a randomized, double-blind, placebo-controlled trial, was conducted to assess the cardiovascular safety of empagliflozin in type 2 diabetic patients with a prior history of cardiovascular events.[10,11] The trial revealed that empagliflozin protected against cardiovascular events in patients with type 2 diabetes. In CANVAS (Canagliflozin Cardiovascular Assessment study), canagliflozin inhibited the development of cardiovascular events in patients with type 2 diabetes, including 30% of patients with no prior history of cardiovascular events.[12] Moreover, the DECLARE-TIMI 58 (Dapagliflozin Effect on Cardiovascular Events) trial also showed suppression of cardiovascular events by an SGLT2 inhibitor.[13] A number of epidemiological studies have suggested that SGLT2 inhibitors exert anti-atherosclerotic effects in patients with type 2 diabetes mellitus.

Animal models with diabetes have been used to investigate the mechanisms by which SGLT2 inhibitors suppress atherosclerosis.[14-16] Dapagliflozin inhibited macrophage foam cell formation and macrophage interleukin-1β (IL-1β) secretion via the reactive oxygen species (ROS)-NLRP3-caspase1 pathway to suppress atherosclerosis in apolipoprotein E-deficient knockout (ApoEKO) mice with

streptozotocin (STZ)-induced diabetes.[17,18] Empagliflozin ameliorated endothelial dysfunction by ameliorating oxidative stress and glucotoxicity, and reducing the inflammatory molecule levels in the perivascular adipose tissue (PVAT), leading to the inhibition of atherosclerosis in diabetic animals.[19,20] However, the precise underlying mechanisms of the anti-atherosclerotic effects of SGLT2 inhibitors still remain unclear.

Tofogliflozin is a highly specific SGLT2 inhibitor; the reported IC50 values of tofogliflozin against rat SGLT1 and rat SGLT2 are 8200 and 15 nM, respectively.[21] Tofogliflozin is used worldwide as a treatment agent for type 2 diabetes mellitus.[22,23] We previously demonstrated that tofogliflozin reduced the degree of body weight gain, mainly via reducing the fat mass associated with a diminished adipocyte size, and improved glucose tolerance and insulin sensitivity.[24]

In the present study, we used tofogliflozin to investigate the mechanisms underlying the anti-atherosclerotic effects of SGLT2 inhibitors. Although tofogliflozin treatment increased the food intake and water intake in the diabetic ApoEKO mice, it also caused a marked decrease of the blood glucose levels. The atherosclerosis was significantly reduced, and the accumulation of macrophages in the atherosclerotic lesions tended to be lower in the diabetic ApoEKO mice treated with tofogliflozin. The expression levels of IL-1β and IL-6 in the peritoneal macrophages were also significantly reduced in the diabetic ApoEKO mice treated with tofogliflozin. Tofogliflozin treatment failed to inhibit atherosclerosis in the nondiabetic ApoEKO mice. No significant difference in the anti-atherosclerotic effects of insulin and tofogliflozin was observed between diabetic ApoEKO mice with equivalent degrees of glycemic control achieved with the two treatments. Insulin treatment significantly reduced the IL-1β and IL-6 expression levels in the peritoneal macrophages of the diabetic ApoEKO mice. Significant decrease of the LPS-stimulated IL-1β concentrations was also observed in the conditioned medium of the peritoneal macrophages collected from insulin- and tofogliflozin-treated diabetic ApoEKO mice. These results suggest that tofogliflozin suppresses atherosclerosis by improving

glucose intolerance associated with inhibition of inflammation in the diabetic ApoEKO mice.

2 | MATERIALS AND METHODS

2.1 | Animals and Ethics statement

The male ApoEKO (6–7 weeks) and male C57BL6 (6–7 weeks) mice were purchased from Charles River Laboratories, Japan, and Taconic Farms Inc, USA, respectively. Three mice were housed per cage, and all the mice were maintained under a 12/12-hour light/dark cycle and had free access to water and chow. The animal care and experimental procedures used in the study were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by The University of Tokyo Animal Care Committee.

2.2 | Experimental protocols

Tofogliflozin was kindly provided by Kowa Company, Ltd. The diabetic ApoEKO mice were separated into two groups: one group was fed a normal chow diet and the other a normal chow diet containing 0.005% tofogliflozin, ad libitum. After 4 weeks, an oral glucose tolerance test (OGTT) was performed, and the plasma levels of insulin, triglyceride (TG), total cholesterol (T-CHO), and non-esterified fatty acid (NEFA) were measured. After 6 weeks, all the mice were administered an intraperitoneal injection of thioglycolate, and 4 days later, peritoneal macrophages were collected to investigate the expression levels of inflammatory cytokines in the macrophages by real-time PCR. Histological changes were evaluated by Sudan IV, Oil Red O, and MOMA-2 staining (Figure S1A). The same experiment was performed in the nondiabetic ApoE KO mice (Figure S1B). The diabetic ApoEKO mice were divided into three groups: a group fed normal chow, a group fed normal chow containing 0.005% tofogliflozin (average 1.71 ± 0.08 mg/kg body weight/day) ad libitum, based on data from previous studies[24], and a group fed normal chow that was given an intraperitoneal injection of insulin. Prior to the study, the optimal insulin dose (Insulin Degludec 0.2 unit/day; Novo) was determined in a preliminary study by administering various doses. After 4 weeks, the blood pressure was measured by the tail-cuff method. Plasma levels of TG, T-CHO, NEFA, and glycoalbumin (GA) were also measured by the enzymatic assay. Plasma IL-1β levels were measured using a mouse IL-1β ELISA kit (Proteintech). After 6 weeks, all mice were dissected and the histological changes were evaluated by Sudan IV (Figure S1C).

2.3 | Mouse model of STZ-induced diabetes

STZ (Sigma-Aldrich) diluted in sodium citrate buffer (pH 4.5) was administered by intraperitoneal injection at 100 mg/kg on days 0 and 4, as previously reported.[24]

2.4 | Blood sample assay

Blood glucose was measured using an automatic glucometer (Glutest mint sensor. Sanwa Chemical Co.). Plasma triglyceride, total cholesterol, and free fatty acid (Wako Pure Chemical Industries, Ltd.) levels were assayed by enzymatic methods, as previously reported, with some modifications.[26]

2.5 | Oral glucose tolerance test

Mice were loaded with oral glucose at 1.5 mg/g body weight after 16 h fasting. Blood samples were taken at 0, 15, 30, 60, 120 min and the blood glucose levels were measured with an automatic glucometer, as previously reported, with some modifications.[26] Blood samples were collected from the tail vein and centrifuged in heparinized tubes, and the separated plasma samples were stored at –20°C. The insulin levels in the plasma samples were measured using a mouse insulin ELISA kit (Morinaga Co., Ltd.).

2.6 | Measurement of blood pressure

The mice were first trained to reduce stress associated with blood pressure measurement. And then, blood pressure was measured with an automatic sphygmomanometer by the tail-cuff method in unanesthetized animals (Natsume Seisakusho Co., Ltd).

2.7 | Histological analysis

The heart and aorta were perfusion-fixed with 4% paraformaldehyde. The aorta was excised from the root to the iliac artery and stained with Sudan IV (Catalog: 194-07652Wako Pure Chemical Industries, Ltd.). The percent Sudan IV-positive areas were measured using the image analyzer software, WinROOF (Mitani Corp), as previously reported, with some modifications.[27] The transverse section of the aortic valve was stained with Oil Red O (Catalog: O0625-25G; Sigma) and MOMA-2 (Catalog: ab33451; Abcam, 1:1000 dilution), and the positively stained areas with the two stains were measured using the image analyzer software, Image J (NIH), and the positively stained areas were calculated, as previously reported, with some modifications.[27]

2.8 | Peritoneal macrophages

The mice were administered an intraperitoneal injection of 3% sodium thioglycolate (Pure Chemical Industries, Ltd.), as previously reported.[25] Peritoneal macrophages were collected after 4 days and the inflammatory cytokine levels were measured in these cells. A proportion of the peritoneal macrophages (4.0×10^5 cells) was cultured in 12-well plates. Then, 24 h after lipopolysaccharide (LPS: 100

ng/mL) stimulation, conditioned medium specimens were collected and the IL-1β concentrations measured.

2.9 | RNA preparation and quantitative PCR

The RNA was extracted using the RNeasy Mini Kit (Qiagen Co.), in accordance with the manufacturer's instructions. cDNA was generated from less than one microgram of RNA using random hexamers with QPCR master mix reagents (ABI). TaqMan quantitative PCR (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min) was then performed with the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems), using TaqMan Gene Expression Assays or SYBR Green PCR Master Mix (Applied Biosystems).[25] The expression level of each of the transcripts was normalized to the mRNA expression level of the housekeeping gene, cyclophilin, as the internal control. The TaqMan MGB probes were purchased from Applied Biosystems: IL-1β: Mm00434228_m1; IL-6: Mm00446190_m1; CCR2: Mm99999051_gH; Arg1: Mm00475988_m1. The primer sequences were designed as follows:

CD36: Fw: TCTGTTGGAACAGAGGATGA, Rv: TGGAACCAAACTGAGGAATG;

TNFα: Fw: CCAGACCCTCACACTCAGATC, Rv: CACTTGGTGGTTTGCTACGAC

Mcp1: Fw: CCTGCTGTTCACAGTTGCC, Rv: ATTGGGATCATCTTGCTGGT

MMP9: Fw: CATTCGCGTGGATAAGGAGT, Rv: CACTGCAGGAGGTCGTAGG

Cyclophilin A: Fw: GAGCTGTTTGCAGACAAAGTTC, Rv: CCCTGGCACATGAATCCTGG

2.10 | Statistical analysis

Data are expressed as means ± SEM. Statistical analyses were assessed with analysis of variance (ANOVA), and post hoc analysis was performed by the Tukey–Kramer method. Differences between two groups were assessed using Wilcoxon signed-rank test. p values of < .05 were considered statistically significant.

3 | RESULTS

3.1 | Tofogliflozin treatment suppressed atherosclerosis with decreasing the blood glucose levels in the diabetic ApoEKO mice

The aortic lesion area was first compared by Sudan IV staining among the wild-type (C57BL6), nondiabetic ApoEKO, and diabetic ApoEKO mice. The area of the atherosclerotic lesions was significantly greater in the nondiabetic ApoE mice as compared with the wild-type mice ($p < .05$, Figure S2A), consistent with a previous report.[28] The area of the atherosclerotic lesions was significantly

greater in the diabetic ApoEKO mice as compared with the nondiabetic ApoEKO mice ($p < .01$, Figure S2A). Treatment of the diabetic ApoEKO mice with tofogliflozin for 6 weeks led to increases in the food and water intakes of the mice ($p < .05$ or $< .01$), but no significant difference in the body weights was observed between the tofogliflozin-treated and control non-treated mice (Figure 1A–C). The diabetic ApoEKO mice began to show significant decreases of the blood glucose level from 1 week after the start of tofogliflozin treatment ($p < .01$, Figure 1D; left panel). The mean blood glucose level at the end of 6 weeks from the study baseline was significantly lower in the tofogliflozin-treated diabetic ApoEKO mice than in the control non-treated group ($p < .01$, Figure 1D; right panel). Consistent with these data, the diabetic ApoEKO mice treated with tofogliflozin showed significantly improved blood glucose levels in the OGTT ($p < .01$, Figure 1E; left panel). The area under the curve (AUC) of the blood glucose levels was significantly reduced after tofogliflozin treatment in the diabetic ApoEKO mice ($p < .01$, Figure 1E; middle panel). Fasting plasma insulin levels were also significantly reduced after tofogliflozin treatment ($p < .05$, Figure 1E; right panel). To confirm that tofogliflozin treatment also suppressed atherosclerosis, we carried out Sudan IV staining, which revealed that tofogliflozin treatment significantly reduced the Sudan IV-positive areas in the diabetic ApoEKO mice ($p < .05$, Figure 1F). There were no differences in the plasma triglyceride, total cholesterol, or free fatty acid levels between the control and tofogliflozin-treated mice (Figure S2). These data suggest that tofogliflozin treatment suppressed atherosclerosis with reducing the blood glucose levels.

3.2 | Tofogliflozin treatment inhibited the expression levels of inflammatory cytokines in the macrophages in atherosclerotic areas in the diabetic ApoEKO mice

As shown in Figure 2A, significant reduction in the oil red O-positive areas in the aortic valves was observed in the tofogliflozin-treated diabetic ApoEKO mice ($p < .05$). A significant reduction in the MOMA-2-positive areas in the aortic valves was also observed in the tofogliflozin-treated diabetic ApoEKO mice ($p < .05$, Figure 2B). We next investigated the expression levels of inflammatory cytokines in the peritoneal macrophages of the mice after tofogliflozin treatment. As shown in Figure 2C, the expression levels of TNFα and IL-1β were significantly decreased in the tofogliflozin-treated diabetic ApoEKO mice ($p < .05$). These data suggest that tofogliflozin treatment inhibited atherosclerosis by reducing macrophage accumulation and inflammation.

3.3 | Tofogliflozin treatment did not suppress atherosclerosis in the nondiabetic ApoEKO mice

In order to investigate whether the anti-atherosclerotic effects of tofogliflozin were entirely mediated by its effect of reducing

FIGURE 1 Tofogliflozin treatment suppressed atherosclerosis in addition to decreasing the blood glucose levels in the STZ-induced diabetic ApoEKO mice. (A) Food intake, (B) water intake, (C) body weight, and (D) plasma blood glucose levels in STZ-induced diabetic ApoEKO mice that received or did not receive tofogliflozin (Tofo) treatment (n = 4–12). *p < .05 and **p < .01 compared with the STZ-induced diabetic ApoEKO mice. (E) Blood glucose and plasma insulin levels during an OGTT in diabetic ApoEKO mice that received or did not receive Tofo treatment (n = 9–12). *p < .05 and **p < .01 compared with the STZ-induced diabetic ApoEKO mice. (F) Sudan IV-positive area in diabetic ApoEKO mice that received or did not receive Tofo treatment (n = 7–12). *p < .05 compared with the STZ-induced diabetic ApoEKO mice. Data are represented as means ± SEM
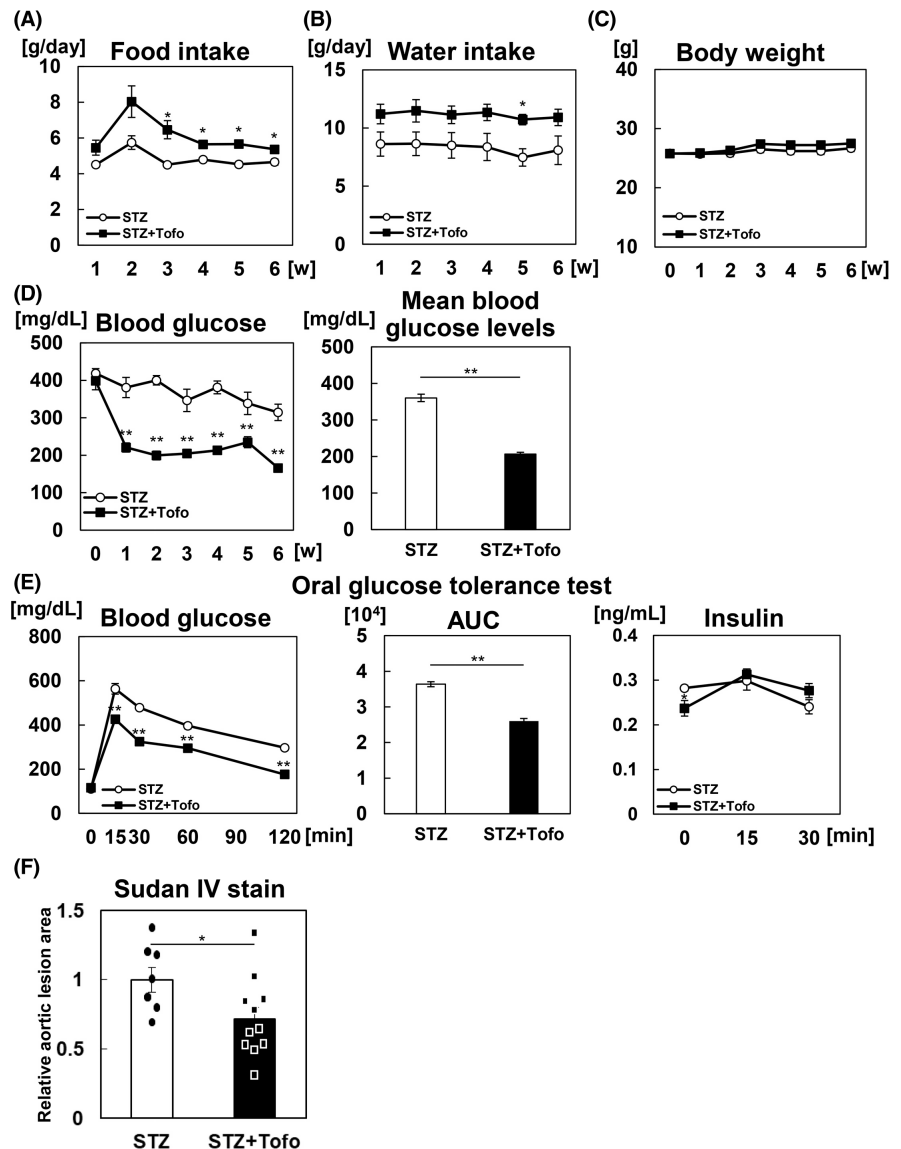
the blood glucose levels, we also examined nondiabetic ApoEKO mice. Although these mice also showed an increase in water intake (p < .05), no significant difference in the food intake or body weight was observed in the tofogliflozin-treated nondiabetic ApoEKO mice (Figure 3A–C). The blood glucose levels were markedly lower in the nondiabetic ApoEKO mice as compared to the diabetic ApoEKO mice (Figures 1D and 3D), while the blood glucose levels were similar between the nondiabetic ApoEKO mice treated and not treated with tofogliflozin (Figure 3D). In the OGTT performed in the nondiabetic ApoEKO mice treated with tofogliflozin, significant reduction of the blood glucose levels at 15 min after glucose loading (p < .05, Figure 3E; left panel) and plasma insulin levels at 30 min after glucose loading was observed (Figure 3F; right panel, p < .05). No reduction of the Sudan IV-positive areas was observed in the tofogliflozin-treated nondiabetic ApoEKO mice (Figure 3F). Also, there were no significant differences in the peritoneal macrophage expression levels of inflammatory cytokines (Figure 3G), or in the plasma triglyceride, total cholesterol, or free fatty acid levels between the

control and tofogliflozin-treated nondiabetic ApoEKO mice (Figure S3). These data suggest that tofogliflozin treatment did not suppress atherosclerosis in the nondiabetic ApoEKO mice.

3.4 | No significant difference in the anti-atherosclerotic effects of insulin and tofogliflozin was observed between diabetic ApoEKO mice with equivalent degrees of glycemic control achieved with the two treatments

In order to investigate whether the anti-atherosclerotic effect of tofogliflozin goes beyond glucose lowering, we treated the diabetic ApoEKO mice with tofogliflozin or insulin. As shown in Figure 4A, equivalent degrees of reduction of the blood glucose levels were obtained by insulin treatment and tofogliflozin treatment in the diabetic ApoEKO mice (p < .01). Consistent with these data, the degrees of reduction of the plasma levels of glycoalbumin (GA) were
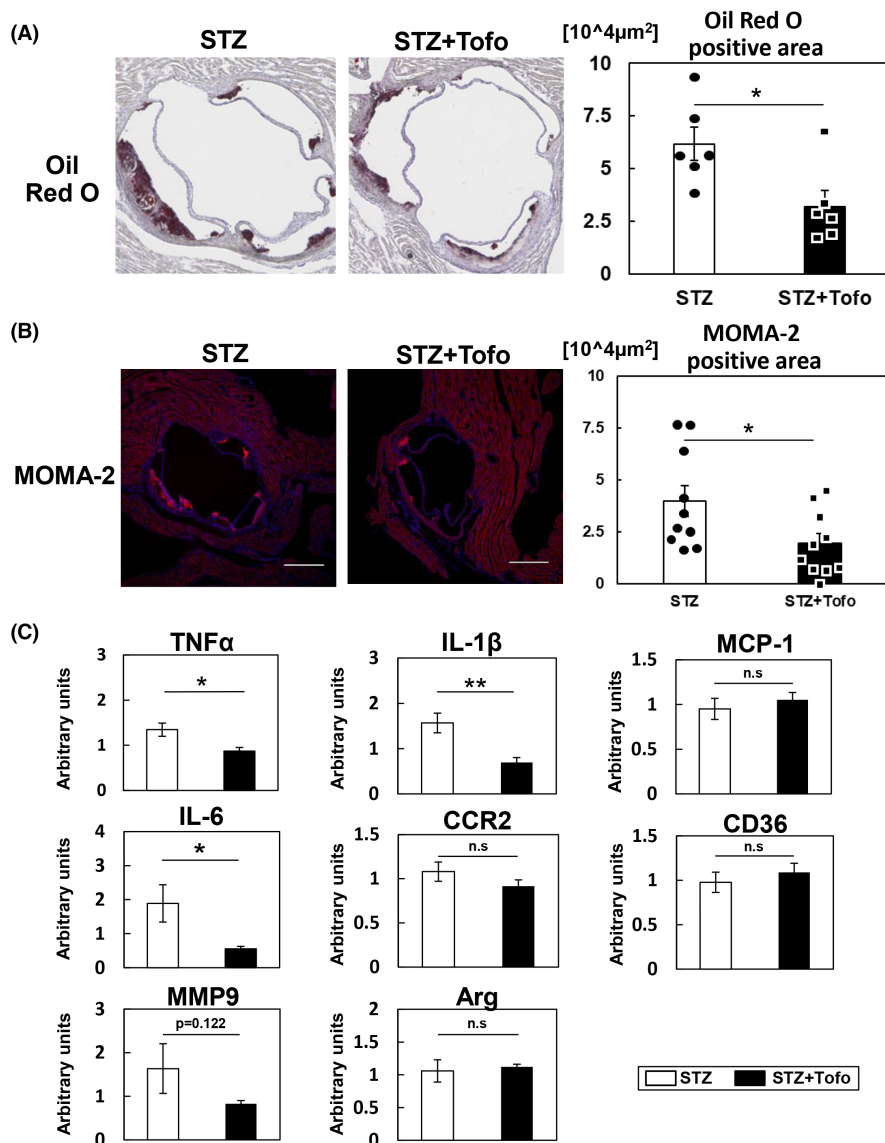
FIGURE 2 Tofogliflozin treatment inhibited the macrophage expression levels of inflammatory cytokines in atherosclerotic lesions in the STZ-induced diabetic ApoEKO mice. (A) Representative immunohistochemical staining and areas of positive staining for Oil Red O in diabetic ApoEKO mice that were or were not treated with Tofo ($n = 6$). *$p < .05$ compared with the STZ-induced diabetic ApoEKO mice. Magnification, ×100. (B) Representative immunofluorescence staining and areas of positive staining for Oil Red O in diabetic ApoEKO mice that received Tofo treatment ($n = 10$). *$p < .05$ compared with the STZ-induced diabetic ApoEKO mice. Magnification, ×40; scale bar = 500 μm (C) Expression levels of inflammatory cytokines in the peritoneal macrophages in diabetic ApoEKO mice that received or did not receive Tofo treatment ($n = 9$–21). *$p < .05$ compared with the STZ-induced diabetic ApoEKO mice. Data are expressed as means ± SEM

also equivalent between the insulin- and tofogliflozin-treated diabetic ApoEKO mice ($p < .01$, Figure 4B). There were no significant differences in the body weight or blood pressure among the three groups (Figure 4C, D). Interestingly, equivalent degrees of reduction of atherosclerotic lesion areas were observed in the tofogliflozin- and insulin-treated diabetic ApoEKO mice ($p < .05$, Figure 4E). Furthermore, there were also no differences in the plasma triglyceride, total cholesterol, or free fatty acid levels among the three groups (Figure S4). These data suggest that the anti-atherosclerotic effects beyond glucose-lowering action of tofogliflozin were not observed in the diabetic ApoEKO mice.

3.5 | Both insulin and tofogliflozin treatment reduced the expression levels of inflammatory cytokines in the peritoneal macrophages

The expression levels of inflammatory cytokines were investigated in the peritoneal macrophages of the untreated, insulin-treated, and tofogliflozin-treated diabetic ApoEKO mice. The expression levels of TNFα, IL-1β, and IL-6 were significantly decreased in the tofogliflozin-treated diabetic ApoEKO mice ($p < .05$ or $< .01$, Figures 5A, S5). Insulin treatment also elicited similar significant reductions of the IL-1β and IL-6 expression levels to same degree of tofogliflozin treatment ($p < .05$, Figures 5A, S5). We measured plasma TNFα and IL-1β levels among the diabetic ApoEKO, insulin- and tofogliflozin-treated diabetic ApoEKO mice. Plasma TNFα was not detected among three groups (data not shown). There was no difference in plasma IL-1β levels among three groups (Figure 5B). We measured the concentrations of TNFα and IL-1β in the peritoneal macrophage-conditioned medium with and without LPS stimulation in all the three groups. No TNFα was detected in the conditioned medium without the LPS stimulation in any of the three groups (data not shown). After LPS stimulation, there were no significant differences in the TNFα concentrations in the conditioned medium among the three groups (data not shown). In regard to the IL-1β concentrations in the peritoneal macrophage-conditioned medium, while there were no significant differences among the three groups prior to the LPS stimulation,

FIGURE 3 Tofogliflozin treatment did not suppress atherosclerosis in the nondiabetic ApoEKO mice. (A) Food intake, (B) water intake, (C) body weight, and (D) plasma blood glucose levels in nondiabetic ApoEKO mice that were or were not treated with Tofo ($n = 3$–5). $*p < .05$ compared with control mice. (E) Blood glucose and plasma insulin levels during an OGTT in nondiabetic ApoEKO mice that received or did not receive Tofo treatment ($n = 5$). $*p < .05$ compared with control mice. (F) Sudan IV-positive area in nondiabetic ApoEKO mice that received or did not receive Tofo treatment ($n = 5$). (G) Expression levels of inflammatory cytokines in the peritoneal macrophages in nondiabetic ApoEKO mice that received or did not receive Tofo treatment ($n = 4$–5). Data are represented as means ± SEM
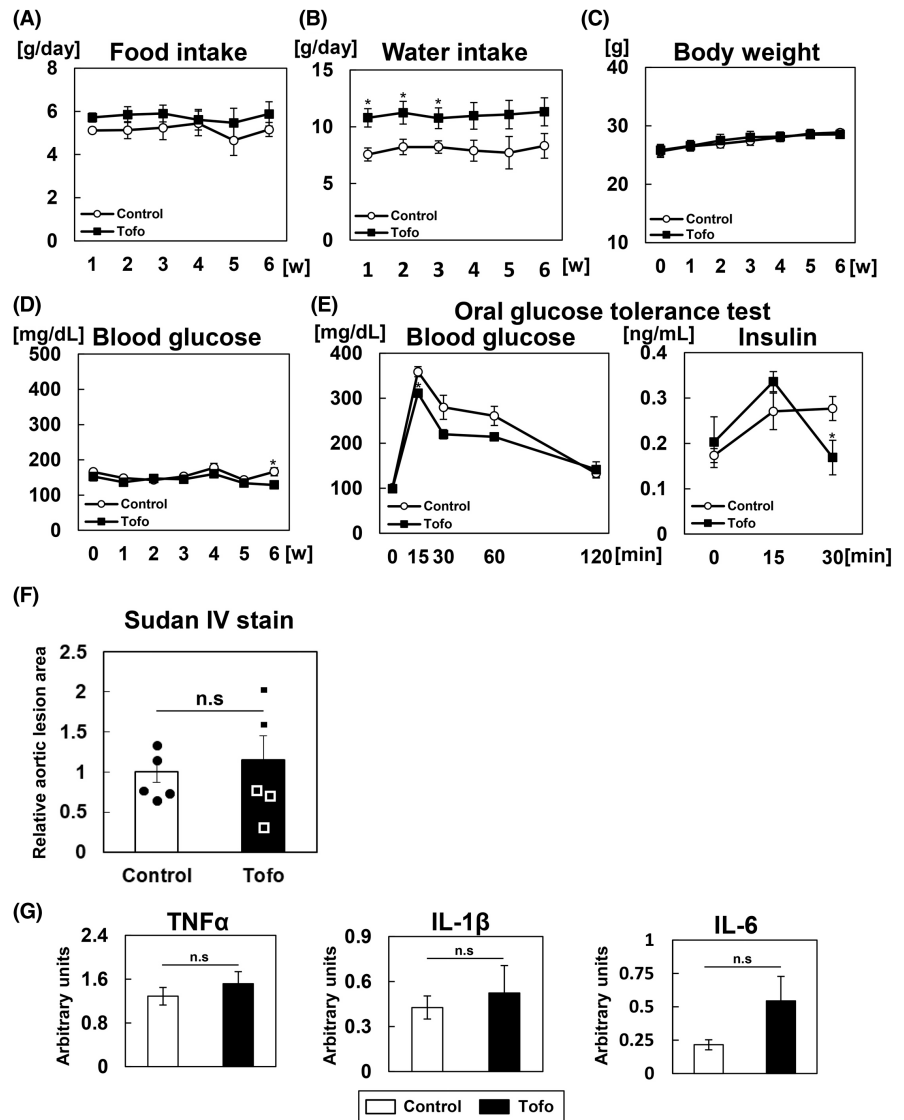
significant reductions of the IL-1β concentrations were observed in the peritoneal macrophage-conditioned medium collected after LPS stimulation from the insulin- and tofogliflozin-treated diabetic ApoEKO mice ($p < .05$, Figure 5C). These data suggest that tofogliflozin treatment also, like insulin, inhibited atherosclerosis by reducing inflammation.

4 | DISCUSSION

In this study, we demonstrated that tofogliflozin treatment had a marked anti-atherosclerotic effects, in addition to eliciting a marked decrease of the blood glucose levels in diabetic ApoEKO mice. Although tofogliflozin treatment also elicited a slight decrease of the blood glucose levels in the nondiabetic ApoEKO mice, it failed to show an atherosclerosis-suppressant effect in these mice. No significant difference in the anti-atherosclerotic effects of insulin and tofogliflozin was observed between diabetic ApoEKO mice with equivalent degrees of glycemic control achieved with the two

treatments. Moreover, tofogliflozin treatment was found to inhibit macrophage accumulation in the atherosclerotic lesions in the diabetic ApoEKO mice. Expression levels of inflammatory cytokines, such as TNFα and IL-1β, in the macrophages were also significantly suppressed in the tofogliflozin-treated ApoEKO mice with STZ-induced diabetes. These data suggest that tofogliflozin treatment reduced the macrophage expressions of inflammatory cytokines via controlling hyperglycemia, thereby exerting anti-atherosclerotic effects in the diabetic ApoEKO mice.

Consistent with our data, Terasaki et al. also revealed that dapagliflozin and ipragliflozin reduced macrophage foam cell formation along with amelioration of hyperglycemia, thereby exerting a suppressive effect against atherosclerosis.[17] They observed a positive correlation between the atherosclerotic lesion areas and HbA1c levels in the diabetic mice.[17] Dapagliflozin suppressed IL-1β secretion via the macrophage ROS-NLRP3-caspase-1 pathway, which is known to be activated by hyperglycemia in the macrophages, thereby exerting a suppressive effect against atherosclerosis.[16] Empagliflozin was demonstrated to exert
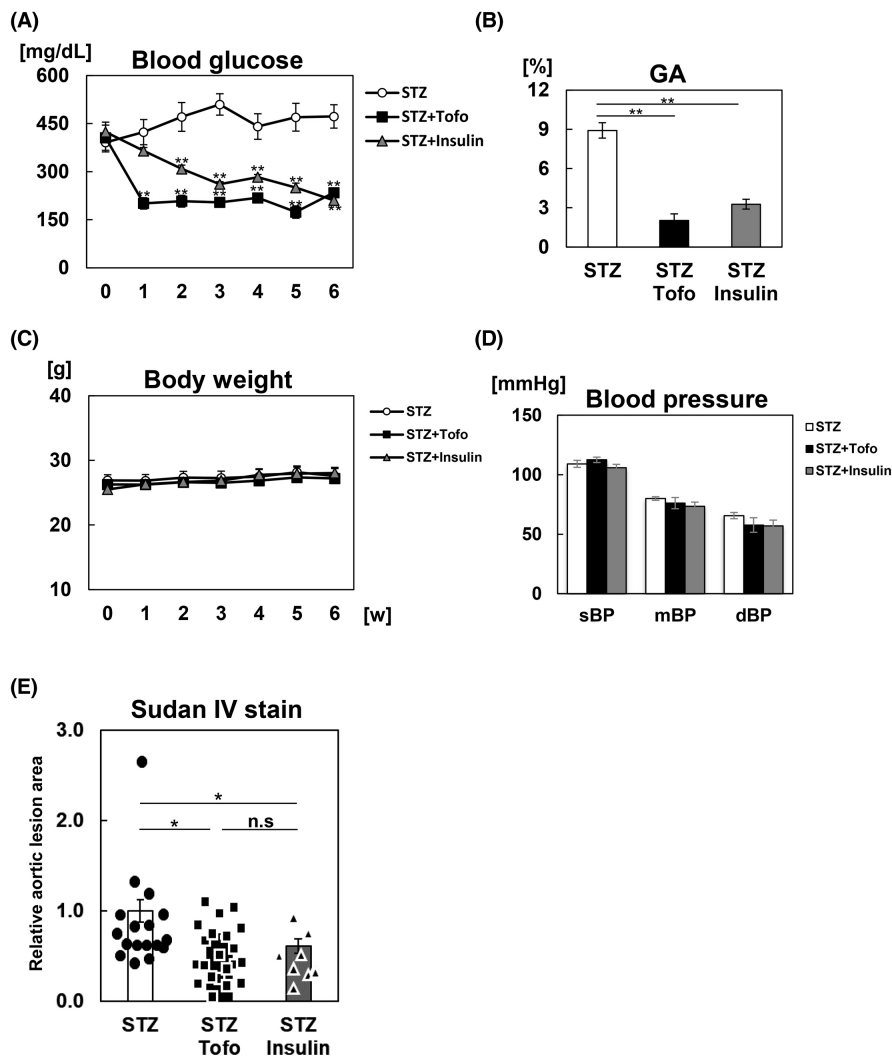
FIGURE 4 No significant difference in the anti-atherosclerotic effects of insulin and tofogliflozin was observed between STZ-induced diabetic ApoEKO mice with equivalent degrees of glycemic control achieved with the two treatments. (A) Blood glucose levels, (B) glycoalbumin (GA), (C) body weight, and (D) blood pressure in the diabetic ApoEKO mice that received no treatment, received 0.005% Tofo, or received insulin (Insulin Degludec 0.2 unit/day) (n = 4–8). **p < .01 compared with the STZ-induced diabetic ApoEKO mice. (E) Sudan IV-positive area in the diabetic ApoEKO mice that received no treatment, received Tofo, or received insulin (n = 9–21). *p < .05 compared with the STZ-induced diabetic ApoEKO mice. Data are means ± SEM

a protective effect on endothelial function, along with reducing the blood glucose levels.[19] Some clinical studies have reported a reduction in the risk of nonfatal MI, stroke, and death from cardiovascular disease, along with the reduction of the blood glucose levels in patients with type 2 diabetes.[29,30] These data suggest that SGLT2 inhibitors may suppress atherosclerosis by ameliorating inflammation and improving endothelial function, along with reducing the blood glucose levels. Canagliflozin and dapagliflozin increased the plasma levels of β-hydroxybutyrate and ketones, leading to suppression of NLRP3 inflammasome-mediated inflammation.[31-33] SGLT-2 inhibitors have also been reported to exert anti-atherosclerotic actions through mechanisms other than glucose lowering. SGLT2 inhibitors, as compared to other antidiabetic medications, have been shown to dramatically reduce major adverse cardiovascular events (MACEs) and hospitalization for heart failure in cardiovascular outcome trials (CVOTs).[10-12,34] The potential mechanisms by which SGLT-2 inhibitors reduce the risk of cardiovascular events, include factors, such as inhibiting the inflammation and sympathetic nervous activity system, increasing autophagy, lysosomal degradation, and decreasing oxidative stress.[34,35] Further studies are required to elucidate the precise contributions of these factors.

Treatment with tofogliflozin inhibited macrophage accumulation and the inflammatory cytokine expression levels in the peritoneal macrophages. Hyperglycemia induces monocyte chemotactic protein 1 (MCP1) and vascular cell adhesion molecule 1(VCAM1) in the endothelial cells, resulting in the accumulation of monocytes.[36-38] Transmigrated monocytes differentiate into macrophages and further exacerbate inflammation by inducing ROS generation in the tissue.[39] Hyperglycemia causes mitochondrial dysfunction in the macrophages and aberrant activation of cytoplasmic NADPH oxidases (NOX), both of which enhance ROS production.[40] The increased ROS subsequently activate NLRP3 inflammasomes to induce IL-1β synthesis.[41-43] Hyperglycemia has been reported to stimulate acute production of TNFα and long-term production of IL-1β during macrophage differentiation in vitro.[44] Moreover, it has been reported that mouse peritoneal macrophages cultured in hyperglycemic media show increased expression levels of pro-inflammatory cytokines, including IL-1β and TNFα, in a time- and dose-dependent manner.[45] Tofogliflozin inhibited the expression levels of IL-1β and TNFα in the diabetic ApoEKO mice. Although we did not investigate the VCAM1, ROS, and NLRP3 expression levels, tofogliflozin may suppress
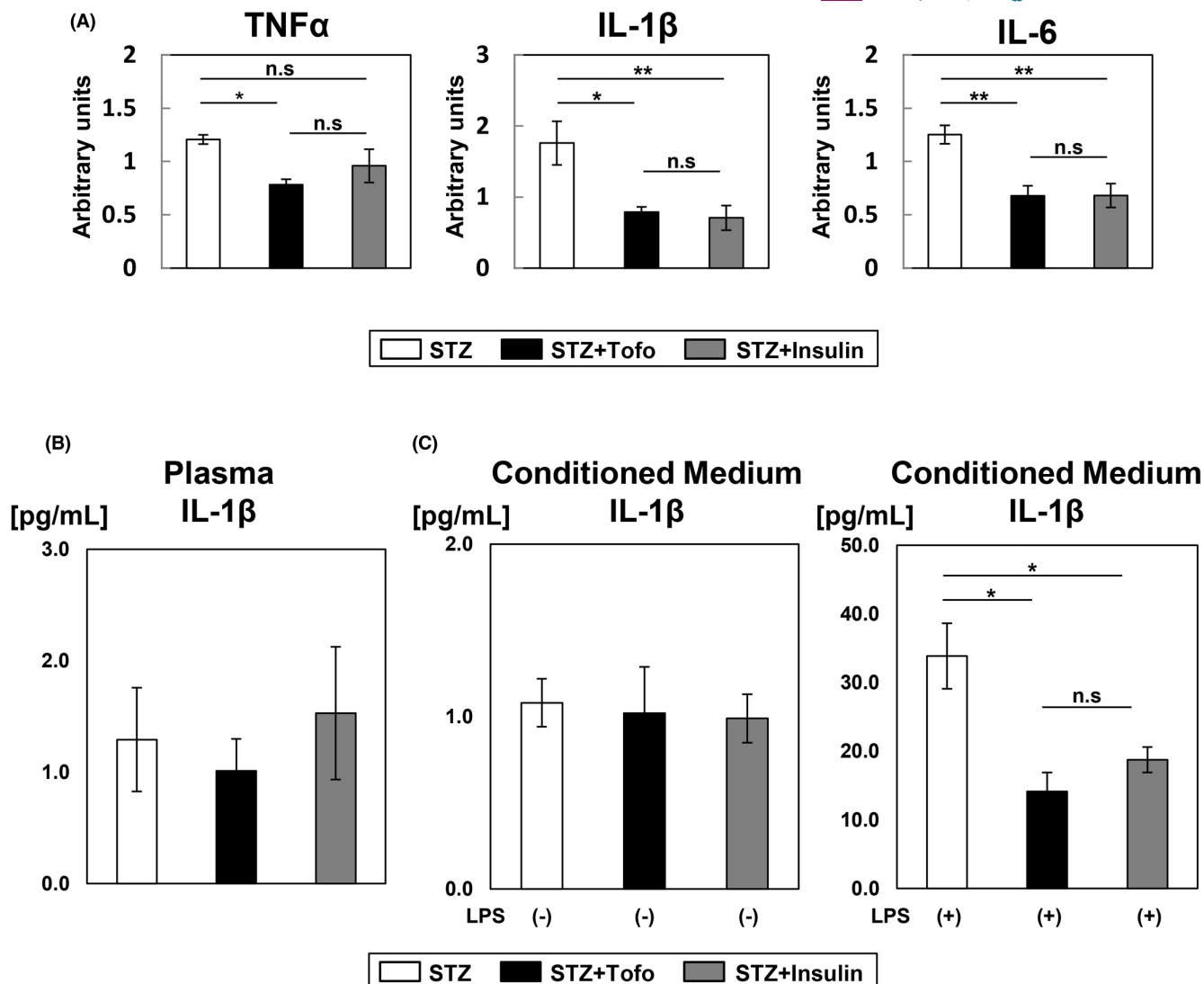
FIGURE 5 Tofogliflozin or insulin treatment inhibited the macrophage expression levels of inflammatory cytokines in the atherosclerotic lesions in the ApoEKO mice with STZ-induced diabetes. (A) Expression levels of TNFα, IL-1β, and IL-6 in the peritoneal macrophages of the in diabetic ApoEKO mice that received/did not receive Tofo or insulin treatment ($n = 5$–6). *$p < .05$ as compared with the diabetic ApoEKO mice. **$p < .01$ as compared with the diabetic ApoEKO mice. (B) Plasma IL-1β levels in the untreated, insulin-treated, and tofogliflozin-treated diabetic ApoEKO mice. (C) Concentrations of IL-1β in the peritoneal macrophage-conditioned medium before and after LPS stimulation in the untreated, insulin-treated, and tofogliflozin-treated diabetic ApoEKO mice. *$p < .05$ as compared with the STZ-induced diabetic ApoEKO mice. Data are represented as means ± SEM

macrophage accumulation and the expression levels of inflammatory cytokines via these mechanisms.

In this study, we found significantly reduced IL-1β concentrations in the peritoneal macrophage-conditioned medium collected after LPS stimulation from the tofogliflozin-treated diabetic ApoEKO mice ($p < .05$, Figure 5C); however, the mechanism by which tofogliflozin suppressed the IL-1β concentrations in the peritoneal macrophage-conditioned medium after LPS stimulation remains unclear. In previous studies, while high glucose caused activation of the NLRP3 inflammasome leading to the generation of IL-1β in the primary macrophages of wild-type mice, this effect was abrogated in NLRP3KO mice.[46]. Dapagliflozin did not inhibit the activation of the NLRP3 inflammasome or generation of IL-1β in macrophages,[46] suggesting that this SGLT2 inhibitor did not exert any direct actions on the macrophages. Empagliflozin caused a greater degree of reduction of macrophage IL-1β secretion, accompanied by increased serum β-hydroxybutyrate (BHB), in subjects with type 2 diabetes. BHB inhibited LPS-stimulated IL-1β secretion in a dose-dependent manner from human macrophages in vitro.[47] Canagliflozin increased the phosphorylation of AMPK (Thr172) and ACC (Ser79) in the macrophages of wild-type mice, but not of AMPKβ1KO mice. IL-1β secretion was reduced by canagliflozin in an AMPKβ1-dependent manner in vitro.[48] These discrepancies may be explained by differences in the effects of various SGLT2 inhibitors or in the experimental conditions.

In conclusion, tofogliflozin suppresses atherosclerosis by improving glucose intolerance associated with inhibition of inflammation in diabetic ApoEKO mice.

DISCLOSURE

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

M.I., N.K., and T.K. participated in research design. M.I., Y.S., N.W., and S.S. conducted experiments. M.I., Y.S., and N.K. performed data analysis. M.I., T.K., T.Y., T.K., and N.K. wrote or contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

ORCID

Tetsuya Kubota https://orcid.org/0000-0003-0341-3137

REFERENCES

1. Patti AM, Rizvi AA, Giglio RV, Stoian AP, Ligi D, Mannello F. Impact of glucose-lowering medications on cardiovascular and metabolic risk in type 2 diabetes. *J Clin Med*. 2020;9:912.
2. Sugden M, Holness M. Pathophysiology of diabetic dyslipidemia: implications for atherogenesis and treatment. *Clin Lipidol*. 2017;6:401–411.
3. García-Carro C, Vergara A, Agraz I, et al. The new era for reno-cardiovascular treatment in type 2 diabetes. *J Clin Med*. 2019;8:864.
4. UK Prospective Diabetes Study (UKPDS) Group. Effect of intensive blood-glucose control with metformin on complications in overweight patients with type 2 diabetes (UKPDS 34). *Lancet*. 1998;352:854–865.
5. Chia CW, Egan JM, Ferrucci L. Age-related changes in glucose metabolism, hyperglycemia, and cardiovascular risk. *Circ Res*. 2018;123:886–904.
6. Nahmias A, Stahel P, Xiao C, Lewis GF. Glycemia and atherosclerotic cardiovascular disease: exploring the gap between risk marker and risk factor. *Front Cardiovasc Med*. 2020;7:100.
7. Sattar N. Revisiting the links between glycaemia, diabetes and cardiovascular disease. *Diabetologia*. 2013;56:686–695.
8. Lopaschuk GD, Verma S. Mechanisms of cardiovascular benefits of sodium glucose co-transporter 2 (SGLT2) inhibitors: a state-of-the-art review. *JACC Basic Transl Sci*. 2020;5:632–644.
9. Gallo LA, Wright EM, Vallon V. Probing SGLT2 as a therapeutic target for diabetes: basic physiology and consequences. *Diab Vasc Dis Res*. 2015;12:78–89.
10. Dardano A, Miccoli R, Bianchi C, Daniele G, Del Prato S. Invited review. Series: implications of the recent CVOTs in type 2 diabetes: which patients for GLP-1RA or SGLT-2 inhibitor? *Diabetes Res Clin Pract*. 2020;162: 108112.
11. Zinman B, Wanner C, Lachin JM, et al. EMPA-REG OUTCOME investigators. empagliflozin, cardiovascular outcomes, and mortality in type 2 diabetes. *N Engl J Med*. 2015;373:2117–2128.
12. Neal B, Perkovic V, Mahaffey KW, et al. CANVAS program collaborative group. canagliflozin and cardiovascular and renal events in type 2 diabetes. *N Engl J Med*. 2017;377:644–657.
13. Wiviott SD, Raz I, Bonaca MP, et al. DECLARE-TIMI 58 investigators. Dapagliflozin and cardiovascular outcomes in type 2 diabetes. *N Engl J Med*. 2019;380:347–357.
14. Salim HM, Fukuda D, Yagi S, Soeki T, Shimabukuro M, Sata M. Glycemic control with ipragliflozin, a novel selective SGLT2 inhibitor, ameliorated endothelial dysfunction in streptozotocin-induced diabetic mouse. *Front Cardiovasc Med*. 2016;3:43.
15. Solini A, Giannini L, Seghieri M, et al. Dapagliflozin acutely improves endothelial dysfunction, reduces aortic stiffness and renal resistive index in type 2 diabetic patients: a pilot study. *Cardiovasc Diabetol*. 2017;16:138.
16. Steven S, Oelze M, Hanf A, et al. The SGLT2 inhibitor empagliflozin improves the primary diabetic complications in ZDF rats. *Redox Biol*. 2017;13:370–385.
17. Leng W, Ouyang X, Lei X, et al. The SGLT-2 inhibitor dapagliflozin has a therapeutic effect on atherosclerosis in diabetic ApoE(-/-) mice. *Mediators Inflamm*. 2016;2016:6305735.
18. Terasaki M, Hiromura M, Mori Y, et al. Amelioration of hyperglycemia with a sodium-glucose Cotransporter 2 inhibitor prevents macrophage-driven atherosclerosis through macrophage foam cell formation suppression in type 1 and type 2 diabetic mice. *PLoS One*. 2015;10:e0143396.
19. Oelze M, Kröller-Schön S, Welschof P, et al. The sodium-glucose cotransporter 2 inhibitor empagliflozin improves diabetes-induced vascular dysfunction in the streptozotocin diabetes rat model by interfering with oxidative stress and glucotoxicity. *PLoS One*. 2014;9:e112394.
20. Ganbaatar B, Fukuda D, Shinohara M, et al. Empagliflozin ameliorates endothelial dysfunction and suppresses atherogenesis in diabetic apolipoprotein E-deficient mice. *Eur J Pharmacol*. 2020;875: 173040.
21. Suzuki M, Honda K, Fukazawa M, et al. Tofogliflozin, a potent and highly specific sodium/glucose cotransporter 2 inhibitor, improves glycemic control in diabetic rats and mice. *J Pharmacol Exp Ther*. 2012;341:692–701.
22. Ikeda S, Takano Y, Cynshi O, et al. A novel and selective sodium-glucose cotransporter-2 inhibitor, tofogliflozin, improves glycaemic control and lowers body weight in patients with type 2 diabetes mellitus. *Diabetes Obes Metab*. 2015;17:984–993.
23. Katakami N, Mita T, Yoshii H, et al. UTOPIA study investigators. Tofoglifozin does not delay progression of carotid atherosclerosis in patients with type 2 diabetes: a prospective, randomized, open-label, parallel-group comparative study. *Cardiovasc Diabetol*. 2020;19:110.
24. Obata A, Kubota N, Kubota T, et al. Tofogliflozin improves insulin resistance in skeletal muscle and accelerates lipolysis in adipose tissue in male mice. *Endocrinology*. 2016;157:1029–1042.
25. Kubota T, Inoue M, Kubota N, et al. Downregulation of macrophage Irs2 by hyperinsulinemia impairs IL-4-induced M2asubtype macrophage activation in obesity. *Nat Commun*. 2018;9:4863.
26. Kubota N, Kubota T, Kajiwara E, et al. Differential hepatic distribution of insulin receptor substrates causes selective insulin resistance in diabetes and obesity. *Nat Commun*. 2016;7:12977.
27. Matsumoto M, Sata M, Fukuda D, et al. Orally administered eicosapentaenoic acid reduces and stabilizes atherosclerotic lesions in ApoE-deficient mice. *Atherosclerosis*. 2008;197:524–533.
28. Meir KS, Leitersdorf E. Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. *Arterioscler Thromb Vasc Biol*. 2004;24:1006–1014.

29. Holman RR, Paul SK, Bethel MA, Matthews DR, Neil HA. 10-year follow-up of intensive glucose control in type 2 diabetes. *N Engl J Med.* 2008;359:1577–1589.

30. Brown A, Reynolds LR, Bruemmer D. Intensive glycemic control and cardiovascular disease: an update. *Nat Rev Cardiol.* 2010;7:369–375.

31. Polidori D, Iijima H, Goda M, Maruyama N, Inagaki N, Crawford PA. Intra- and inter-subject variability for increases in serum ketone bodies in patients with type 2 diabetes treated with the sodium glucose co-transporter 2 inhibitor canagliflozin. *Diabetes Obes Metab.* 2018;20:1321–1326.

32. Nishitani S, Fukuhara A, Shin J, et al. Metabolomic and microarray analyses of adipose tissue of dapagliflozin-treated mice, and effects of 3-hydroxybutyrate on induction of adiponectin in adipocytes. *Sci Rep.* 2018;8:8805.

33. Youm Y-H, Nguyen KY, Grant RW, et al. The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome-mediated inflammatory disease. *Nat Med.* 2015;21:263–269.

34. Cowie MR, Fisher M. SGLT2 inhibitors: mechanisms of cardiovascular benefit beyond glycaemic control. *Nat Rev Cardiol.* 2020;17:761–772.

35. Perkovic V, Jardine MJ, Neal B, et al. Canagliflozin and renal outcomes in Type 2 diabetes and nephropathy. *N Engl J Med.* 2019;380:2295–2306.

36. De Rosa S, Arcidiacono B, Chiefari E, Foti D, Indolfi C, Brunetti A. Type 2 diabetes mellitus and cardiovascular disease: genetic and epigenetic links. *Front. Endocrinol.* 2018;9:2.

37. Poznyak A, Grechko AV, Poggio P, Myasoedova VA, Alfieri V, Orekhov AN. The diabetes mellitus-atherosclerosis connection: the role of lipid and glucose metabolism and chronic inflammation. *Int J Mol Sci.* 2020;21:1835.

38. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: Molecular mechanisms and clinical implications. *Rev Endocr Metab Disord.* 2010;11:61–74.

39. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res.* 2014;2:1.

40. Rendra E, Riabov V, Mossel DM, Sevastyanova T, Harmsen MC, Kzhyshkowska J. Reactive oxygen species (ROS) in macrophage activation and function in diabetes. *Immunobiology.* 2019;224:242–253.

41. Abais JM, Xia M, Zhang Y, Boini KM, Li PL. Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector? *Antioxid Redox Signal.* 2015;22:1111–1129.

42. He Y, Hara H, Nunez G. Mechanism and regulation of NLRP3 inflammasome activation. *Trends Biochem Sci.* 2016;41:1012–1021.

43. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. *Cell Mol Immunol.* 2016;13:148–159.

44. Moganti K, Li F, Schmuttermaier C, et al. Hyperglycemia induces mixed M1/M2 cytokine profile in primary human monocyte derived macrophages. *Immunobiology.* 2017;222:952–959.

45. Wen Y, Gu J, Li SL, Reddy MA, Natarajan R, Nadler JL. Elevated glucose and diabetes promote interleukin-12 cytokine gene expression in mouse macrophages. *Endocrinology.* 2006;147:2518–2525.

46. Kim SR, Lee S-G, Kim SH, et al. SGLT2 inhibition modulates NLRP3 inflammasome activity via ketones and insulin in diabetes with cardiovascular disease. *Nat Commun.* 2020;11:2127.

47. Leng W, Ouyang X, Lei X, et al. The SGLT-2 inhibitor dapagliflozin has a therapeutic effect on atherosclerosis in diabetic ApoE-/-mice. *Mediators Inflamm.* 2016;2016:6305735.

48. Day EA, Ford RJ, Lu JH, et al. The SGLT2 inhibitor canagliflozin suppresses lipid synthesis and interleukin-1 beta in ApoE deficient mice. *Biochem J.* 2020;477:2347–2361.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.