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A Selective Mineralocorticoid Receptor Blocker, Esaxerenone, Attenuates Vascular Dysfunction in Diabetic C57BL/6 Mice

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Aims: Pharmacological blockade of mineralocorticoid receptors (MRs) is a potential therapeutic approach to reduce cardiovascular complications since MRs play a crucial role in cardiovascular regulation. Recent studies suggest that MR antagonists affect several extrarenal tissues, including vessel function. We investigated the effect of a novel nonsteroidal selective MR blocker, esaxerenone, on diabetes-induced vascular dysfunction.

Methods: Diabetes was induced by a single dose of streptozotocin in 8-week-old male C57BL/6 mice. Esaxerenone (3 mg/kg/day) or a vehicle was administered by gavage to diabetic mice for 3 weeks. Metabolic parameters, plasma aldosterone levels, and parameters related to renal function were measured. Endothelium-dependent or -independent vascular responses of the aortic segments were analyzed with acetylcholine or sodium nitroprusside, respectively. Human umbilical vein endothelial cells (HUVECs) were used for the in vitro study.

Results: Induction of diabetes elevated plasma aldosterone level ($P<0.05$) and impaired endothelium-dependent vascular relaxation ($P<0.05$). The administration of esaxerenone ameliorated the endothelial dysfunction ($P<0.01$) without the alteration of metabolic parameters, blood pressure, and renal function. Esaxerenone improved the $eNOS^{Ser1177}$ phosphorylation in the aorta obtained from diabetic mice ($P<0.05$) compared with that in the vehicle-treated group. Furthermore, a major MR agonist, aldosterone, decreased $eNOS^{Ser1177}$ phosphorylation and increased $eNOS^{Thr495}$ phosphorylation in HUVECs, which recovered with esaxerenone. Esaxerenone ameliorated the endothelium-dependent vascular relaxation caused by aldosterone in the aortic segments obtained from C57BL/6 mice ($P<0.001$).

Conclusion: Esaxerenone attenuates the development of diabetes-induced endothelial dysfunction in mice. These results suggest that esaxerenone has potential vascular protective effects in individuals with diabetes.

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Key words: Esaxerenone, Endothelial dysfunction, Mineralocorticoid receptor, Aldosterone

Abbreviation list: Ach: acetylcholine, ANOVA: analysis of variance, BUN: blood urea nitrogen, CVD: cardiovascular diseases, eNOS: endothelial nitric oxide synthase, HUVEC: human umbilical vein endothelial cells, MR: mineralocorticoid receptors, MRAs: mineralocorticoid receptor antagonists, NO: nitric oxide, SEM: standard error of the mean, SNP: sodium nitroprusside, STZ: streptozotocin.

Introduction

Despite accumulating knowledge and advancing therapeutics, cardiovascular disease is still responsible for a large proportion of mortality worldwide[1]. The pathophysiological role of aldosterone in cardiovascular disease has been demonstrated[2]. Volume expansion and/or a hypertensive effect via mineralocorticoid receptors (MRs) expressed in the kidney is its potent underlying mechanism[3]. Moreover, elevated plasma

aldosterone levels correlate with increased mortality. Pharmacological blockade of MRs significantly reduced morbidity, improved survival in patients with heart failure, and decreased hospitalization in postmyocardial infarction in several clinical trials[4-6]. Extrarenal effects of mineralocorticoid receptor antagonists (MRAs) have recently gained attention. Several studies have been conducted to clarify the effects of MRAs on other tissues/organs, such as the heart, vessels, and metabolic organs[7].

Endothelium dysfunction is a major underlying pathophysiology of cardiovascular complications in diabetic patients[8, 9]. Recent clinical studies have shown that MRs play a crucial role in cardiovascular regulation, particularly in the development of vascular dysfunction in diabetic patients[10, 11]. Moreover, MRAs reverse this vascular complication in diabetic individuals[12-15]. Preclinical studies also show that the diabetic condition impairs endothelium-dependent vasodilation, and MRAs prevent it by increasing $eNOS^{Ser1177}$ phosphorylation and reducing oxidative stress in mice aorta[16-21].

Spironolactone and eplerenone are traditionally available MRAs; however, the clinical use of these MRAs is limited because of their relatively low MR selectivity and steroidal structure[22]. Recently, esaxerenone, a new nonsteroidal MR blocker with higher potency and selectivity to MR, was introduced in Japan[23]. Several clinical and preclinical studies have reported the great antihypertensive and renoprotective effects of esaxerenone compared with spironolactone or eplerenone. Pharmacological studies clarified that esaxerenone has more than 1,000-fold affinity to MRs over other NR3C nuclear receptors due to its flipped side chain constructure. Moreover, the binding site of esaxerenone is larger and intruded into the protein core. Therefore, the suppressive effect is more potent and longer lasting than those of spironolactone and eplerenone[24-28]. However, the effect of esaxerenone on vascular function in diabetes has not been fully investigated. Thus, here, we investigated whether esaxerenone ameliorates diabetes-induced endothelial dysfunction in diabetic mice.

2. Methods

2.1. Animals and Drug Administration

C57BL/6J wild-type mice were obtained from Japan SLC, Inc. Esaxerenone was supplied by Daiichi Sankyo Co., Ltd., Japan. Eight-week-old male mice were injected with a single dose of streptozotocin (STZ, 150 mg/kg, Santa Cruz) or vehicle intraperitoneally to examine the effect on diabetes-induced endothelial dysfunction. Three days after the injection, diabetic mice were randomly divided into esaxerenone (3 mg/kg/day) or vehicle (carboxymethyl cellulose) groups and treated by oral gavage once daily for three weeks. The *ex vivo* vascular reactivity assay used aortic segments obtained from 8-week-old male C57BL/6J mice. Mice were maintained under controlled temperature (23℃ ± 1℃) with a 12-h artificial light and dark cycle. All experimental procedures conformed with the guidelines for animal experimentation of the Tokushima University. The Animal Care and Use Committee of Tokushima University reviewed and approved the protocol under #T2020-127.

2.2. Measurement of Plasma Aldosterone Levels and Metabolic Parameters

Blood pressure was measured by a tail-cuff system in conscious mice (Softron). At the time of sacrifice, blood was collected from the heart. Plasma was separated by centrifugation (9,000 rpm for 15 min) at 4℃ and stored until further analyses at −80℃. Plasma lipid levels (total cholesterol, high density lipoprotein cholesterol, and triglyceride), blood urea nitrogen (BUN), creatinine, and glycoalbumin were measured at the Sanritsu Zelkova examination center (Japan). Plasma aldosterone level was measured by using a commercial available kit according to the manufacturer's recommendations (R&D Systems, Inc., USA).

2.3. Vascular Reactivity Assay

Vascular reactivity was analyzed as previously documented[29]. After three weeks of esaxerenone administration, the descending thoracic aorta was isolated and cut into 2-mm rings with special care. The aortic segments were mounted between two parallel wires in the organ bath filled with modified Krebs–Henseleit buffer (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11.1 mM glucose) that was aerated (95% O_2 and 5% CO_2) and warmed (37℃). Changes in isometric tension were recorded on a polygraph (LabChart). After 60 min of stabilization, the aortic segments were exposed to 31.4 mM KCl. Endothelial relaxation was then assessed with acetylcholine (Ach; 10^{-9}–10^{-4} M) in the aortic

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Table 1. Effect of esaxerenone on metabolic parameters after 3 weeks of treatment

	CTRL	STZ	Esax	*P*-value
Body weight, g	25.7 ± 0.5†††	22.7 ± 0.7	22.1 ± 0.3	< 0.001
Blood glucose, mg/dl	136.4 ± 3.9†††	563.0 ± 42.8	658.3 ± 51.8	< 0.001
Systolic blood pressure, mmHg	105.1 ± 1.9	109.2 ± 4.1	106.6 ± 2.1	0.59
Diastolic blood pressure, mmHg	69.6 ± 2.3	63.5 ± 4.1	66.1 ± 2.3	0.37
Total-cholesterol, mg/dl	105.6 ± 2.7††	161.8 ± 15.7	144.4 ± 11.1	0.004
Triglycerides, mg/dl	145.5 ± 14.2††	408.0 ± 65.1	353.9 ± 61.2	0.003
HDL-cholesterol, mg/dl	59.7 ± 2.0†	77.5 ± 7.7	77.5 ± 4.8	0.04
Aldosterone, pg/ml	293.7 ± 42.2†	696.2 ± 108.0	1244.6 ± 179.9††	< 0.001
BUN, mg/dl	29.4 ± 1.0	34.7 ± 2.2	34.6 ± 3.4	0.20
Creatinine, mg/dl	0.15 ± 0.004††	0.11 ± 0.008	0.13 ± 0.006	0.004
Glycoalbumin, mg/dl	3.1 ± 0.2†††	14.6 ± 0.9	15.0 ± 0.5	< 0.001
Food intake, g/day/head	2.8 ± 0.3††	5.5 ± 0.4	6.0 ± 0.6	0.004

CTRL: non-diabetic control, Esax: esaxerenone, HDL: high density lipoprotein, STZ: streptozotocin.
All values are mean ± SEM. †; $P<0.05$, ††; $P<0.01$, †††; $P<0.001$ vs. STZ group

segments, previously contracted by phenylephrine (60% of maximum). Endothelium-independent relaxation was examined with increasing concentrations of sodium nitroprusside (SNP; 10^{-9}–10^{-4} M). An *ex vivo* experiment was performed with the same protocol. The aortic rings used in the *ex vivo* experiment were treated with 1,000 nM aldosterone (Sigma–Aldrich) with or without 30-min pretreatment with 10 nM esaxerenone.

2.4. Cell Culture Experiments

Human umbilical vein endothelial cells (HUVECs) were purchased from Life Technologies and cultured in EGM-2 (Lonza). HUVECs (passages 4–6) were stimulated with aldosterone for 3 h in EBM-2 (Lonza) containing 2% charcoal/dextran-treated fetal bovine serum (Cytiva) with or without 4-h pretreatment with 10 nM esaxerenone.

2.5. Western Blot Analysis

Cells and tissues were lysed with RIPA buffer (Wako Pure Chemical Industries, Ltd.) containing a protease inhibitor cocktail (Takara Bio Inc.) and phosphatase inhibitors (Roche) on ice. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (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℃ with primary antibody against either phosphorylated-eNOS^{Ser1177}, phosphorylated-eNOS^{Thr495}, eNOS (BD Biosciences), phosphorylated-Akt^{Ser473}, Akt (Cell Signaling Technology), or β-actin (Sigma). After five washings with TBS-T buffer, each membrane was incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. The signal was detected using a luminescent image analyzer (LAS-1000, Fuji Film) with ECL-plus reagent (GE Healthcare). The ratio of phosphorylated-eNOS^{Ser1177} to phosphorylated-eNOS^{Thr495} was calculated as another marker for endothelial function[30].

2.6. Statistical Analysis

All numerical values are expressed as means ± standard error of the mean (SEM). An unpaired Student's *t*-test analyzed the parameter comparisons between the two groups. Differences between multiple groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Comparison of dose-response curves was performed by two-factor repeated measures ANOVA, followed by Dunnett's post hoc test for comparison between groups, and *P*-value <0.05 was considered significant.

3. Results

3-1. Effect of Esaxerenone on Metabolic Parameters

Diabetes induction by STZ significantly increased the blood glucose level, which was accompanied with elevation of plasma glycoalbumin level. Induction of diabetes by STZ also elevated the plasma lipid levels as in previous studies[31]. The induction of diabetes increased the plasma aldosterone level, which was further enhanced by esaxerenone treatment. In this study, esaxerenone did not lower blood pressure in diabetic mice. Furthermore, esaxerenone did not affect blood lipid levels, glucose levels, renal functions as determined by creatinine and BUN levels, and body weight in diabetic mice. These data are summarized in **Table 1**.

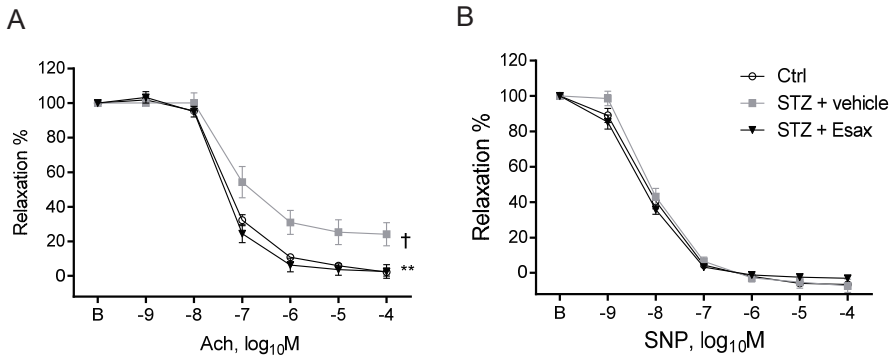

Fig. 1. Esaxerenone administration attenuated endothelial dysfunction in diabetic mice

Endothelium-dependent or -independent vascular relaxation to Ach (A) and SNP (B), respectively, was determined in the aortic segments of nondiabetic mice and diabetic mice treated with esaxerenone or vehicle. (A) Induction of diabetes by STZ injection impaired endothelium-dependent vascular relaxation compared with that in nondiabetic mice. Esaxerenone treatment ameliorated this response. (B) There was no difference in the endothelium-independent vascular response between the three groups: n=9–11 (per group). †; $P<0.05$ vs. nondiabetic group, **$P<0.01$ vs. vehicle-treated group. Ctrl, nondiabetic control, Esax, esaxerenone. All values are means ± SEM

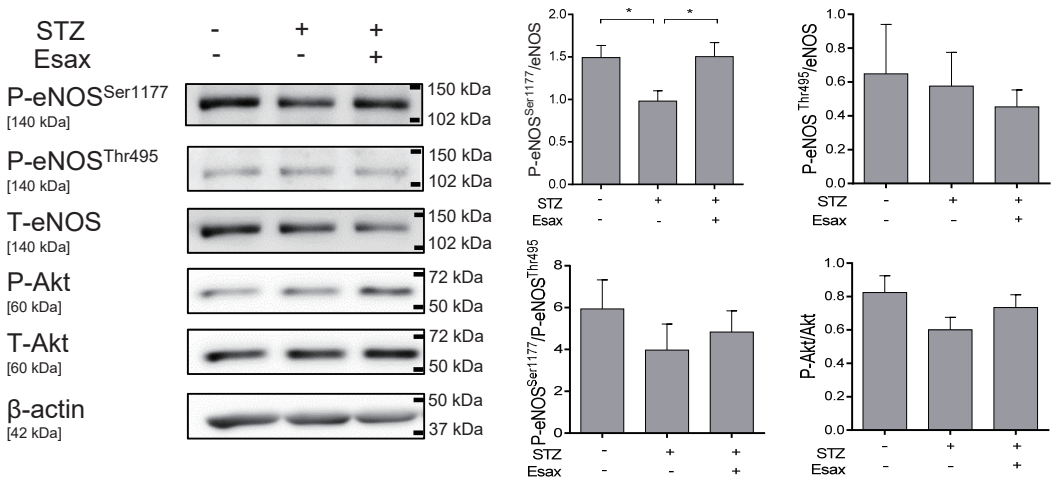

Fig. 2. Esaxerenone administration restored eNOS^{Ser1177} phosphorylation in diabetic mice

Western blot analysis demonstrated that induction of diabetes by STZ decreased eNOS^{Ser1177} phosphorylation in the aorta while esaxerenone treatment restored this effect. The induction of diabetes and esaxerenone did not significantly affect eNOS^{Thr495} and Akt phosphorylation in our mice. n=11 (per group). *$P<0.05$ vs. vehicle-treated diabetic group. Esax, esaxerenone. All values are means ± SEM.

3.2. Esaxerenone Ameliorated the Endothelial Dysfunction in Diabetic Mice

The vascular reactivity assay, which used aortic rings obtained from our mice, demonstrated that induction of diabetes by STZ impaired vasodilatation in response to Ach, suggesting impairment of endothelial function ($P<0.05$). However, three weeks of esaxerenone administration ameliorated endothelium-dependent vascular dysfunction **(Fig. 1A)** ($P<0.01$). Conversely, in our experiment, endothelium-independent vasodilation in response to SNP did not differ among the groups **(Fig. 1B)**. eNOS^{Ser1177} phosphorylation was reduced in diabetic mice ($P<0.05$), whereas esaxerenone treatment restored this response ($P<0.05$). In this experiment, we did not observe significant effects of esaxerenone on eNOS^{Thr495} and Akt phosphorylation **(Fig. 2)**.

3.3. Esaxerenone Improved the Aldosterone-Induced Impairment of eNOS Phosphorylation in HUVECs

In the *in vivo* experiments, esaxerenone abolished the endothelial dysfunction by improving eNOS^{Ser1177} phosphorylation. Therefore, HUVECs were used to elucidate the underlying mechanism. Aldosterone significantly reduced eNOS^{Ser1177} phosphorylation in a dose-dependent manner and increased eNOS^{Thr495}

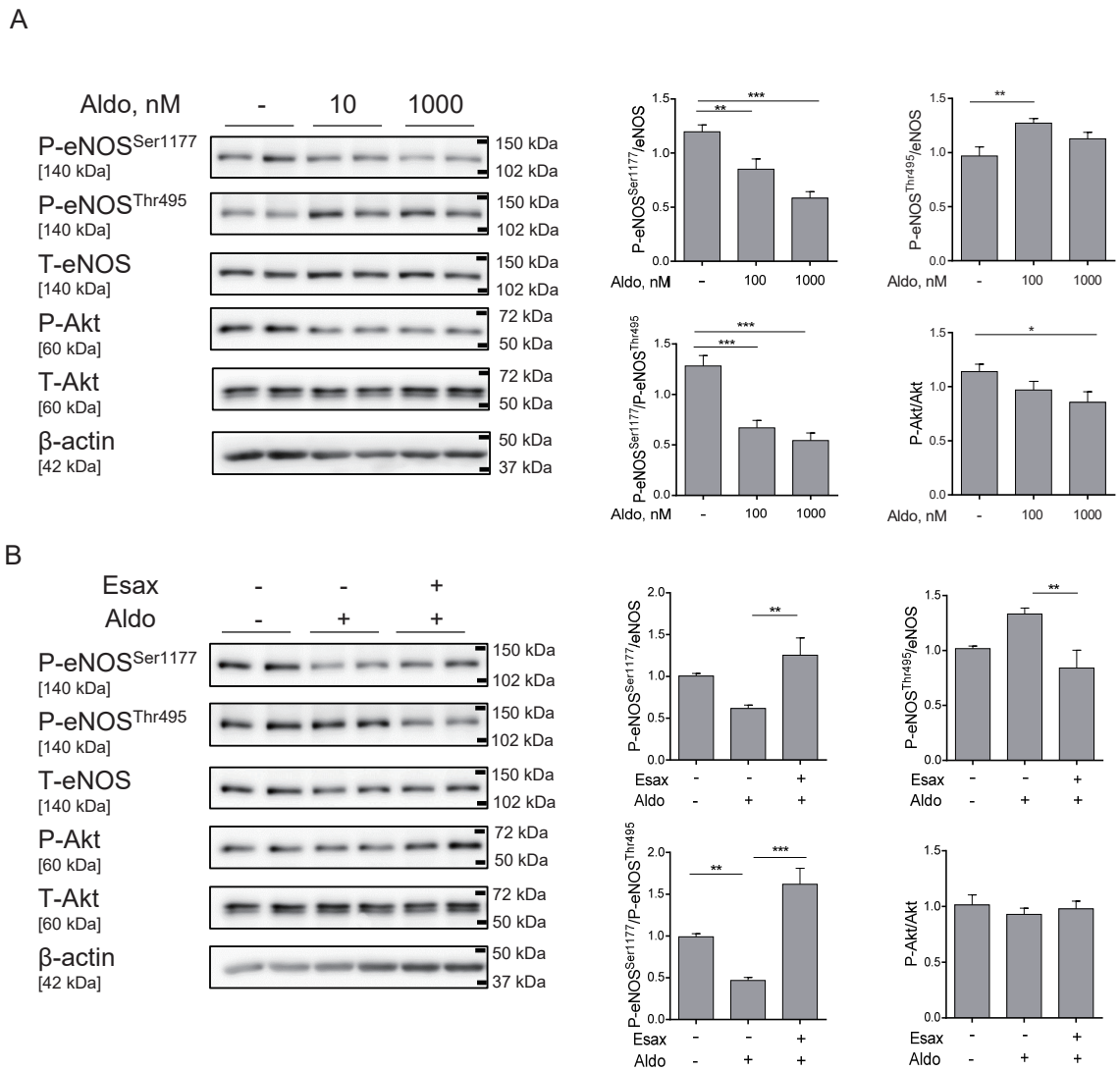

Fig. 3. Esaxerenone ameliorated aldosterone-induced impairment of eNOS phosphorylation in HUVECs

(A) Aldosterone attenuated eNOS^{Ser1177} and Akt phosphorylation and promoted eNOS^{Thr495} phosphorylation in HUVECs. According to this, the eNOS^{Ser1177}/eNOS^{Thr495} phosphorylation ratio was decreased by aldosterone in a dose-dependent manner (n=8, per group). (B) Pretreatment with esaxerenone attenuated the decrease in eNOS^{Ser1177} phosphorylation and inhibited the increase in the phosphorylation of eNOS^{Thr495} phosphorylation induced by aldosterone. The eNOS^{Ser1177}/ eNOS^{Thr495} phosphorylation ratio recovered in the presence of esaxerenone (n=8, per group). *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$. Aldo, aldosterone, NT, nontreatment, Esax, esaxerenone. All values are means ± SEM.

phosphorylation **(Fig. 3A)**. In addition, another marker for endothelial function, the eNOS^{Ser1177}/eNOS^{Thr495} phosphorylation ratio, decreased with aldosterone treatment ($P<0.001$). Akt phosphorylation also decreased in the presence of aldosterone. However, the pretreatment with esaxerenone increased eNOS^{Ser1177} phosphorylation ($P<0.01$) and the eNOS^{Ser1177}/eNOS^{Thr495} phosphorylation ratio ($P<0.001$) and decreased the eNOS^{Thr495} phosphorylation ($P<0.01$) **(Fig. 3B)**.

3.4. Esaxerenone Reduced the Aldosterone-Induced Vascular Dysfunction in the Aortic Segments

The direct effect of aldosterone on vascular relaxation was examined by vascular reactivity assay using aortic rings obtained from C57BL/6 mice. Aldosterone impaired Ach-induced vasodilatation ($P<0.05$) while preincubation with esaxerenone attenuated this impairment ($P<0.001$) **(Fig. 4A)**. Both esaxerenone and aldosterone did not affect the SNP-induced vasodilation **(Fig. 4B)**.

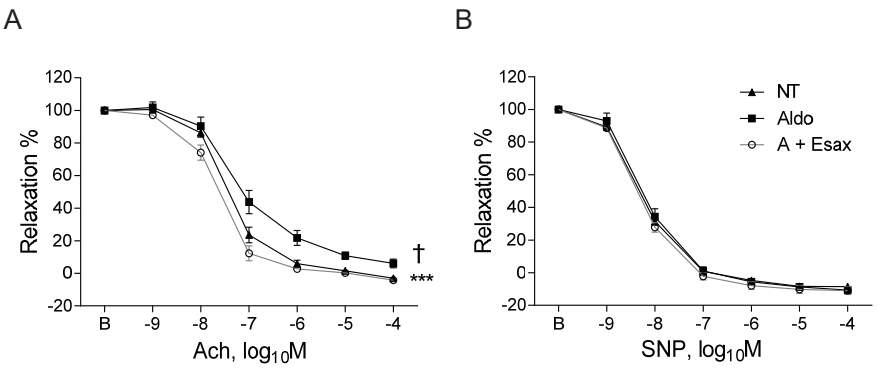

Fig. 4. Esaxerenone ameliorated endothelium-dependent vascular relaxation impaired by aldosterone

(A) Aldosterone significantly impaired endothelium-dependent vascular relaxation in the aortic segments obtained from C57BL/6 mice as determined by the response to Ach. Simultaneously, pretreatment with esaxerenone ameliorated aldosterone-induced endothelial dysfunction. (B) Both aldosterone and esaxerenone did not affect vascular response to SNP. $n=8$ (per group). †; $P<0.05$ vs. NT group, ***; $P<0.001$ vs. Aldo group. NT, nontreatment, Aldo, aldosterone, Esax, esaxerenone. All values are means ± SEM.

4. Discussion

Our results showed that MR blockade by esaxerenone ameliorated Ach-induced vascular relaxation by enhancing eNOS^{Ser1177} phosphorylation that was impaired by the induction of diabetes by STZ without affecting blood pressure, renal function, and metabolic parameters in C57BL/6 mice. Neither the induction of diabetes nor esaxerenone affected SNP-induced vascular relaxation in our condition. *In vitro* experiments using HUVECs demonstrated that aldosterone decreased eNOS^{Ser1177} phosphorylation, which was recovered in the presence of esaxerenone. These results suggest that esaxerenone ameliorates diabetes-induced endothelial dysfunction.

Vascular dysfunction is a primary contributor to CVD-associated mortality and morbidity in diabetic individuals[32-34]. Identical with our findings, preclinical and clinical studies have demonstrated that aldosterone plasma levels, a major agonist of MR, lead to an increase in diabetes[10, 11, 35]; further, MRAs ameliorated vascular dysfunction in diabetic models[15, 20, 35]. This supports the concept that MR antagonism with esaxerenone is a potential vasoprotective treatment in diabetes.

In this study, we used esaxerenone, a recently approved MR blocker in Japan. Esaxerenone has higher MR-binding specificity and nonsteroidal structure[23]. Previous preclinical and clinical studies have demonstrated the superior potency of esaxerenone to that of spironolactone or eplerenone for treating hypertension[23, 24]. The extrarenal effects of MR antagonists have been gaining attention. Among them, several studies have shown the antiatherosclerotic effects of MRAs targeting vascular cells[36, 37]. Endothelium dysfunction is a major underlying pathophysiology of cardiovascular complications in diabetic patients[8, 9]. The prevention of endothelial dysfunction is indispensable to avoid vascular complications. Therefore, we focused on the effects of esaxerenone on vascular function in diabetes. Previous studies have already demonstrated that both spironolactone and eplerenone prevent endothelial dysfunction in diabetes[15, 35]. However, the use of traditionally available MRAs is limited, owing to their relatively low selectivity and steroidal structure[22]. In this study, esaxerenone clearly had protective effects on endothelial function in diabetic mice. Esaxerenone also restored decreased eNOS^{Ser1177} phosphorylation caused by STZ injection in the aorta. In our *in vitro* experiments, aldosterone abolished the eNOS^{Ser1177} phosphorylation and promoted eNOS^{Thr495} phosphorylation in HUVECs. The presence of esaxerenone inhibited these effects. Further, these results suggest that esaxerenone increased nitric oxide (NO) production in this cell type, leading to the improvement of endothelial function. Previous studies have reported that aldosterone infusion promoted NAD(P)H oxidase activity, promoting oxidative stress[38, 39]. Moreover, aldosterone is suggested to affect NO production and bioavailability through various pathways[16-18, 40-45]. By contrast, previous studies have reported that MRAs reduce vascular dysfunction by promoting eNOS activity and NO bioavailability in diabetic mice[14, 15]. These results were confirmed by a study that used endothelium-specific MR-deleted mice[35]. NO is a principal vascular tone regulator synthesized primarily by eNOS in endothelial cells[46]. Diabetes-associated eNOS dysfunction has also been known. Therefore, esaxerenone administration could be a potential strategy for this central mechanism of diabetic vascular complications. To the best of our

knowledge, this is the first study to report the protective effects of esaxerenone on diabetes-induced endothelial function. Previous studies have demonstrated amelioration of endothelial dysfunction by traditionally available MRAs; however, esaxerenone is expected to have more beneficial effects because of its higher affinity to MR and nonsteroidal structure. Further studies are needed to clarify the underlying mechanisms of esaxerenone for vascular protection.

Esaxerenone did not lower blood pressure in our study condition. Previous studies suggest that the effects of MRAs on blood pressure depend on the mouse model[47]. In mice given a high-salt diet or water, MRAs, including esaxerenone, lowered blood pressure; however, in mice kept under normal salt conditions, MRAs did not affect blood pressure. In fact, in diabetic mice, esaxerenone and other MRAs did not lower blood pressure[15, 48, 49]. The present study is consistent with the previous studies, indicating that the effects of esaxerenone on endothelium are independent of blood pressure, at least partially. The results of our *in vitro* studies, which demonstrated a direct effect of aldosterone and esaxerenone on HUVECs, partially support this finding.

This study has several limitations. First, we injected STZ to induce diabetes. This model is widely used for diabetic research; however, this model does not completely represent type 2 diabetes, a common pattern of diabetes. Second, the aldosterone dose used in this study was higher than in human hyperaldosteronism. However, previous studies used similar doses to show its effects on eNOS in HUVECs[16, 18]. Third, in this study, esaxerenone did not lower blood pressure in our diabetic mice. Esaxerenone is approved for the treatment of patients with hypertension in Japan. In future, further studies using hypertension models may be needed when we consider the clinical setting.

In conclusion, our data demonstrated that esaxerenone administration ameliorates diabetes-induced impairment of endothelial function by promoting eNOS phosphorylation in diabetic mice. Moreover, aldosterone-induced vascular dysfunction was ameliorated by esaxerenone in the aortic segments and HUVECs. Our results strengthen the concept that MR plays a crucial role in vascular dysfunction, and MR blockade by esaxerenone is a promising therapeutic approach for vascular dysfunction in diabetes.

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Competing Interests

The authors declare that they have no conflict of interest. Esaxerenone was supplied by Daiichi Sankyo Co., Ltd.

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