

SURAT KETERANGAN

Nomor: 2668/UNUSA-LPPM/Adm.I/XII/2022

Lembaga Penelitian dan Pengabdian Kepada Masyarakat (LPPM) Universitas Nahdlatul Ulama Surabaya menerangkan telah selesai melakukan pemeriksaan duplikasi dengan membandingkan artikel-artikel lain menggunakan perangkat lunak **Turnitin** pada tanggal 27 November 2022.

Judul : Canagliflozin Prevents Diabetes-Induced Vascular Dysfunction in ApoE-Deficient Mice

Penulis : Arief Rahadian, Daiju Fukuda, Hotimah Masdan Salim, Shusuke Yagi, Kenya Kusunose, Hirotsugu Yamada, Takeshi Soeki, and Masataka Sata

No. Pemeriksaan : 2022.12.09.1085

Dengan Hasil sebagai Berikut:

Tingkat Kesamaan diseluruh artikel (*Similarity Index*) yaitu 14%

Demikian surat keterangan ini dibuat untuk digunakan sebagaimana mestinya.

Surabaya, 09 Desember 2022

Ketua LPPM



UNUSA
LPPM

Achmad Syafiuddin, Ph.D

NPP: 20071300

LPPM Universitas Nahdlatul Ulama Surabaya

Website : lppm.unusa.ac.id

Email : lppm@unusa.ac.id

Hotline : 0838.5706.3867

Canagliflozin Prevents Diabetes-Induced Vascular Dysfunction in ApoE-Deficient Mice

by HotimahHotimah

Submission date: 27-Nov-2022 01:37PM (UTC+0700)

Submission ID: 1963932676

File name: Art._Canagliflozin.pdf (888.1K)

Word count: 5939

Character count: 35260

Canagliflozin Prevents Diabetes-Induced Vascular Dysfunction in ApoE-Deficient Mice

Arief Rahadian¹, Daiju Fukuda², Hotimah Masdan Salim¹, Shusuke Yagi¹, Kenya Kusunose¹,
Hirotugu Yamada¹, Takeshi Soeki¹ and Masataka Sata¹

¹Department of Cardiovascular Medicine, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan

²Department of Cardio-Diabetes Medicine, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan

Aim: Recent studies have demonstrated that selective sodium–glucose cotransporter 2 inhibitors (SGLT2i) reduce cardiovascular events, although their mechanism remains obscure. We examined the effect of canagliflozin, an SGLT2i, on atherogenesis and investigated its underlying mechanism.

Method: Canagliflozin (30 mg/kg/day) was administered by gavage to streptozotocin-induced diabetic apolipoprotein E-deficient (ApoE^{-/-}) mice. Sudan IV staining was performed at the aortic arch. Immunostaining, quantitative RT-PCR, and vascular reactivity assay were performed using the aorta. *In vitro* experiments using human umbilical vein endothelial cells (HUVECs) were also performed.

Result: Canagliflozin decreased blood glucose ($P < 0.001$) and total cholesterol ($P < 0.05$) levels. Sudan IV staining showed that 12-week canagliflozin treatment decreased atherosclerotic lesions ($P < 0.05$). Further, 8-week canagliflozin treatment ameliorated endothelial dysfunction, as determined by acetylcholine-induced vasodilation ($P < 0.05$), and significantly reduced the expressions of inflammatory molecules such as ICAM-1 and VCAM-1 in the aorta at the RNA and protein levels. Canagliflozin also reduced the expressions of NADPH oxidase subunits such as NOX2 and p22phox in the aorta and reduced urinary excretion of 8-OHdG, suggesting a reduction in oxidative stress. Methylglyoxal, a precursor of advanced glycation end products, increased the expressions of ICAM-1 and p22phox in HUVECs ($P < 0.05$, both). Methylglyoxal also decreased the phosphorylation of eNOS^{Ser1177} and Akt but increased the phosphorylation of eNOS^{Thr495} and p38 MAPK in HUVECs.

Conclusion: Canagliflozin prevents endothelial dysfunction and atherogenesis in diabetic ApoE^{-/-} mice. Anti-inflammatory and antioxidative potential due to reduced glucose toxicity to endothelial cells might be its underlying mechanisms.

See editorial vol. 27: 1139-1140

Key words: SGLT2 inhibitor, Canagliflozin, Atherosclerosis, Endothelial dysfunction

Abbreviation: 8-OHdG: 8-hydroxy-2-deoxyguanosine, Ach: acetylcholine, AGE: advanced glycation end products, ApoE^{-/-}: apolipoprotein e-deficient, CMC: carboxymethyl cellulose, HDL: high-density lipoprotein, HUVEC: human umbilical vein endothelial cell, ICAM-1: intercellular adhesion molecule-1, IL: interleukin, Mac-3: macrophage antigen-3, MGO: methylglyoxal, RAGE: receptor for AGEs, SGLT2i: sodium-dependent glucose transport 2 inhibitor, SNP: sodium nitroprusside, STZ: streptozotocin, T2DM: type 2 diabetes mellitus, TC: total cholesterol, TG: triglyceride, TNF- α : tumor Necrosis Factor- α , VCAM-1: vascular cell adhesion molecule-1, WTD: Western-type diet

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) in the world is increasing¹⁻³. Even after

using medical treatment and lifestyle modifications, it is still hard to achieve the goal of reducing blood glucose to an optimal level^{4,5}. In T2DM, atherosclerosis plays an important role in cardiovascular disease com-

Address for correspondence: Daiju Fukuda, Department of Cardio-Diabetes Medicine, Tokushima University Graduate School of Biomedical Sciences, 3-18-15, Kuramoto-cho, Tokushima 770-8503, Japan E-mail: daiju.fukuda@tokushima-u.ac.jp

Received: July 20, 2019 Accepted for publication: December 23, 2019

Copyright©2020 Japan Atherosclerosis Society

This article is distributed under the terms of the latest version of CC BY-NC-SA defined by the Creative Commons Attribution License.

plications, and vascular inflammation due to hyperglycemia is one of the mechanisms of atherosclerosis⁶⁻¹⁰.

Recent randomized placebo-control studies found that sodium-dependent glucose transport 2 inhibitors (SGLT2is) (e.g., canagliflozin) reduced cardiovascular events in addition to their effect as antidiabetic drugs¹¹⁻¹⁴. Previous studies demonstrated that SGLT2is reduce blood glucose levels, blood pressure, and body weight in T2DM patients, all of which play roles in atherogenesis^{11, 15-17}. However, the mechanisms by which SGLT2is prevent cardiovascular events are not completely understood and need to be explored to achieve better treatment in T2DM patients with risk of cardiovascular events.

Previous preclinical studies have demonstrated that SGLT2is reduced vascular inflammation and atherogenesis in apolipoprotein e-deficient (ApoE^{-/-}) mice¹⁸⁻²². Oxidative stress that is induced in a hyperglycemic state causes vascular inflammation, thus leading to the development of endothelial dysfunction, which is an initial step in atherogenesis²³⁻²⁵. However, few studies have investigated the effects of canagliflozin on atherogenesis, particularly on endothelial cells^{26, 27}. Therefore, in the current study, we examined whether canagliflozin ameliorates endothelial dysfunction and prevents atherogenesis in diabetic ApoE^{-/-} mice.

Methods

Animals and Treatment

ApoE^{-/-} (C57BL/6J background) mice were originally purchased from The Jackson Laboratory. Male, 8-week-old ApoE^{-/-} mice were treated with streptozotocin (STZ) (75 mg/kg/day) on three consecutive days to induce diabetes. A Western-type diet (Harlan Teklad Western diet TD.88137) was started from 9 weeks old and continued throughout the study period. From 9 weeks old, canagliflozin (30 mg/kg/day), which was supplied by Mitsubishi Tanabe Pharma, was orally administered for 12 weeks or 8 weeks to investigate its effect on atherosclerosis or endothelial function, respectively. Canagliflozin was suspended in 0.5% carboxymethyl cellulose (CMC) solution. The control group received only CMC. Mice were maintained under a 12-hour light/dark cycle. All experimental procedures conformed to the guidelines for the animal experimentation of Tokushima University.

Measurement of Metabolic Parameters

At the time of sacrifice, blood was collected from the heart into EDTA-containing tubes, and plasma

was separated by centrifugation (9,000 rpm for 15 min) at 4°C. Plasma lipid levels (total cholesterol [TC], high-density lipoprotein [HDL] cholesterol, and triglycerides [TG]) were measured at LSI Medicine Corporation (Japan). Plasma levels of nonfasting insulin (FUJIFILM Wako Shibayagi Corporation) and advanced glycation end products (AGEs; Cell Biolabs, Inc.) were measured using commercially available kits. Urinary 8-hydroxy-2-deoxyguanosine (8-OHdG) concentration in urine collected for 16 hours was determined using a commercially available kit (Japan Institute for the Control of Aging, Nikken SEIL Co., Ltd.) and was corrected by creatinine level.

Analysis of Atherosclerotic Lesions

The thoracic aorta was opened longitudinally, and atherosclerotic lesions in the aortic arch were visualized using Sudan IV staining after fixation with 10% neutral buffered formalin. The luminal aspect of the stained aorta was photographed. The quantification of atherosclerotic lesions in the aortic arch was performed with Adobe Photoshop™ CS3 image analysis software. The thoracic aorta and abdominal aorta were collected and snap-frozen immediately for gene and/or protein analyses.

Histological and Immunohistochemical Analyses

Each heart was cut along a horizontal plane between the lower tips of the right and left atria. The upper portion was snap-frozen in OCT compound (TissueTeck). Sections (5 µm intervals) were stained with oil red O to detect lipid deposition. Other sections were incubated overnight with antivascular cell adhesion molecule-1 (VCAM-1) antibody, anti-intercellular adhesion molecule-1 (ICAM-1) antibody (Abcam), or antimacrophage antigen-3 (Mac-3) antibody (BD Biosciences). Thereafter, sections were incubated with biotinylated secondary antibody (VECTOR Laboratories, Inc.), followed by VECTASTAIN ABC-AP Kit (VECTOR Laboratories, Inc.) and were stained using a VectorRed AP Substrate Kit (VECTOR Laboratories, Inc.). All sections were counterstained with hematoxylin. The ratio of positive area to plaque area was then calculated²⁸.

Vascular Reactivity Assay

The analysis of vascular reactivity was performed as described previously²⁹. The descending thoracic aorta was cut into 2 mm rings with special care to preserve the endothelium, and the rings were mounted on a force transducer in an organ bath filled with modified Krebs-Henseleit buffer (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PH₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 11.1 mM glu-

Table 1. Primer sequences

Name	Foward	Reverse
Mouse		
ICAM-1	TTCACACTGAATGCCAGCTC	GTCTGCTGAGACCCCTCTTG
VCAM-1	CCCGTCATTGAGGATATTGG	GGTCATTGTCACAGCACCAC
MCP-1	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGAGCTCTCC
TNF- α	ACCCTCACACTCAGATCATCTTC	TGGTGGTTTGCTACGACGT
F4/80	TGCATCTAGCAATGGACAGC	GCCTTCTGGATCCATTTGAA
iNOS	CACCTTGGAGTTCACCCAGT	ACCCTCGTACTTTGGGATGC
IL6	ACAACCACGGCCTTCCCTACTT	CACGATTTCACAGAGAACATGTG
NOX2	ACTCCTTGGGTCAGCACTGG	GTTCTGTCCAGTTGTCTTCG
NOX4	TGTTGGGCTAGGATTGTGTT	AGGGACCTTCTGTGATCCTCG
p22phox	CATGTGGGCCAACGAACA	CACTGTGTGAAACGTCCAGCAGTA
P47phox	AGACGGCTCCTATCCCTATCTCTG	TGCTAGCAATACCGGTGGAGATTA
RAGE	ACTACCGAGTCCGAGTCTACC	GTAGCTTCCCTCAGACACACA
β -actin	CCTGAGCGCAAGTACTCTGTGT	GCTGATCCACATCTGCTGGAA
Human		
ICAM-1	TGATGGGCAGTCAACAGCTA	GGGTAAGGTTCTTGCCCACT
p22phox	CCAGTGGTACTTTGGTGCCT	CCAGTGGTACTTTGGTGCCT
RAGE	ACCAGTTATGTAAGTCCCTGGATCA	CGTGTACGGCTGCTTGGAAATAG
GAPDH	TGGGTGTGAACCATGAGAAG	GCTAAGCAGTTGGTGGTGC

ose) aerated with 95% O₂ and 5% CO₂ at 37°C. Vessel rings were primed with 31.4 mM KCl and precontracted with phenylephrine to produce submaximal (60% of maximum) contraction. After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) or sodium nitropruside (SNP) to obtain cumulative concentration–response curves. Isometric tension was recorded on a polygraph.

Cell Culture Experiments

Human umbilical vein endothelial cells (HUVECs) were purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVECs (passages 5–8) were treated with 500 μ M methylglyoxal (MGO; Sigma-Aldrich) in the presence or absence of 1 mM canagliflozin in EBM-2 (Lonza) containing 2% FBS for 30 minutes or 8 hours.

Quantitative RT-PCR

Total RNA was extracted from tissues and cells by using an illustra RNAspin RNA isolation kit (GE Healthcare). Reverse transcription was performed using a QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR was performed on an Mx3000P (Agilent Technologies) by using gene-specific primers (Table 1) and Power SYBR Green PCR Master Mix (Applied Biosystems). Data are expressed in arbitrary units normalized by β -actin or GAPDH.

Western Blot Analysis

Cell lysates were prepared using RIPA buffer (Wako Pure Chemical Industries, Ltd.) containing a protease inhibitor cocktail (Takara Bio Inc.) and phosphatase inhibitors (Roche). Proteins were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare). After blocking with 5% bovine serum albumin or 5% skimmed milk, the membranes were incubated overnight at 4°C with primary antibody against either phosphorylated-eNOS^{Ser1177}, eNOS (BD Biosciences), phosphorylated-eNOS^{Thr495}, phosphorylated-Akt^{Ser473}, Akt, phosphorylated-p38 MAPK, p38 MAPK (Cell Signaling Technology), or β -actin (Sigma). Horseradish peroxidase-conjugated antimouse Ig antibody or antirabbit Ig antibody (Cell Signaling Technology) was then used as the secondary antibody. Antibody distribution was visualized with ECL-plus reagent (GE Healthcare) by using a luminescent image analyzer (LAS-1000, Fuji Film).

Statistical Analysis

All numerical values are expressed as mean \pm SEM. Student's *t*-test was used to compare the two groups. Comparisons between multiple groups were performed with analysis of variance (ANOVA), followed by Scheffe's post hoc test for comparison between groups. The comparison of vascular response curves was performed by two-factor repeated measurement ANOVA. A value of $P < 0.05$ was considered

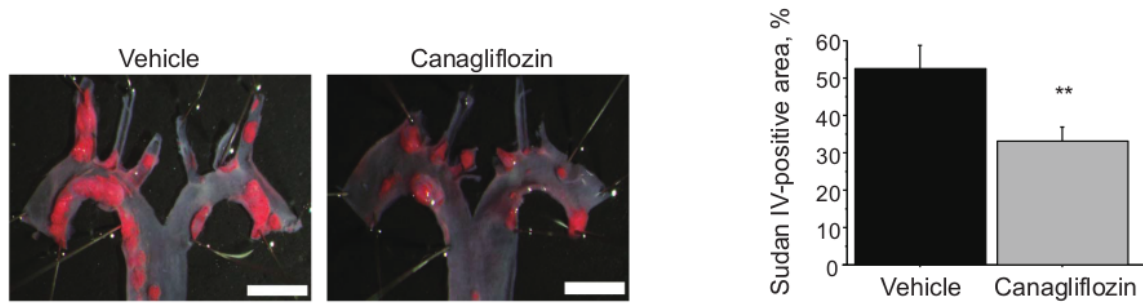


Fig. 1. Canagliflozin attenuated atherosclerosis progression in diabetic ApoE^{-/-} mice

En face Sudan IV staining showed that the administration of canagliflozin for 12 weeks significantly reduced atherosclerotic lesions in the aortic arch ($n=13$ –17 per group). Bar: 2 mm. **, $P<0.01$. All values are mean \pm SEM.

Table 2. Effects of canagliflozin after 12-week treatment

	Veh ($n=13$)	Cana ($n=17$)
Body weight, g	27.5 \pm 1.5	32.1 \pm 1.3
Blood glucose, mg/dl	612.6 \pm 43.9	218.9 \pm 7.7***
Total cholesterol, mg/dl	2215.5 \pm 338.3	1333.2 \pm 145.5*
Triglyceride, mg/dl	294.7 \pm 53.9	186.0 \pm 44.2
HDL cholesterol, mg/dl	45.1 \pm 12.3	21.65 \pm 3.8*
Insulin, pg/ml	668.3 \pm 162.0	1125.1 \pm 207.7
Heart rate, bpm	652.2 \pm 23.1	678.4 \pm 13.9
Systolic blood pressure, mmHg	101.5 \pm 5.7	92.9 \pm 3.3
Diastolic blood pressure, mmHg	65.4 \pm 3.3	59.6 \pm 1.9

Veh; vehicle, Cana; canagliflozin, HDL; high density lipoprotein. All values are mean \pm SEM. *, $P<0.05$ and ***, $P<0.001$ vs. Veh.

significant.

Results

Canagliflozin Decreased Atherosclerotic Lesions in Diabetic ApoE^{-/-} Mice

After 12 weeks of canagliflozin administration, we examined atherosclerosis lesions in the aortic arch in diabetic ApoE^{-/-} mice. Canagliflozin administration significantly reduced atherosclerotic lesions compared with the vehicle group ($P<0.01$) (Fig. 1). The administration of canagliflozin for 12 weeks significantly reduced blood glucose level. Canagliflozin significantly reduced TC and HDL cholesterol levels and tended to reduce TG level after 12 weeks of treatment. The canagliflozin-treated group and nontreated group had no differences in blood pressure levels (Table 2).

Canagliflozin Ameliorated Endothelial Dysfunction in Diabetic ApoE^{-/-} Mice

We examined the effects of canagliflozin on endothelial dysfunction, which is an initial step of atherogenesis. Canagliflozin administration for 8

weeks significantly ameliorated Ach-dependent vasodilation in diabetic ApoE^{-/-} mice, thus suggesting the improvement of endothelial function ($P<0.05$) (Fig. 2A). Our study showed that there were no significant differences in the endothelial-independent vasodilation, as determined by the vascular response to SNP (Fig. 2B). As observed with 12-week treatment, canagliflozin treatment for 8 weeks significantly reduced blood glucose levels. The plasma AGE level in the canagliflozin-treated group was lower than that of the vehicle-treated group (vehicle vs. canagliflozin; 9.8 ± 4.0 vs. 7.9 ± 2.1 mg/ml, $P=0.12$); however, it did not reach statistical significance. Canagliflozin also reduced TC level and HDL cholesterol level with this treatment protocol (Table 3). Throughout this study protocol, canagliflozin reduced food intake, water intake, and urine volume compared with the vehicle group (Table 4).

Canagliflozin Reduced Inflammatory Molecule Expression and Oxidative Stress in Diabetic ApoE^{-/-} Mice

Canagliflozin administration for 8 weeks signifi-

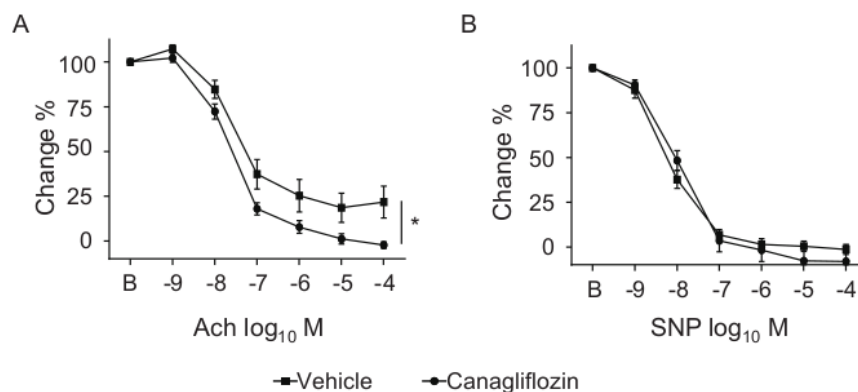


Fig. 2. Canagliflozin ameliorated endothelium-dependent vasodilation in diabetic ApoE^{-/-} mice

(A) After 8 weeks of treatment with canagliflozin, the vascular response to Ach was ameliorated in canagliflozin-treated diabetic ApoE^{-/-} mice compared with the nontreated group. (B) There was no difference in vascular response to SNP between the treated group and nontreated group ($n=10-16$, per group). *, $P<0.05$. All values are mean \pm SEM.

Table 3. Effects of canagliflozin after 8-week treatment

	Veh ($n=10$)	Cana ($n=16$)
Body weight, g	21.5 \pm 0.6	21.6 \pm 0.5
Blood glucose, mg/dl	578.4 \pm 51.9	214.8 \pm 26.2***
Total cholesterol, mg/dl	3138.6 \pm 356.3	2114.0 \pm 177.3**
Triglyceride, mg/dl	315.9 \pm 75.5	187.6 \pm 80.9
HDL cholesterol, mg/dl	125.0 \pm 26.0	52.9 \pm 14.5*
Insulin, pg/ml	260.3 \pm 97.9	427.6 \pm 183.8
Heart rate, bpm	682.8 \pm 18.3	696.4 \pm 8.9
Systolic blood pressure, mmHg	86.0 \pm 3.8	89.6 \pm 3.0
Diastolic blood pressure, mmHg	49.0 \pm 1.3	57.3 \pm 2.4*

Veh; vehicle, Cana; canagliflozin. HDL; high density lipoprotein.

All values are mean \pm SEM. *, $P<0.05$, **, $P<0.01$, and ***, $P<0.001$ vs. Veh.

Table 4. Food intake, water intake, and urine output after canagliflozin treatment

	Veh ($n=10$)		Cana ($n=16$)	
	4 th week	8 th week	4 th week	8 th week
Food intake, g	3.21 \pm 0.27	3.87 \pm 0.35	2.44 \pm 0.17*	2.7 \pm 0.13**
Water intake, ml	14.00 \pm 1.8	14.05 \pm 2.34	9.38 \pm 1.09*	9.84 \pm 1.70
Urine output, ml	11.72 \pm 1.83	14.46 \pm 2.18	7.03 \pm 1.29*	7.12 \pm 1.47**

Veh; vehicle, Cana; canagliflozin. All values are mean \pm SEM. *, $P<0.05$, **, $P<0.01$ vs. Veh.

cantly reduced the expression of inflammatory molecules such as ICAM-1, VCAM-1, MCP-1, F4/80, and IL-6 in the aorta. Canagliflozin also decreased the expression of tumor necrosis factor- α (TNF- α) and receptor for AGEs (RAGE) in the aorta; however, it did not reach statistical significance. Furthermore, canagliflozin also reduced the expression of molecules related to oxidative stress such as iNOS and NADPH oxidase subunits (e.g., NOX2, NOX4, p22phox, and

p47phox) (Fig. 3A). Canagliflozin significantly decreased the urinary excretion of 8-OHdG, which is an oxidative stress marker, compared with the vehicle group ($P<0.01$) (Fig. 3B). Canagliflozin also reduced lipid deposition in atherosclerotic plaques in the aortic root (Fig. 4A). The results of immunostaining demonstrated that canagliflozin decreased the expression of inflammatory molecules such as VCAM-1 ($P<0.05$) and ICAM-1 ($P<0.01$) and decreased the accumula-

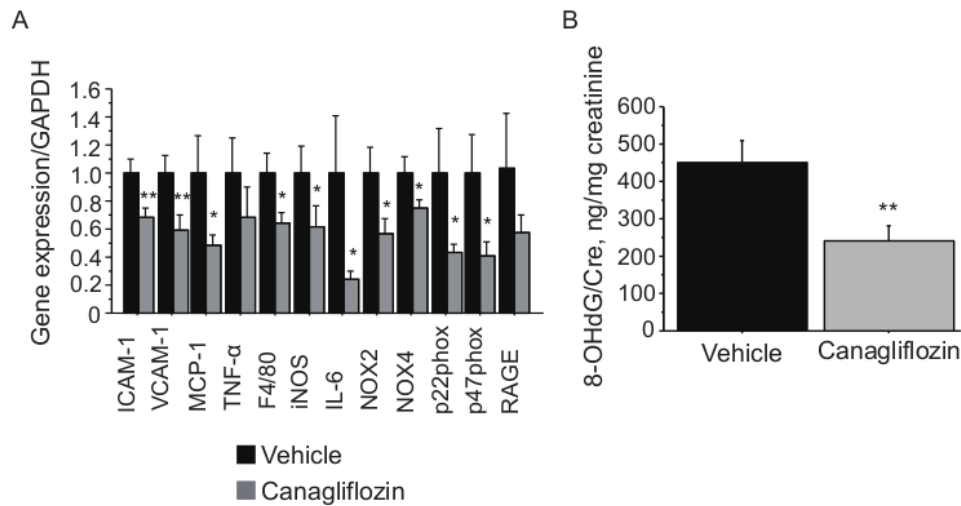


Fig. 3. Canagliflozin reduced the expression of inflammatory molecules and oxidative stress-related molecules in diabetic ApoE^{-/-} mice

(A) Canagliflozin treatment for 8 weeks reduced the expression of a macrophage marker, namely, F4/80, and inflammatory molecules (ICAM-1, VCAM-1, and MCP-1, and IL-6) in the aorta of diabetic ApoE^{-/-} mice. Canagliflozin also reduced the expression of molecules that contribute to the production of oxidative stress, such as iNOS, NOX2, NOX4, p22phox, and p47phox, in the aorta. Canagliflozin tended to reduce RAGE. (B) Canagliflozin treatment for 8 weeks reduced the urinary excretion of 8-OHdG in diabetic ApoE^{-/-} mice ($n = 10-14$, per group); $P < 0.05$ and $**$; $P < 0.01$. All values are mean \pm SEM.

tion of macrophages ($P < 0.01$) in atherosclerotic plaques in the aortic root (Figs. 4B-4D).

Hyperglycemic Condition Contributed to Endothelial Dysfunction

Our *in vivo* data suggested that canagliflozin suppressed the glucose toxicity represented by the AGE-RAGE system. Thus, we treated HUVECs with MGO, which is a major precursor of AGEs, to investigate the effects of canagliflozin on endothelial cells. MGO significantly increased the expression of inflammatory molecules such as ICAM-1, RAGE, and p22phox. The presence of canagliflozin in culture medium did not affect the expressions promoted by MGO (Fig. 5A). MGO decreased the phosphorylation of eNOS^{Ser1177} and Akt^{Ser473} ($P < 0.05$) (Fig. 5B). On the contrary, MGO increased the phosphorylation of eNOS^{Thr495} and p38 MAPK in HUVECs ($P < 0.05$) (Fig. 5C).

Discussion

We demonstrated that canagliflozin attenuated the development of endothelial dysfunction and atherogenesis in diabetic ApoE^{-/-} mice. Canagliflozin reduced the expression of inflammatory molecules and

oxidative stress, thus suggesting that this drug reduced vascular inflammation. The results of *in vitro* experiments using HUVECs suggested that the glucose-lowering effects of canagliflozin contribute to the inhibition of inflammatory activation of endothelial cells.

Canagliflozin is a selective SGLT2i that inhibits glucose reabsorption and increases glucose excretion into urine¹¹. Although there are numerous antidiabetic drugs, large-scale randomized trials have proved that only a few drugs decrease cardiovascular events in diabetic patients. By contrast, recent studies demonstrated that SGLT2i, including canagliflozin, reduced cardiovascular events in diabetic patients^{11, 12}. Several studies demonstrated that canagliflozin reduced albuminuria, plasma lipid levels, blood pressure, and body weight, in addition to its effect as an antidiabetic drug, thus suggesting that these metabolic actions of canagliflozin contribute to cardiovascular protection^{11, 15-17}. However, the mechanisms by which SGLT2is reduce cardiovascular events are not well established.

In this study, we demonstrated that the administration of canagliflozin to diabetic ApoE^{-/-} mice for 12 weeks reduced atherosclerotic lesions in the aorta. Several previous studies also reported that SGLT2is reduced atherosclerotic lesion development in diabetic and nondiabetic mice^{18, 19, 26}. Our results are consis-

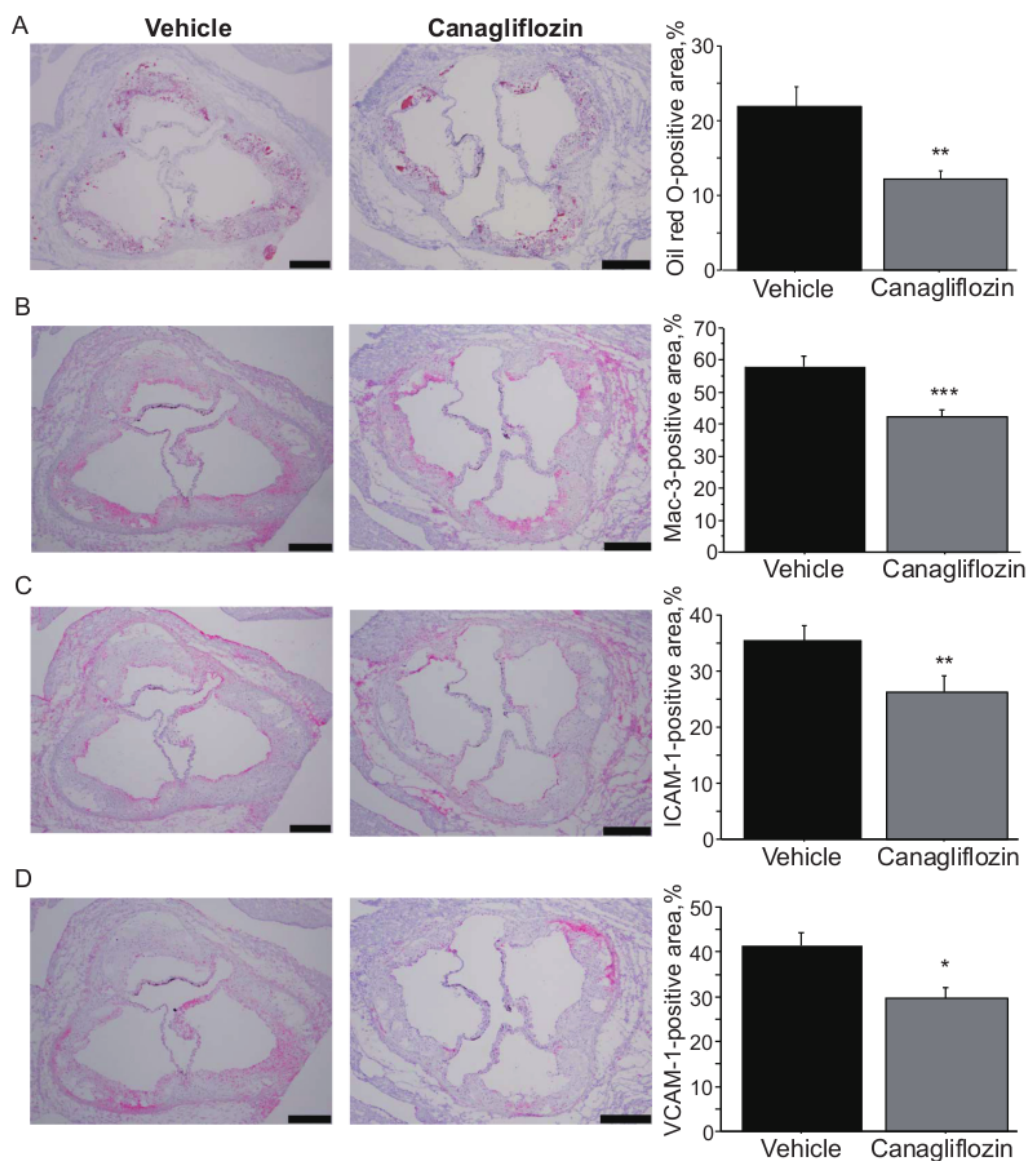


Fig. 4. Effects of canagliflozin on the characteristics of atherosclerotic plaques

(A) Oil red O staining showed that canagliflozin treatment for 8 weeks reduced lipid deposition in atherosclerotic plaques in the aortic root compared with the nontreatment group. (B–D) Immunostaining against Mac-3 (B), ICAM-1 (C), and VCAM-1 (D). Canagliflozin treatment for 8 weeks reduced the accumulation of macrophages and the expression of adhesion molecules compared with the nontreatment group ($n = 10$ per group). Bar: 200 μm ; *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$. All values are mean \pm SEM.

tent with these studies. We also showed that canagliflozin significantly reduced lipid deposition, macrophage accumulation, and expression of adhesion molecules such as ICAM-1 and VCAM-1. These results indicate that canagliflozin attenuated vascular inflammation, which is a major cause of atherosclerosis, in

diabetes.

One of the pathological processes of atherogenesis is endothelial dysfunction. Endothelial dysfunction is an initial step of atherosclerosis. Our study showed that canagliflozin treatment improved endothelial dysfunction in diabetic ApoE^{-/-} mice compared with the

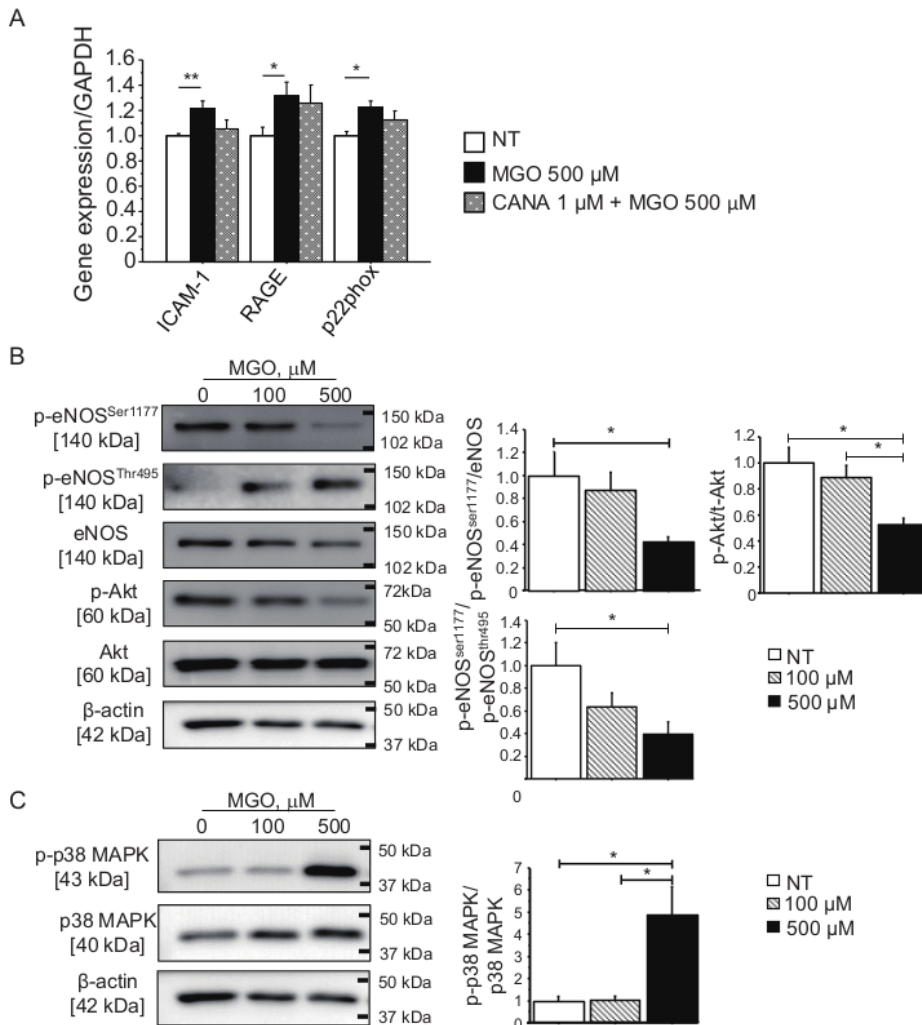


Fig. 5. MGO induced endothelial dysfunction

(A) The incubation of HUVECs with MGO, which is one of the major precursors of AGEs, for 8 hours increased the expression of inflammatory molecules such as ICAM-1, RAGE, and p22phox, which is an NADPH oxidase subunit ($n=5$, per group). The presence of canagliflozin did not affect these expressions. (B) The incubation of HUVECs with MGO (500 μ M) for 30 minutes reduced the phosphorylation of eNOS^{Ser1177} and increased the phosphorylation of the eNOS inhibitory site, namely, eNOS^{Thr495}. MGO also reduced the phosphorylation of Akt^{Ser473} ($n=8$, per group). (C) MGO increased the phosphorylation of p38 MAPK in HUVECs ($n=8$, per group). *, $P<0.05$ and **, $P<0.01$. All values are mean \pm SEM.

nontreatment group. The improvement of endothelial dysfunction by SGLT2is has been shown in other studies^{26, 27}. In addition to these studies, our results demonstrated that canagliflozin ameliorated endothelial dysfunction in a diabetic atherosclerotic mouse model. Vascular inflammation promotes endothelial dysfunction, thus leading to the development of atherosclerosis³⁰. In the current study, together with the amelioration of endothelial dysfunction, canagliflozin-

treated mice showed lower expressions of inflammatory molecules such as VCAM-1, ICAM-1, and MCP-1 in the aorta. In addition to these inflammatory molecules, we observed that canagliflozin reduced the expression of molecules related to the elevation of oxidative stress. Metabolic disorders influence the production of oxidative stress, and a hyperglycemic state is one of them³¹. Numerous studies have reported that oxidative stress promotes various processes of vas-

cular inflammation and decreases NO bioavailability, thus leading to endothelial dysfunction³². Therefore, several studies have indicated that oxidative stress is one of the therapeutic targets for vascular diseases³³. In the current study, canagliflozin reduced iNOS; NOX2; NOX4; p22phox, and p47phox expression in the aorta of diabetic ApoE^{-/-} mice. Previous studies have reported that these NADPH oxidase subunits contribute to the development of atherosclerosis³⁴. Furthermore, we found that canagliflozin decreased the excretion of 8-OHdG, which is a marker for oxidative stress, thus suggesting that canagliflozin reduced oxidative stress in diabetic ApoE^{-/-} mice.

We further examined the mechanism by which canagliflozin attenuated endothelial dysfunction and atherosclerosis. In this study, we focused on the role of the AGE–RAGE system. AGEs are generated in a hyperglycemic condition³⁵. There is a growing body of evidence indicating that AGEs elicit oxidative stress via its receptor, namely, RAGE, and evoke inflammation in vascular cells, thus leading to the development of microvascular and/or macrovascular diabetic complications^{36, 37}. In the present study, despite having no significance, canagliflozin decreased plasma AGE levels and RAGE expression. HUVECs treated with MGO, which is the main precursor of AGEs³⁸, showed increased expression of inflammatory molecules such as ICAM-1, RAGE, and p22phox. However, canagliflozin did not reduce their expression possibly because SGLT2 is exclusively expressed on proximal tubules and because endothelial cells rarely express SGLT2³⁹. MGO also decreased the phosphorylation of eNOS^{Ser1177} and Akt and increased the phosphorylation of an inhibitory site of eNOS, namely, eNOS^{Thr495}. These results suggest that a hyperglycemic condition affects endothelial function. Furthermore, MGO promoted the phosphorylation of p38 MAPK, thus suggesting activation of this pathway. The p38 MAPK pathway is associated with the promotion of inflammation⁴⁰ and regulation of oxidation⁵. The activation of MAPK by MGO might affect endothelial function in a diabetic condition⁴¹. Our results suggest that the glucose-lowering effect of canagliflozin promotes the Akt–eNOS pathway and inhibits p38 MAPK activation, thus conferring atheroprotective effects.

Our study demonstrated that a glucose-lowering effect that subsequently suppresses the AGE–RAGE system was the one of the mechanisms by which canagliflozin attenuated the development of endothelial dysfunction and atherosclerosis. From this point of view, all antidiabetic drugs may have the same effects. However, several studies proposed the specific effects of SGLT2i. A recent study demonstrated that treat-

ment with canagliflozin favorably affects the levels of adipokines and inflammatory molecules, which are associated with impaired adipose tissue function and cardiovascular disease, compared with glimepiride⁴². Furthermore, many studies demonstrated that SGLT2is decrease body weight, blood pressure, and TG. These results support the hypothesis that SGLT2is have specific cardioprotective effects^{16, 17}. Further studies are needed to understand the cardioprotective effects of SGLT2is.

This study has several limitations. First, we used STZ-induced diabetic mice. This model does not reflect T2DM completely. Second, canagliflozin significantly reduced TC and HDL cholesterol levels and tended to reduce TG level. Several previous studies have demonstrated similar effects of SGLT2is^{18–21}. These effects on lipid levels might have affected the development of atherogenesis and endothelial dysfunction in the treated group. Third, the glucose-lowering effect of canagliflozin contributed largely to the inhibition of atherogenesis in our mouse model. To investigate the SGLT2i-specific effects, we needed to incorporate other groups that received different classes of antidiabetic drug. Fourth, the dose of canagliflozin used in this study was higher than that for humans. In a previous study, the same dose of canagliflozin effectively increased urinary glucose excretion and decreased blood glucose levels without any harmful effects⁴³. The difference in metabolic speed of medical substances between mice and humans may explain higher dose for mice. Finally, several studies have reported that SGLT2is have a small diuretic effect and reduce body weight; however, in the current study, the treated group did not show these effects. Furthermore, canagliflozin reduced food intake, water intake, and urine volume in this study. These might have been caused by the improvement of a hyperglycemic condition by canagliflozin. Unexpectedly, canagliflozin increased diastolic blood pressure after 8 weeks of treatment. Although the results of animal studies cannot be directly applied to humans, further research is needed to elucidate the mechanism of action of SGLT2is on both metabolic and cardiovascular systems. Despite these limitations, our research suggests that canagliflozin improves endothelial function and attenuates atherosclerosis by reducing inflammation and oxidative stress.

Conclusion

Canagliflozin ameliorates endothelial dysfunction and prevents atherogenesis in diabetic ApoE^{-/-} mice. Anti-inflammatory and antioxidative effects that are potentially caused by the reduction of glucose tox-

icity might be involved in the cardioprotective effects of canagliflozin.

Competing Interests

The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School is supported in part by unrestricted research grants from Boehringer Ingelheim. Dr. Sata received research funding and lecture fees from Mitsubishi Tanabe Pharma. Other authors declare that they have no conflicts of interest.

Sources of Funding

This work was partially supported by JSPS Kakuhhi Grants (No. 19K08584 to D.F. and No. 19H03654 to M.S.), SENSHIN Medical Research Foundation (D.F.), Takeda Science Foundation (M.S.), and the Vehicle Racing Commemorative Foundation (M.S.). The funders had no role in the study design, data collection and analysis, or manuscript preparation.

Acknowledgements

The authors would like to thank Shintaro Okamoto and Etsuko Uematsu (Tokushima University) for their technical assistance.

References

- 1) Collaboration NCDRF. Worldwide trends in diabetes since 1980: a pooled analysis of 751 population-based studies with 4.4 million participants. *Lancet*, 2016; 387: 1513-1530
- 2) Shaw JE, Sicree RA and Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract*, 2010; 87: 4-14
- 3) Whiting DR, Guariguata L, Weil C and Shaw J. IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res Clin Pract*, 2011; 94: 311-321
- 4) Zimmet P and Alberti KGMM. Surgery or Medical Therapy for Obese Patients with Type 2 Diabetes? *N Engl J Med*, 2012; 366: 1635-1636
- 5) Ali MK, Bullard KM, Saaddine JB, Cowie CC, Imperatore G and Gregg EW. Achievement of Goals in U.S. Diabetes Care, 1999–2010. *N Engl J Med*, 2013; 368: 1613-1624
- 6) Kralova Lesna I, Tonar Z, Malek I, Maluskova J, Nedorost L, Pirk J, Pitha J, Lanska V and Poledne R. Is the amount of coronary perivascular fat related to atherosclerosis? *Physiol Res*, 2015; 64 Suppl 3: S435-S443
- 7) Park I, Kassiteridi C and Monaco C. Functional diversity of macrophages in vascular biology and disease. *Vascul Pharmacol*, 2017; 99: 13-22
- 8) Ross R. Atherosclerosis — An Inflammatory Disease. *N Engl J Med*, 1999; 340: 115-126
- 9) Fenyó IM and Gafencu AV. The involvement of the monocytes/macrophages in chronic inflammation associated with atherosclerosis. *Immunobiology*, 2013; 218: 1376-1384
- 10) Maki K, Rieko T, Koh O, Sayaka S, Hiromichi W, Hajime Y, Noriko S-A, Tatsuya M, Akira S, Yuko T and Koji H. Association between monocyte chemoattractant protein-1 and blood pressure in smokers. *J Int Med Res*, 2017; 46: 965-974
- 11) Neal B, Perkovic V, Mahaffey KW, de Zeeuw D, Fulcher G, Erondú N, Shaw W, Law G, Desai M and Matthews DR. Canagliflozin and Cardiovascular and Renal Events in Type 2 Diabetes. *N Engl J Med*, 2017; 377: 644-657
- 12) Wu JHY, Foote C, Blomster J, Toyama T, Perkovic V, Sundström J and Neal B. Effects of sodium-glucose cotransporter-2 inhibitors on cardiovascular events, death, and major safety outcomes in adults with type 2 diabetes: a systematic review and meta-analysis. *Lancet Diabetes Endocrinol*, 2016; 4: 411-419
- 13) Chao EC and Henry RR. SGLT2 inhibition—a novel strategy for diabetes treatment. *Nat Rev Drug Discov*, 2010; 9: 551-559
- 14) Zinman B, Wanner C, Lachin JM, Fitchett D, Bluhmki E, Hantel S, Mattheus M, Devins T, Johansen OE, Woerle HJ, Broedl UC and Inzucchi SE. Empagliflozin, Cardiovascular Outcomes, and Mortality in Type 2 Diabetes. *N Engl J Med*, 2015; 373: 2117-2128
- 15) Budoff MJ and Wilding JPH. Effects of canagliflozin on cardiovascular risk factors in patients with type 2 diabetes mellitus. *Int J Clin Pract*, 2017; 71: e12948
- 16) Davies MJ, Merton K, Vijapurkar U, Yee J and Qiu R. Efficacy and safety of canagliflozin in patients with type 2 diabetes based on history of cardiovascular disease or cardiovascular risk factors: a post hoc analysis of pooled data. *Cardiovasc Diabetol*, 2017; 16: 40
- 17) Stenlof K, Cefalu WT, Kim KA, Alba M, Usiskin K, Tong C, Canovatchel W and Meininger G. Efficacy and safety of canagliflozin monotherapy in subjects with type 2 diabetes mellitus inadequately controlled with diet and exercise. *Diabetes Obes Metab*, 2013; 15: 372-382
- 18) Han JH, Oh TJ, Lee G, Maeng HJ, Lee DH, Kim KM, Choi SH, Jang HC, Lee HS, Park KS, Kim Y-B and Lim S. The beneficial effects of empagliflozin, an SGLT2 inhibitor, on atherosclerosis in ApoE^{-/-} mice fed a western diet. *Diabetologia*, 2017; 60: 364-376
- 19) Leng W, Ouyang X, Lei X, Wu M, Chen L, Wu Q, Deng W and Liang Z. The SGLT-2 Inhibitor Dapagliflozin Has a Therapeutic Effect on Atherosclerosis in Diabetic ApoE^{-/-} Mice. *Mediators Inflamm*, 2016; 2016: 6305735
- 20) Terasaki M, Hiromura M, Mori Y, Kohashi K, Nagashima M, Kushima H, Watanabe T and Hirano T. 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
- 21) Nasiri-Ansari N, Dimitriadis GK, Agrogiannis G, Perrea D, Kostakis ID, Kaltsas G, Papavasiliou AG, Randevas HS and Kassi E. Canagliflozin attenuates the progression

- of atherosclerosis and inflammation process in APOE knockout mice. *Cardiovasc Diabetol*, 2018; 17: 106
- 22) Nakatsu Y, Kokubo H, Bumdelger B, Yoshizumi M, Yamamotoya T, Matsunaga Y, Ueda K, Inoue Y, Inoue M-K, Fujishiro M, Kushiya A, Ono H, Sakoda H and Asano T. The SGLT2 Inhibitor Luseogliflozin Rapidly Normalizes Aortic mRNA Levels of Inflammation-Related but Not Lipid-Metabolism-Related Genes and Suppresses Atherosclerosis in Diabetic ApoE KO Mice. *Int J Mol Sci*, 2017; 18: 1704
 - 23) Dasu MR, Devaraj S and Jialal I. High glucose induces IL-1 β expression in human monocytes: mechanistic insights. *Am J Physiol Endocrinol Metab*, 2007; 293: E337-E346
 - 24) Devaraj S, Venugopal SK, Singh U and Jialal I. Hyperglycemia Induces Monocytic Release of Interleukin-6 via Induction of Protein Kinase C- α and - β . *Diabetes*, 2005; 54: 85-91
 - 25) Piga R, Naito Y, Kokura S, Handa O and Yoshikawa T. Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells. *Atherosclerosis*, 2007; 193: 328-334
 - 26) Oelze M, Kröller-Schön S, Welschof P, Jansen T, Hausding M, Mikhed Y, Stamm P, Mader M, Zinßius E, Agduletova S, Gottschlich A, Steven S, Schulz E, Bottari SP, Mayoux E, Münzel T and Daiber A. The Sodium-Glucose Co-Transporter 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
 - 27) Salim HM, Fukuda D, Yagi S, Soeki T, Shimabukuro M and 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
 - 28) Hara T, Fukuda D, Tanaka K, Higashikuni Y, Hirata Y, Nishimoto S, Yagi S, Yamada H, Soeki T, Wakatsuki T, Shimabukuro M and Sata M. Rivaroxaban, a novel oral anticoagulant, attenuates atherosclerotic plaque progression and destabilization in ApoE-deficient mice. *Atherosclerosis*, 2015; 242: 639-646
 - 29) Gao YJ, Lu C, Su LY, Sharma AM and Lee RM. Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br J Pharmacol*, 2007; 151: 323-331
 - 30) Lusis AJ. Atherosclerosis. *Nature*, 2000; 407: 233-241
 - 31) Teresa Vanessa F, Annamaria P, Pengou Z and Franco F. Hyperglycemia-induced Oxidative Stress and its Role in Diabetes Mellitus Related Cardiovascular Diseases. *Curr Pharm Des*, 2013; 19: 5695-5703
 - 32) Higashi Y, Noma K, Yoshizumi M and Kihara Y. Endothelial function and oxidative stress in cardiovascular diseases. *Circ J*, 2009; 73: 411-448
 - 33) Munzel T, Gori T, Bruno RM and Taddei S. Is oxidative stress a therapeutic target in cardiovascular disease? *Eur Heart J*, 2010; 31: 2741-2748
 - 34) Kigawa Y, Miyazaki T, Lei XF, Kim-Kaneyama JR and Miyazaki A. Functional Heterogeneity of NADPH Oxidases in Atherosclerotic and Aneurysmal Diseases. *J Atheroscler Thromb*, 2017; 24: 1-13
 - 35) Yamagishi SI, Nakamura N and Matsui T. Glycation and cardiovascular disease in diabetes: A perspective on the concept of metabolic memory. *J Diabetes*, 2017; 9: 141-148
 - 36) Aronson D and Rayfield EJ. How hyperglycemia promotes atherosclerosis: molecular mechanisms. *Cardiovascular Diabetology*, 2002; 1: 1
 - 37) Singh R, Barden A, Mori T and Beilin L. Advanced glycation end-products: a review. *Diabetologia*, 2001; 44: 129-146
 - 38) Allaman I, Bélanger M and Magistretti PJ. Methylglyoxal, the dark side of glycolysis. *Front Neurosci*, 2015; 9: 23
 - 39) Han Y, Cho YE, Ayon R, Guo R, Youssef KD, Pan M, Dai A, Yuan JX and Makino A. SGLT inhibitors attenuate NO-dependent vascular relaxation in the pulmonary artery but not in the coronary artery. *Am J Physiol Lung Cell Mol Physiol*, 2015; 309: L1027-L1036
 - 40) Pietersma A, Tilly BC, Gaestel M, de Jong N, Lee JC, Koster JF and Sluiter W. p38 Mitogen Activated Protein Kinase Regulates Endothelial VCAM-1 Expression at the Post-transcriptional Level. *Biochem Biophys Res Commun*, 1997; 230: 44-48
 - 41) Peng X-q, Damarla M, Skirball J, Nonas S, Wang X-y, Han EJ, Hasan EJ, Cao X, Boueiz A, Damico R, Tudor RM, Sciuto AM, Anderson DR, Garcia JGN, Kass DA, Hassoun PM and Zhang J-t. Protective role of PI3-kinase/Akt/eNOS signaling in mechanical stress through inhibition of p38 mitogen-activated protein kinase in mouse lung. *Acta Pharmacologica Sinica*, 2010; 31: 175-183
 - 42) Garvey WT, Van Gaal L, Leiter LA, Vijapurkar U, List J, Cuddihy R, Ren J and Davies MJ. Effects of canagliflozin versus glimepiride on adipokines and inflammatory biomarkers in type 2 diabetes. *Metabolism*, 2018; 85: 32-37
 - 43) Liang Y, Arakawa K, Ueta K, Matsushita Y, Kuriyama C, Martin T, Du F, Liu Y, Xu J, Conway B, Conway J, Polidori D, Ways K and Demarest K. Effect of canagliflozin on renal threshold for glucose, glycemia, and body weight in normal and diabetic animal models. *PLoS One*, 2012; 7: e30555

Canagliflozin Prevents Diabetes-Induced Vascular Dysfunction in ApoE-Deficient Mice

ORIGINALITY REPORT

14%

SIMILARITY INDEX

10%

INTERNET SOURCES

19%

PUBLICATIONS

5%

STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

6%

★ www.mdpi.com

Internet Source

Exclude quotes On

Exclude matches < 1%

Exclude bibliography On