Vasodilatory effect of dapagliflozin in rat coronary arteries through inhibition of extracellular Ca^{2+} influx

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Vasodilatory effect of dapagliflozin in rat coronary arteries through inhibition of extracellular Ca^{2+} influx

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ABSTRACT

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Dapagliflozin, a sodium–glucose co-transporter 2 (SGLT2) inhibitor, is an antidiabetic medication that reduces blood glucose. Although it is well known that dapagliflozin has additional benefits beyond glycemic control, such as reducing blood pressure and lowering the risk of cardiovascular events, no sufficient research data are available on the direct effect of dapagliflozin on cardiovascular function. Thus, in this study, we investigated the direct vascular effect of dapagliflozin on isolated rat coronary arteries. The left descending coronary arteries of 13-week-old male Sprague Dawley rats were cut into segments 2–3 mm long and mounted in a multi-wire myography system to measure isometric tension. Dapagliflozin effectively reduced blood vessel constriction induced by U-46619 (500 nM) in coronary arteries regardless of the endothelium. Treatment with an eNOS inhibitor (L-NNA, 100 μM), sGC inhibitor (ODQ, 5 μM), or COX inhibitor (indomethacin, 3 μM) did not affect the vasodilation induced by dapagliflozin. The application of a Ca^{2+}-activated K^+ channel (K_{Ca}) blocker (TEA, 2 mM), voltage-dependent K^+ channel (K_V) blocker (4-AP, 2 mM), ATP-sensitive K^+ channel (K_{ATP}) blocker glibenclamide (3 μM), and inward-rectifier

K^+ channel (K_{IR}) blocker ($BaCl_2$, 30 μM) did not affect the dapagliflozin-induced vasodilation either. The treatment with dapagliflozin decreased contractile responses induced by the addition of Ca^{2+}, which suggested that the extracellular Ca^{2+} influx was inhibited by dapagliflozin. Treatment with dapagliflozin decreased the phosphorylation level of the 20 kDa myosin light chain (MLC_{20}) in vascular smooth muscle cells. In the present study, we found that dapagliflozin has a significant vasodilatory effect on rat coronary arteries. Our findings suggest a novel pharmacologic approach for the treatment of cardiovascular diseases in diabetic patients through the modulation of Ca^{2+} homeostasis via dapagliflozin administration.

Key words : dapagliflozin; coronary artery; vasodilation; cardiovascular disease; SGLT2 inhibitor

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I. INTRODUCTION

Type 2 diabetes (T2D) is characterized by a chronic elevation of blood sugar levels due to insulin resistance and impaired insulin secretion, resulting in disturbances in the regulation of carbohydrate, lipid, and protein metabolism.[1] Type 2 diabetes is one of the prevalent global health issues, accounting for nearly 90% of approximately 530 million cases of diabetes worldwide.[2] In addition to the well-known defects in blood sugar control, T2D also involves an increased risk of various cardiovascular complications, including coronary artery disease, heart failure, stroke, and peripheral artery disease.[3] Notably, cardiovascular diseases (CVD) are a leading cause of morbidity and mortality in individuals with T2D. These findings emphasize the need for comprehensive treatment strategies

considering glycemic control and cardiovascular risk factors.[4]

In recent years, a new class of antidiabetic medications, sodium-glucose cotransporter 2 (SGLT2) inhibitors, has emerged with the potential to offer cardiovascular benefits beyond glucose management. Up to now, U.S. Food and Drug Administration (FDA) approved for the usage of various SGLT2 inhibitors, including canagliflozin, dapagliflozin, and empagliflozin, in patients with T2D.[5] Among these inhibitors, dapagliflozin stands out as a promising member of this class.

Dapagliflozin's primary mode of action involves inhibiting glucose reabsorption in the kidneys, leading to increased urinary glucose excretion and improved glycemic control.[6] Beyond its glucose-lowering effects, dapagliflozin has garnered significant attention for its intriguing cardiovascular effects, making it a subject of interest for both basic researchers and clinicians.

Various clinical trials, including the DECLARE-TIMI 58 (Dapagliflozin Effect on Cardiovascular Events) and Dapa-HF (Dapagliflozin and the prevention of adverse outcome in Heart Failure) study trials, have revealed dapagliflozin's potential cardioprotective effects for patients with T2D.[7] These trials demonstrated that dapagliflozin treatment significantly reduced major adverse cardiovascular events, including cardiovascular death, non-fatal myocardial infarction, and stroke.[4, 8] Consequently, an increasing number of studies are being conducted to explore the underlying mechanisms explaining the observed cardioprotective effects of dapagliflozin. Recent research suggested that treatment with dapagliflozin can improve endothelial function, reduce arterial stiffness, and exhibit anti-inflammatory effects in patients with T2D.[9] These multifaceted cardiovascular benefits establish dapagliflozin as a promising adjunctive therapy to alleviate the CVD burden in individuals with T2D. Although evidence supports dapaglifozin's cardiovascular benefits, further research is necessary to comprehensively

understand these underlying mechanisms.[10] Thus, it is necessary to elucidate the direct effect of dapagliflozin in blood vessels, which contributes to understanding the pleiotropic effects of dapagliflozin. Therefore, this study aims to investigate the direct effect of dapagliflozin on the cardiovascular system, with a specific focus on vascular function in coronary arteries.

II. MATERIALS AND METHODS

1. Chemicals and reagents

U-46619, acetylcholine (ACh), N^{ω}-Nitro-L-arginine (L-NNA), 1H-(1,2,4) oxadiazolo[4,3-a] quinoxaline-1-one (ODQ), tetraethylammonium chloride (TEA), barium chloride ($BaCl_2$), 4-Aminopyridine (4-AP), dimethyl sulfoxide (DMSO), ethylene glycol bis (2-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA), and phlorizin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Indomethacin was obtained from Calbiochem (Darmstadt, Germany). Glibenclamide was purchased from Tocris Bioscience (Avonmouth, Bristol, UK). Cyclopiazonic acid (CPA) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Dapagliflozin was purchased from MCE (Monmouth Junction, NJ, USA). Dapagliflozin exhibited a purity of 99.87%, and phlorizin showed a purity of ≥99%. All other chemicals used in this study were of analytical reagent grade.

2. Animals and tissue preparation

All experiments were conducted following the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85–23, 2011), and were approved by the Ethics Committee and the Institutional Animal Care and Use Committee of Yonsei University College of Medicine (Approval number: 2022– 0164). A total of 68 rats were used in this study, specifically 13-week-old male Sprague Dawley rats. After euthanasia, the hearts were excised, and the coronary arteries were quickly dissected. The left descending coronary arteries were cut into 2–3 mm long segments and placed in an ice-cold Krebs-Henseleit (K-H) solution (composition mmol/L: NaCl 119, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1) that was bubbled with 95% O_2 and 5% CO_2. Adipose and connective tissue were removed from the coronary

arteries using a microscope (model SZ-40, Olympus, Tokyo, Japan). In the endothelium-denuded experiments, the endothelium was removed by perfusing vessels with 0.1% Triton X-100 for 10 seconds, followed by an additional 10 sec of perfusion with K-H solution.[11] Endothelium-denudation was confirmed by the absence of relaxation induced by ACh (10 μM).

3. Measurement of isometric tension in coronary arteries

The coronary artery segments were mounted in a wire myograph (model 620M, Danish Myotechnology, Aarhus, Denmark) to measure isometric tension. Arterial rings were bathed in a 37°C K-H solution, aerated with 95% O_2 and 5% CO_2. Arteries were equilibrated for 20 min and stretched to their optimal resting tension (2 mN). Contractility of the vessels was evaluated by incubating the arteries three times in a high K^+ solution (70 mM, composition mmol/L: NaCl 53.6, KCl 70, $MgSO_4$ 1.2, KH_2PO_2 1.4, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11.1). The response was recorded by stabilizing the vessel, contracting the arteries to high K^+ (70 mM) or U-46619, followed by a cumulative addition of dapagliflozin (500 μM) or vehicle (dimethyl sulfoxide: DMSO, 0.0006–0.284%). In addition, to investigate the relaxation effect induced by the other SGLT2 inhibitor, coronary arteries were pre-constricted using U-46619 (500 nM), and then phlorizin (10 μM) was administrated. To investigate dapagliflozin's induced vascular relaxation, L-NNA, indomethacin, ODQ, TEA, BaCl2, glibenclamide, or 4-AP were pre-treated for 20 min, and then the relaxation response of dapagliflozin (500 μM) in U-46619 (500 nM)-induced contraction was recorded. To determine the involvement of Ca^{2+} influx in dapagliflozin-induced vasodilation, the K-H solution was replaced with a Ca^{2+}-free K-H (composition mmol/L: NaCl 119, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1, and EGTA 1) solution containing high K^+ (70 mM) and the sarcoplasmic reticulum Ca^{2+}-

ATPase (SERCA) inhibitor, CPA (5 μM), to empty the intracellular Ca^{2+}. Then, $CaCl_2$ was cumulatively added. After a 20 min pre-treatment with dapagliflozin (500 μM) in the same arteries, the changes in contractility by another cumulative addition of $CaCl_2$ were compared to those observed before dapagliflozin pre-treatment. The $CaCl_2$-induced contraction was calculated as the percentage of the maximum contraction recorded from the KCl contraction.

4. Cell culture

Human aortic smooth muscle cells (AoSMCs) were purchased from Lonza (Walkersville, MD, USA). AoSMCs were cultured in smooth muscle cell basal media supplemented with various growth factors (SmGM™-2, insulin, fibroblast growth factors, epidermal growth factors, and 2% serum). The cell cultures were maintained in an incubator at 37°C with a 5% CO_2 humidified atmosphere. Cells were used for experiments between four and six passages. Prior to experimentation, cultured AoSMCs were serum-starved for 18 h in smooth muscle cell basal medium (smBM™) without FBS.

5. Measurement of MLC_{20} phosphorylation level (western blot analysis)

The cultured AoSMCs were treated with a vehicle (0.1% DMSO), U-46619 (100 nM), or U-46619 (100 nM) with dapagliflozin (50 μM), then homogenized in an ice-cold lysis buffer, as previously described.[12] Cell lysates were centrifuged at 15,000 rpm for 20 min at 4°C, supernatants were collected, and protein concentrations were normalized using the bicinchoninic acid (BCA) method. Approximately 65 μg protein for each sample was processed for western blotting. After resolving protein on a 15% SDS-PAGE gel, the protein was transferred onto a nitrocellulose (NC) membrane using a transfer method.[13, 14] NC membranes were blocked with 5% skim milk in TBST (tris-buffered saline with 0.1%

Tween 20). Blots were incubated overnight at 4°C with phosphorylated 20 kDa myosin light chain (MLC_{20}) and total MLC_{20} antibodies (Cell Signaling, Boston, MA, USA) at a 1:500 dilution. Blots were washed thrice and incubated with mouse anti-rabbit lgG-HRP secondary antibodies (1:1000 dilution) for 1 h at room temperature. Following the secondary antibody incubation, NC membranes were washed thrice. Membranes were developed using enhanced chemiluminescence (ECL; Cytiva, Amersham, UK) detection solution, and protein bands were imaged with AGFA CP 1000. Blots were stripped and reprobed with the β-actin antibody (1:3000 dilution; Santa Cruz Biotechnology, CA, USA) to verify equal loading between samples.

6. Statistical analysis

Data is presented as mean values ± standard deviation. Normal distribution of data was confirmed using Shapiro–Wilk's test. One-way or two-way ANOVA was used to compare groups, followed by multiple comparison testing with Bonferroni post-hoc tests. In all experiments assessing vascular tension, the '*n*-values' denote the number of arteries derived from distinct animals. The '*n*-value' means numbers of independent experiments in western blot analysis. Statistical analysis was performed using GraphPad Prism Version 10.0.0 (GraphPad Software Inc., San Diego, CA, USA).

III. RESULTS

1. Effect of dapagliflozin on high-K^+- or U-46619-induced contraction in rat coronary arteries

To investigate the effect of dapagliflozin on vascular function, we cumulatively administered dapagliflozin on coronary arteries pre-constricted with high K^+ (70 mM) or U-46619 (500 nM). Dapagliflozin (1–500 μM) demonstrated a concentration-dependent vasodilation in these coronary arteries (Figure 2A, B). The dapagliflozin-induced vasodilation was 94.91 ± 7.05% in arteries constricted with high K^+ and 92.41 ± 6.80% in those constricted with U-46619. The effective concentration of dapagliflozin at 50% of its maximum vasodilation (EC_{50}) was determined as 70.2 μM.

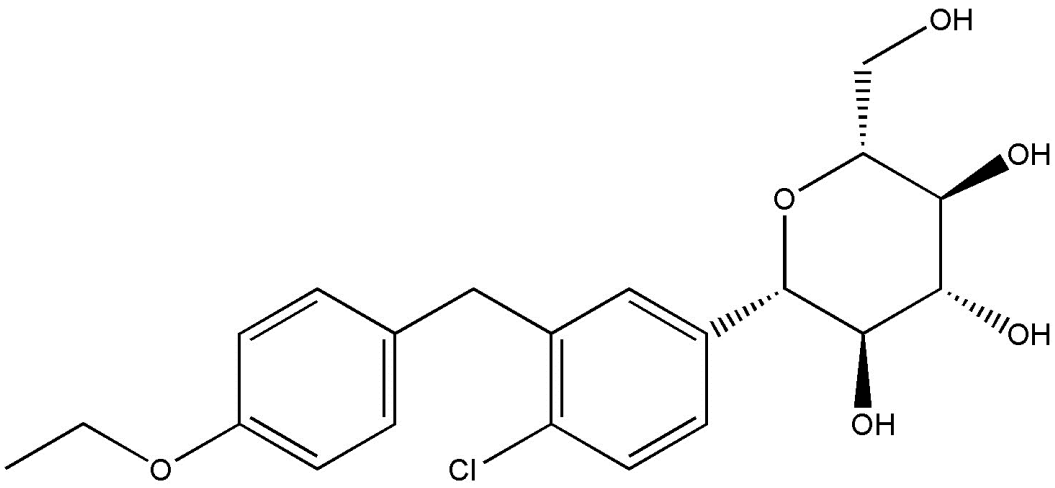

Figure 1. Chemical structure of dapagliflozin. Dapagliflozin is chemically described as (2S,3R,4R,5S,6R)-2-[4-chloro-3-[(4-ethoxyphenyl)methyl]phenyl]-6-(hydroxylmethyl)oxane-3,4,5-triol. The molecular weight of dapagliflozin is 408.87 g/mol and molecular formula is $C_{21}H_{25}ClO_6$.

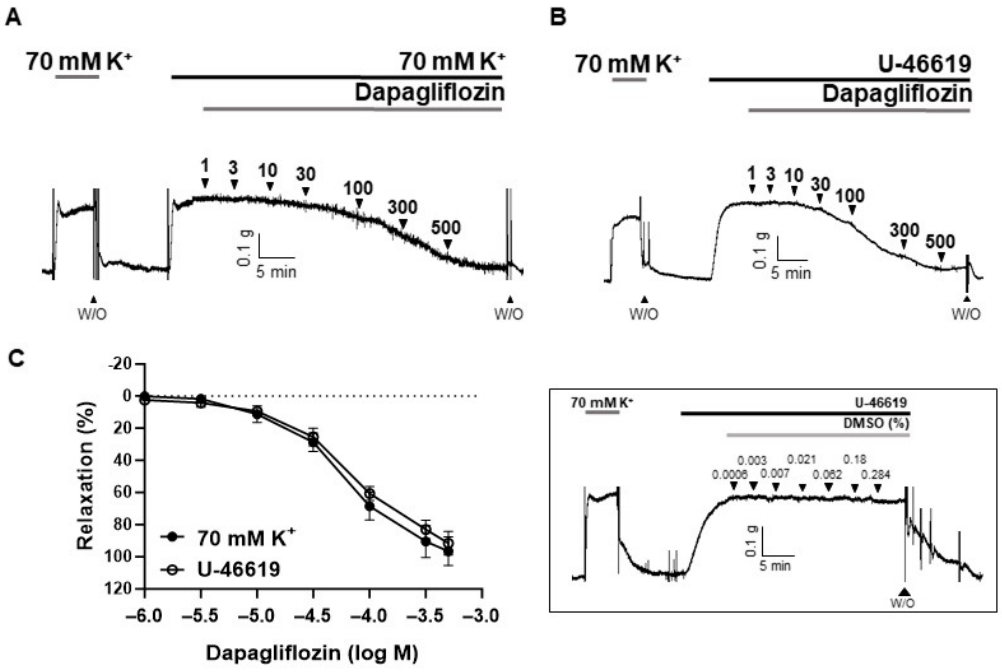

Figure 2. Dapagliflozin-induced vasodilation in rat coronary arteries. Representative traces showing response to cumulative administration of dapagliflozin (1–500 μM) on high K^+ (**A**) or U-46619 (500 nM)-induced contractions (**B**). Statistical analysis of the relaxation response to dapagliflozin (**C**). Mean ± SD (n = 6). Inset, representative trace showing responses to the vehicle, DMSO (0.0006-0.284%). W/O, wash out.

2. Effects of SGLT2 inhibition on rat coronary arteries

To investigate whether dapagliflozin-induced vasodilation was due to SGLT inhibition, we applied phlorizin, a non-selective SGLT1 and SGLT2 inhibitor, to coronary arteries and analyzed its vasodilatory response. Our data indicated that dapagliflozin (500 μM) caused a 81.25 ± 8.25% relaxation in U-46619-contracted coronary arteries. In contrast, phlorizin (10 μM) exhibited no vasodilatory effect (-2.06 ± 4.93%, Figure 3B). The combined administration of dapagliflozin (500 μM) and phlorizin (10 μM) resulted in a substantial vasodilation (82.57 ± 3.19%, Figure 3C) similar to the relaxation degree induced by dapagliflozin (500 μM) alone (81.25 ± 8.25%).

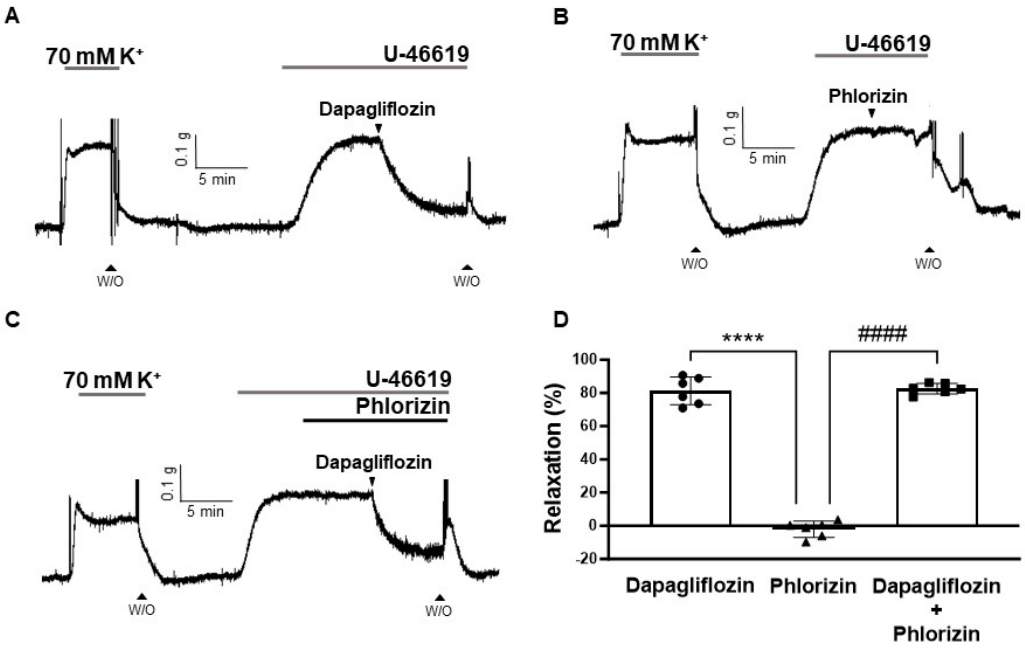

Figure 3. Comparison of vasorelaxant effects induced by dapagliflozin and phlorizin. Original traces illustrating vasodilation induced by dapagliflozin (500 μM, **A**), phlorizin (10 μM, **B**), and a combination of dapagliflozin (500 μM) and phlorizin (10 μM, **C**). Mean ± SD (n = 6). **** $p < 0.0001$, dapagliflozin vs. phlorizin; $^{\#\#\#\#}$ $p < 0.0001$, dapagliflozin + phlorizin vs. phlorizin. W/O, wash out.

3. Dapagliflozin-induced endothelium-independent relaxation

To determine whether the vasodilation induced by dapagliflozin is dependent on the presence of the endothelium, we applied dapagliflozin to endothelium-intact (Figure 4A) and endothelium-denuded coronary arteries (Figure 4B). The maximal value of dapagliflozin-induced vasodilation was 81.25 ± 8.25% in the endothelium intact arteries, and 82.50 ± 7.78% in the endothelium-denuded arteries. There was no significant difference in the vasodilatory effects of dapagliflozin between endothelium-intact and endothelium-denuded coronary arteries. (Figure 4C).

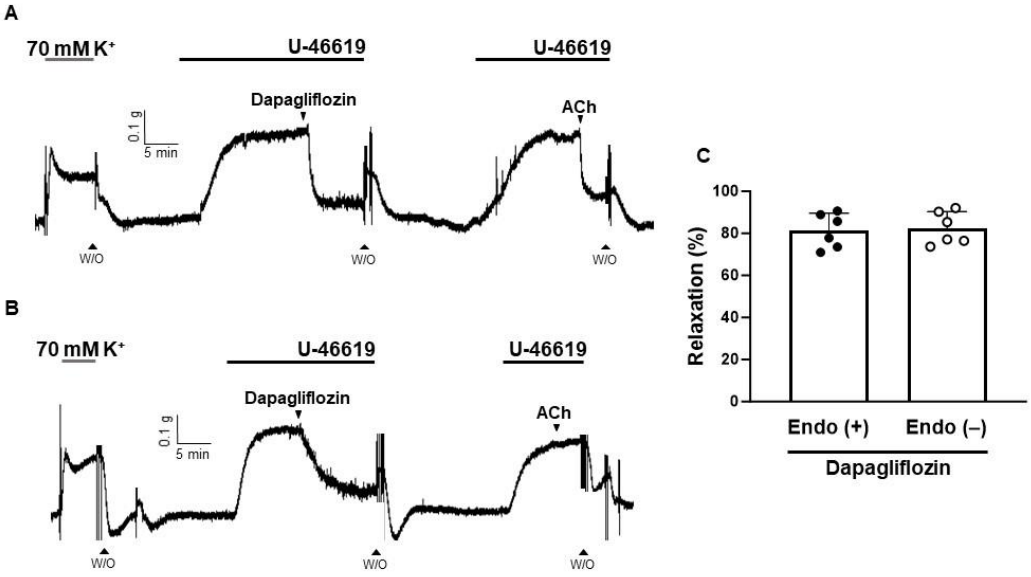

Figure 4. Endothelium-independent vasodilation induced by dapagliflozin. Representative trace showing dapagliflozin (500 μM)-induced vasodilation in the endothelium-intact (**A**) and endothelium-denuded coronary arteries (**B**). Statistical of dapagliflozin-induced vasodilation (**C**). Mean ± SD (*n* = 6). ACh, acetylcholine; W/O, wash out.

4. Effects of L-NNA, ODQ, and indomethacin on dapagliflozin-induced vasodilation

To confirm that dapagliflozin-induced vasodilation is endothelium-independent, and to further investigate the involvement of the nitric oxide (NO) / cyclic guanosine monophosphate (cGMP) pathways and cyclooxygenase (COX) / prostacyclin (PGI_2), the arteries were exposed to a 20 min incubation period with an endothelial nitric oxide synthase (eNOS) inhibitor [N^{ω}-Nitro-L-arginine (L-NNA, 100 μM)]; a soluble guanylyl cyclase (sGC) inhibitor [1H-(1,2,4) oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 5 μM)]; or a COX inhibitor (indomethacin, 3 μM), prior to contraction with U-46619 (500 nM). The vasodilatory responses induced by dapagliflozin were 86.00 ± 5.84%, 79.64 ± 3.80% and 87.76 ± 7.94%, in the presence of L-NNA, ODQ and indomethacin, respectively (Figure 5).

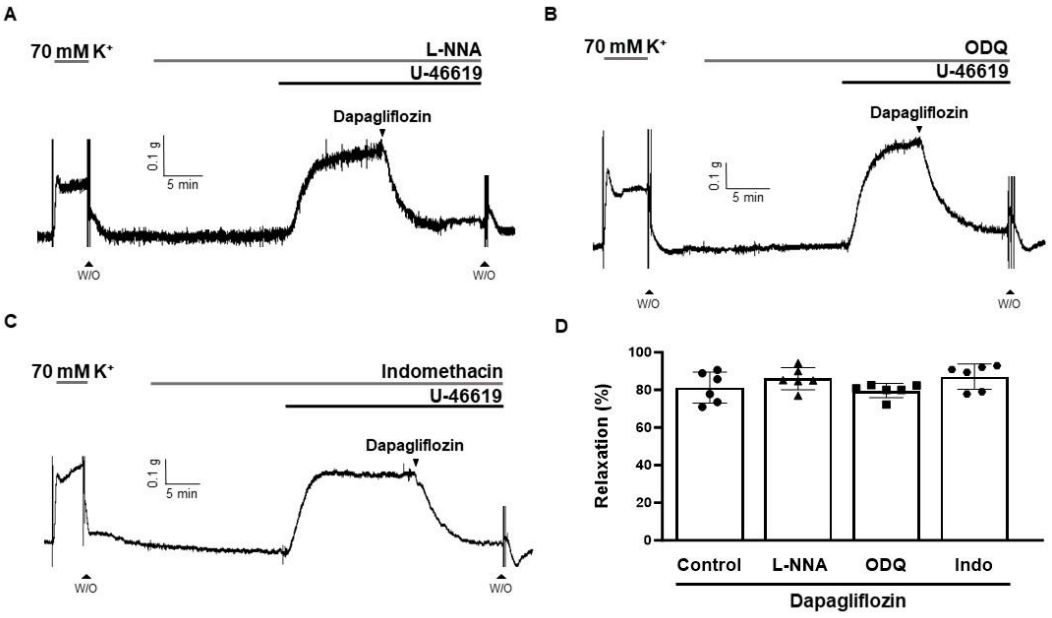

Figure 5. Effects of L-NNA, ODQ and indomethacin on dapagliflozin-induced vasodilation. Representative traces showing dapagliflozin (500 μM)-induced vasodilation in the presence of L-NNA (100 μM, **A**), ODQ (5 μM, **B**) and indomethacin (3 μM, **C**). Statistical analysis of the relaxation response of dapagliflozin in the presence of L-NNA, ODQ and indomethacin (**D**). Relaxation of arteries is expressed as the percentage of the contraction induced by U-46619 (500 nM). Mean ± SD (n = 6). L-NNA, N^{ω}-Nitro-L-arginine; ODQ, 1H-[1,2,4]-oxadiazolo-[4,3-α]-quinoxalin-1-one; Indo, Indomethacin; W/O, wash out.

5. Effects of K^+ channel blockers on dapagliflozin-induced vascular relaxation

To determine whether of K^+ channels are involved in the vasodilatory response induced by dapagliflozin, we pre-treated the arteries with various K^+ channel blockers: a non-selective K^+ channels blocker, TEA (2 mM); a voltage-dependent K^+ channels blocker, 4-AP (2 mM); an ATP-sensitive K^+ channels blocker, glibenclamide (3 μM); and an inward rectifier K^+ channels blocker, $BaCl_2$ (30 μM). These blockers were administered 20 min prior to treatment with U-46619 (500 nM). The relaxation response of dapagliflozin were 88.12 ± 4.77%, 82.78 ± 7.88%, 81.47 ± 7.23% and 84.35 ± 8.04%, in the presence of TEA, 4-AP, glibenclamide and $BaCl_2$, respectively (Figure 6). Treatment with K^+ channel blockers did not affect the dapagliflozin-induced relaxation response.

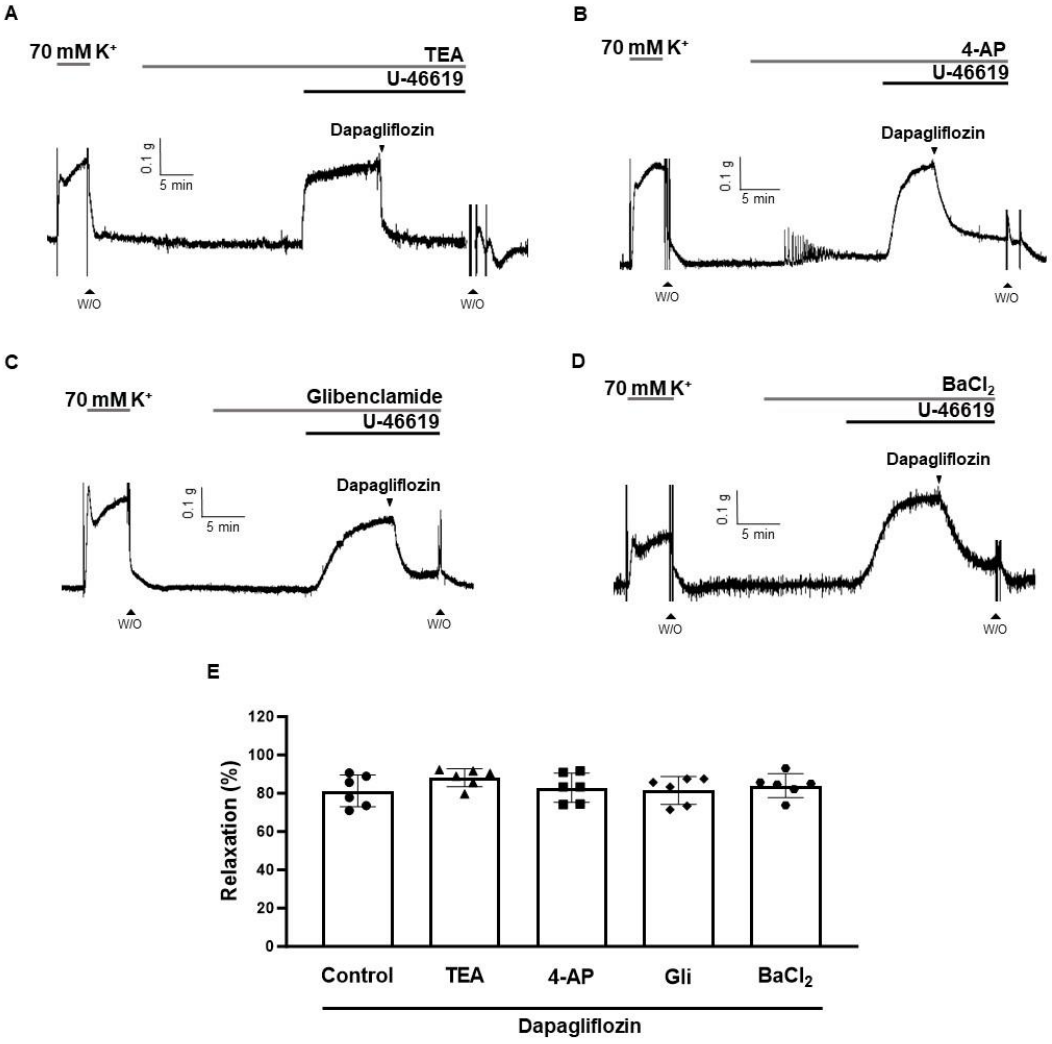

Figure 6. Effects of K^+ channel blockers on dapagliflozin-induced vasodilation. Effects of dapagliflozin (500 μM) in the coronary arteries pre-contracted with U-46619 (500 nM) in the presence of TEA (2 mM, **A**), 4-AP (2 mM, **B**), glibenclamide (3 μM, **C**) or $BaCl_2$ (30 μM, **D**). Statistical analysis of the relaxation response of dapagliflozin in the presence of K^+ blockers. Relaxation of arteries is expressed as the percentage of the contraction induced by U-46619 (**E**). Mean ± SD (n = 6). TEA, tetraethylammonium; 4-AP, 4-aminopyridine; Gli, glibenclamide; $BaCl_2$, barium chloride; W/O, wash out.

6. Effect of dapagliflozin on extracellular Ca^{2+}-induced contraction

To investigate whether dapagliflozin's vasodilatory effects are associated with the inhibition of extracellular Ca^{2+} influx, we examined the effect of dapagliflozin on contractile responses induced by the cumulative addition of $CaCl_2$ (0.1–2.0 mM). These responses were observed in arteries that had been incubated in a Ca^{2+}-free K-H solution containing a sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA, 5 μM), and high K^+ (70 mM). We confirmed that the contractile responses elicited by the repetitive addition of $CaCl_2$ remained unaffected (Figure 7A). Pre-treatment with dapagliflozin (500 μM) significantly decreased the contractile responses elicited by the cumulative addition of $CaCl_2$ (Figure 7B, C).

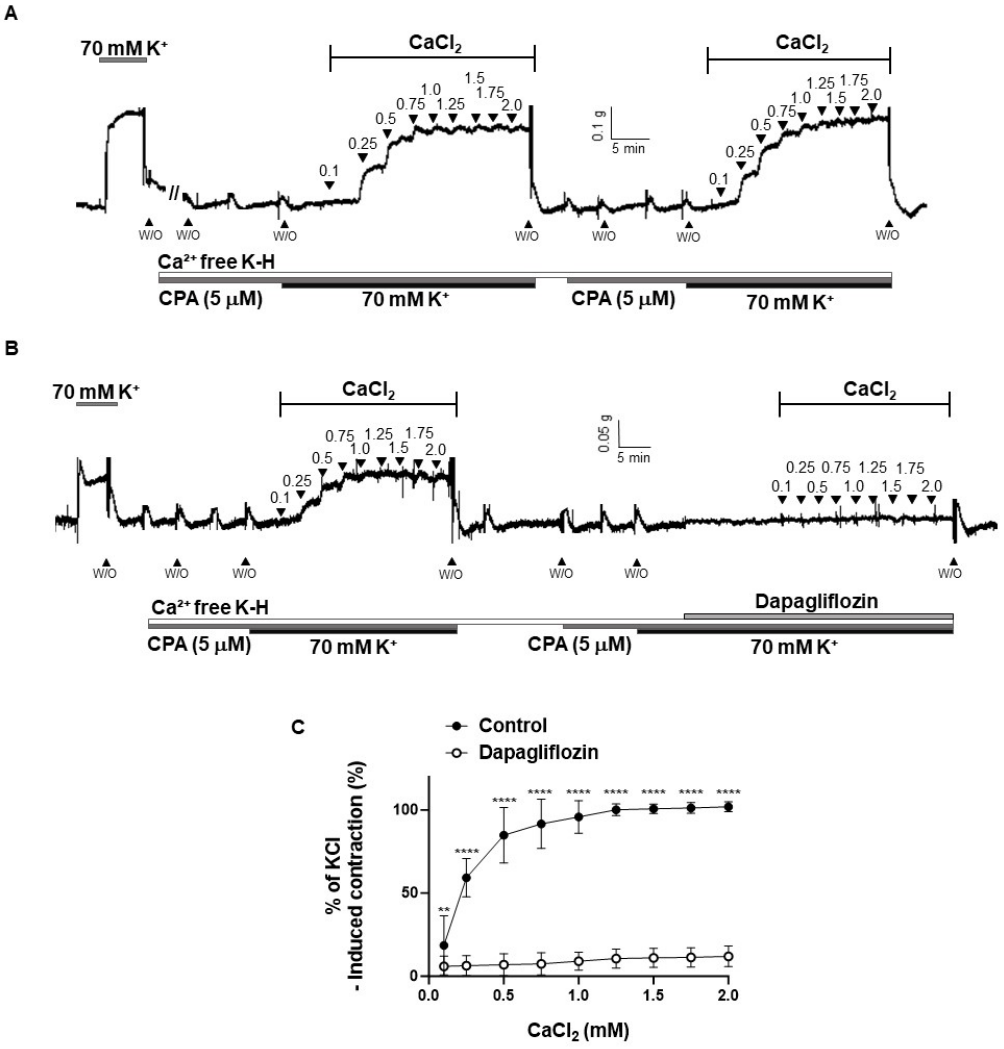

Figure 7. Inhibition of extracellular Ca^{2+}-induced vasoconstriction by dapagliflozin. Representative traces showing the contraction responses by repeated addition of Ca^{2+} (0.1–2.0 mM) in the absence of dapagliflozin (**A**) and in the presence of dapagliflozin (500 μM, **B**). Statistical analysis of contraction induced by $CaCl_2$ in the coronary arteries with or without dapagliflozin (**C**). Mean ± SD (n = 5). ** $p < 0.01$; **** $p < 0.0001$, control vs. dapagliflozin. CPA, cyclopiazonic acid; W/O, wash out.

7. Decrease in phosphorylation level of MLC_{20} by dapagliflozin in human aortic smooth muscle cells

To investigate whether the relaxation induced by dapagliflozin resulted from reduced MLC_{20} phosphorylation, we assessed the phosphorylation and total expression level of MLC_{20} in AoSMCs (Figure 8). The administration of U-46619 (100 nM) increased the phosphorylation level of MLC_{20} in AoSMCs, which was significantly reduced by the treatment with dapagliflozin (50 μM).

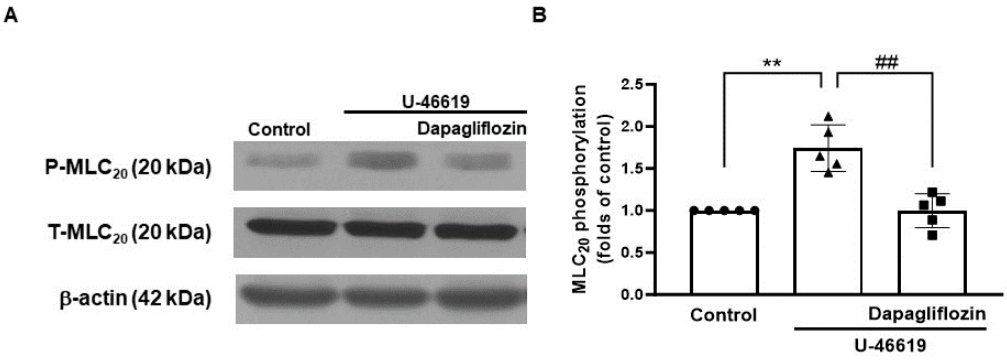

Figure 8. Effect of dapagliflozin on the phosphorylation of 20 kDa myosin light chain (MLC_{20}). Representative western blot analysis for phosphorylated MLC_{20} (P-MLC_{20}) and total MLC_{20} (T-MLC_{20}) in control, AoSMC treated with U-46619 (100 nM), and AoSMC co-treated with U-46619 and dapagliflozin (50 μM, **A**). Quantitative data for phosphorylated MLC_{20}. Mean ± SD (n = 5). $^{**}p < 0.01$, Control vs. U-46619; $^{\#\#}p < 0.01$, Control vs. U-46619 + dapagliflozin.

IV. DISCUSSION

In this study, we have demonstrated for the first time that dapagliflozin induces vasodilation in rat coronary arteries. Furthermore, our findings indicate that the vasodilatory response induced by dapagliflozin is not mediated by endothelium-dependent mechanism. Our results suggest that dapagliflozin-induced vasodilation is associated with decrease in extracellular Ca^{2+} influx, occurring independently of SGLT2 inhibition. Moreover, phosphorylation level of MLC_{20} was decreased by the treatment with dapagliflozin in AoSMCs.

Recent clinical trials reported that dapagliflozin treatment can reduce the risk of death from cardiovascular disease by improving glycemic and blood pressure control.[7, 15, 16] However, the exact mechanism on blood pressure lowering effect of dapagliflozin was not studied yet. In the current study, we propose that the potential vasodilatory effect of dapagliflozin may play a role in contributing to its beneficial effects on blood pressure. This discovery holds notable implications, as it could potentially lead to blood pressure reduction and enhanced cardiovascular outcomes. Consequently, this may contribute to reducing cardiovascular morbidity and mortality.

In this study, we observed the vasorelaxant effect of dapagliflozin in rat coronary arteries contracted with high K^+ and U-46619 (Figure 2A, B). Using these vasoconstrictor agents, we investigated whether dapagliflozin could induce vasodilation in constricted arteries through direct membrane depolarization and/or agonist stimulation. In either case, we found no notable distinction in the vasodilatory response induced by dapagliflozin, implying that dapagliflozin induces relaxation regardless of the type of stimulation. Phlorizin is a non-specific SGLT1 and SGLT2 inhibitor and is the first SGLT inhibitor reported to lower blood glucose and normalize insulin sensitivity.[17] In this study, we

investigated whether dapagliflozin-induced vasodilation was resulted from the inhibition of SGLT2. If the effect of dapagliflozin is attributed to SGLT2 inhibition, similar outcomes should be observed with other SGLT2 inhibitors. Therefore, we also employed a different inhibitor to corroborate our findings. We confirmed that phlorizin did not induce vasodilation in rat coronary arteries (Figure 3B). These findings propose that the vasodilation triggered by dapagliflozin is not a consequence of SGLT2 inhibition, but rather an intrinsic characteristic of this compound, accountable for its diverse vascular effects.

Vasodilation occurs through the relaxation of smooth muscle cells within blood vessel walls. This relaxation can result from the removal of a contractile stimulus or the direct influence of vasodilatory agents.[18] The luminal surface of vessels is covered by a monolayer of endothelial cells, comprising the vascular endothelium.[19, 20] When the endothelium is exposed to different stimuli, it releases substances that induce vasodilation, including nitric oxide (NO), prostacyclin (prostaglandin I_2; PGI_2), endothelium-derived hyperpolarizing factor (EDHF).[21–24] In this research, we assessed whether dapagliflozin induces vasodilation by acting through the endothelium. We found that even after the removal of the endothelium, the vasodilatory effect of consistently maintained in rat coronary arteries (Figure 4). To confirm that dapagliflozin-induced relaxation is independent on endothelium, we used L-NNA, ODQ, indomethacin. Nitric oxide is produced by eNOS within the endothelium, diffusing subsequently into smooth muscle cells and initiating sGC activation to elevate intracellular cyclic guanosine monophosphate (cGMP) levels, thereby inducing relaxation.[25] The eNOS inhibitor (L-NNA) was used to confirm the endothelium-independent vasodilatory effect of dapagliflozin (Figure 5A). Treatment with L-NNA had no effect on dapagliflozin-induced vasodilation in coronary arteries. We determined that the sGC inhibitor (ODQ) did not alter the vasodilation induced by dapagliflozin (Figure 5B). Our results indicate that the NO/cGMP pathway is not involved in dapagliflozin-

induced vascular relaxation. The results align with a previous investigation conducted by Ahasanul Hasan *et al*., which demonstrated dapagliflozin induced endothelium-independent vasodilation in rat mesenteric arteries.[26] They also found that treatment with L-NNA and endothelial removal did not affect dapagliflozin-induced vasodilation.

Furthermore, we investigated whether the vasodilatory effect of dapagliflozin is affected by another vasoactive substance, PGI_2 which is generated by COX.[27, 28] The binding of PGI_2 to the prostaglandin I_2 receptor (IP receptor) on smooth muscle cells triggers the activation of adenylate cyclase, leading to the production of cyclic adenosine monophosphate (cAMP).[29] Then, cAMP activates protein kinase A, leading to the relaxation of smooth muscle in the same way as NO.[30] We observed that the non-selective COX inhibitor (indomethacin) did not affect the dapagliflozin-induced vasodilation (Figure 5C). These findings confirmed that vasodilatory effect induced by dapagliflozin is through an endothelium-independent pathway. After we defined that endothelium is not involved in the dapagliflozin-induced vasodilation, we investigated whether dapagliflozin directly acts on smooth muscle cells to induce relaxation.[31, 32] The relaxation of vascular smooth muscle initiates with a decrease in intracellular Ca^{2+}, which results from reduction of extracellular Ca^{2+} influx or re-uptake of Ca^{2+} to sarcoplasmic reticulum (SR).[18] Arterial smooth muscle and endothelial cells contain several K^+ channels involved in membrane potential and vascular tone regulation.[33, 34] Activation of K^+ channels induces efflux of K^+, leading to membrane hyperpolarization. Consequently, this results in the closure of voltage-dependent Ca^{2+} channels, blocking extracellular Ca^{2+} influx, and inducing relaxation of smooth muscle cells.[35] In this study, we examined whether dapagliflozin-induced vasodilation involves K^+ channel activation. A non-selective K^+ channel inhibitor, TEA, had no effect on the dapagliflozin-induced vasodilation (Figure 6A). To verify these results, we incubated arteries with various blockers for different types of K^+ channels. We found that treatment

with 4-AP, glibenclamide, and $BaCl_2$ did not alter the effect of dapagliflozin, suggesting that K^+ channels, including the voltage-gated potassium channel blocker, ATP-sensitive K^+ channel blocker, and inward rectifier K^+ channel, were not involved in the vasodilatory response induced by dapagliflozin (Figure 6B–D). Our data does not align with previous studies, as earlier research indicated the involvement of K_V channels in the vascular dilation response to dapagliflozin in the mesenteric artery.[26] We assume that these differences may arise from differences in the type of animals or type of blood vessels.

Since we have confirmed that endothelial cells and K^+ channels are not involved in the vasodilation induced by dapagliflozin, we investigated whether dapagliflozin directly inhibits the increase of intracellular Ca^{2+} levels in smooth muscle cells. Since Ca^{2+} channel blockade did not induce arterial contraction, we were unable to test the relaxing effect of dapagliflozin in the presence of Ca^{2+} channel blockers. Therefore, we employed alternative methods to test the effects of dapagliflozin. We incubated coronary arteries in a Ca^{2+}-free K-H solution containing CPA, which depletes intracellular Ca^{2+}. Then, the solution was switched to one containing 70 mM K^+ to enable the opening of voltage-dependent Ca^{2+} channels. The cumulative addition of Ca^{2+} elicited a contraction response in coronary arteries, which was attenuated by the administration of dapagliflozin (Figure 7A). Based on these results, we assumed that dapagliflozin treatment leads to the inhibition of extracellular Ca^{2+} influx. Our results are in accordance with a previous study showed that dapagliflozin attenuates diabetic cardiomyopathy through reduction of intracellular Ca^{2+} overload.[36] Although a direct structural relationship between dapagliflozin and Ca^{2+} channels was not suggested in the current study, these data suggest a possibility that dapagliflozin may affect Ca^{2+} channels.

When the intracellular Ca^{2+} concentration increases in vascular smooth muscle cells, myosin light chain kinase (MLCK) which phosphorylates MLC_{20}, is activated.

Phosphorylation and dephosphorylation of MLC_{20} are regarded as significant events in the regulation of smooth muscle contraction.[37] Therefore, molecules regulating MLC_{20} phosphorylation are considered key determinants of muscle contraction. Furthermore, Ca^{2+} binds with calmodulin, forming the Ca^{2+}-calmodulin complex, which activates MLCK responsible for phosphorylating serine 19 (Ser^{19}) of MLC_{20}. Phosphorylated MLC_{20} triggers the activation of myosin ATPase, resulting in muscle contraction.[38] Therefore, the phosphorylation of MLC_{20} is vital for smooth muscle contraction. In contrast, the reduction of intracellular Ca^{2+} enables an opposing response. Given the observed reduction in contraction induced by Ca^{2+} in coronary arteries due to dapagliflozin, we proceeded to investigate whether dapagliflozin affects the phosphorylation level of MLC_{20} in vascular smooth muscle cells. We discovered that treatment with U-46619 leads to an increase in the phosphorylation level of MLC_{20} in AoSMC. The combined treatment with U-46619 and dapagliflozin significantly reduced the phosphorylation level of MLC_{20} compared to U-46619 treatment alone (Figure 8A). Even though we did not directly measure changes in intracellular Ca^{2+} concentration induced by dapagliflozin, the above findings suggest that dapagliflozin reduces MLC_{20} phosphorylation, ultimately leading to vascular relaxation.[39–41]

Our findings support the potential multifaceted cardiovascular benefits of dapagliflozin including decrease in vascular tone, attenuation of vascular inflammation and atherosclerosis, modulation of sympathetic nerve activity, regulation of natriuretic peptides, and inhibition of sodium-hydrogen exchange.[1] This suggests the possibility that dapagliflozin may have additional molecular targets beyond blocking SGLT2 in the renal tubules, which could result in beneficial cardiovascular effects across various cells, tissues, and organ systems. Although dapagliflozin has a significant vasodilator effect, there has not yet been sufficient research regarding its improved cardiovascular function.

Nevertheless, further investigations are needed to investigate whether chronic dapagliflozin administration triggers vasodilation in resistance arteries and subsequently decrease systemic blood pressure *in vivo*.

V. CONCLUSION

In this study, we discovered that dapagliflozin induces concentration-dependent vascular relaxation in rat coronary arteries, which is associated with endothelium-independent mechanism. Furthermore, we also found that the NO/cGMP pathway, and prostacyclin are not involved in the vasodilatory effect induced by dapagliflozin. The inhibition of extracellular Ca^{2+} influx was found to be associated with the vasodilation induced by dapagliflozin. Our data contribute to provide basic knowledge to build therapeutic potential of dapagliflozin as an anti-hypertension.

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ABSTRACT(IN KOREAN)

쥐 관상동맥에서 세포 외 Ca^{2+} 유입 억제를 통한 다파글리플로진의 혈관 확장 효과

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최 수 연

다파글리플로진은 혈당을 낮추는 항당뇨 약물로서, 소듐-글루코스 공동수송체-2 (sodium-glucose cotransporter-2: SGLT-2) 억제제로 알려져 있다. 다파글리플로진은 혈압을 낮추고 심혈관 위험을 줄이는 등 혈당 조절 이외의 추가적인 이점이 보고되어 있지만, 심혈관 기능 개선에 대한 연구는 충분하지 않은 실정이다. 따라서 본 연구에서는 쥐 심장 관상 동맥에서 다파글리플로진을 처리한 경우에 나타나는 혈관 효과를 조사하였다.

본 연구에서 다파글리플로진은 내피 세포의 유무와 관계없이 U-46619 (500 nM)에 의해 유발된 혈관 수축을 효과적으로 감소 시키는 것으로 나타났다. 또한, eNOS 억제제 (L-NNA, 100 μM), sGC 억제제 (ODQ, 5 μM) 또는 COX 억제제 (indomethacin, 3 μM)의 전처리는 다파글리플로진에 의한

혈관 확장에 영향을 미치지 않는 것으로 나타났다. 마찬가지로 포타슘 이온 통로 (K_{Ca}) 차단제 (TEA, 2 mM), 전압의존형 포타슘 통로 (K_V) 차단제 (4-AP, 2 mM), ATP-민감성 포타슘 통로 (K_{ATP}) 차단제 (glibenclamide, 3 μM) 및 내향성 포타슘 통로 (K_{IR}) 차단제 ($BaCl_2$, 30 μM)의 전처리는 다파글리플로진에 의해 유도된 혈관 확장에 영향을 미치지 않는 것으로 나타났다. 매커니즘을 추가로 확인하기 위해 Ca^{2+}이 없는 K-H 용액에서 배양된 동맥에 $CaCl_2$ (0.1-2.0 mM)를 첨가함으로써 발생하는 수축 반응을 조사하였다. 이때 다파글리플로진의 전처리는 Ca^{2+} 첨가에 의해 유도된 수축 반응을 감소시켰는데, 이는 세포 외 Ca^{2+} 유입이 다파글리플로진에 의해 억제되는 것으로 사료된다. 또한, 다파글리플로진은 혈관 평활근 세포에서 20 kDa 마이오신 경쇄 (MLC_{20})의 인산화 수준을 감소시키는 것으로 나타났다.

본 연구에서는 다파글리플로진이 쥐 관상 동맥에서 유의미한 혈관 확장 효과를 가지고 있음을 보여주었다. 이 연구 결과는 다파글리플로진 투여를 통한 Ca^{2+} 항상성 조절을 통해 당뇨병 환자의 심혈관 질환 치료에 대한 새로운 약리학적 접근 방법을 제시할 수 있을 것으로 사료된다.

핵심되는 말: 다파글리플로진; 관상 동맥; 혈관 확장; 심혈관 질환; SGLT2 억제제

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