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LCZ696, an Angiotensin Receptor-Neprilysin Inhibitor, Ameliorates Endothelial Dysfunction in Diabetic C57BL/6 Mice

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Aims: LCZ696 (sacubitril/valsartan) exerts cardioprotective effects. Recent studies have suggested that it improves the endothelial function; however, the underlying mechanisms have not been thoroughly investigated. We investigated whether LCZ696 ameliorates diabetes-induced endothelial dysfunction.

Methods: Diabetes was induced using streptozotocin in 8-week-old male C57BL/6 mice. Diabetic mice were randomly assigned to receive LCZ696 (100 mg/kg/day), valsartan (50 mg/kg/day), or a vehicle for three weeks. The endothelium-dependent and endothelium-independent vascular responses of the aortic segments were determined based on the response to acetylcholine and sodium nitroprusside, respectively. Human umbilical vein endothelial cells (HUVEC) and aortic segments obtained from C57BL/6 mice were used to perform *in vitro* and *ex vivo* experiments, respectively.

Results: LCZ696 and valsartan reduced the blood pressure in diabetic mice ($P<0.05$). The administration of LCZ696 ($P<0.001$) and valsartan ($P<0.01$) ameliorated endothelium-dependent vascular relaxation, but not endothelium-independent vascular relaxation, under diabetic conditions. LCZ696, but not valsartan, increased $eNOS^{Ser1177}$ ($P=0.06$) and Akt ($P<0.05$) phosphorylation in the aorta. In HUVEC, methylglyoxal (MGO), a major precursor of advanced glycation end products, decreased $eNOS^{Ser1177}$ phosphorylation ($P<0.05$) and increased $eNOS^{Thr495}$ phosphorylation ($P<0.001$). However, atrial natriuretic peptide (ANP) reversed these effects. ANP also ameliorated the MGO-induced impairment of endothelium-dependent vascular relaxation in the aortic segments ($P<0.05$), although L-NAME completely blocked this effect ($P<0.001$).

Conclusion: LCZ696 ameliorated diabetes-induced endothelial dysfunction by increasing the bioavailability of ANP. Our findings suggest that LCZ696 has a vascular protective effect in a diabetic model and highlight that it may be more effective than valsartan.

Key words: Diabetes, Sacubitril/valsartan, Natriuretic peptide, Endothelial dysfunction

Abbreviation list: ACE, angiotensin-converting enzyme; Ach, acetylcholine; AGEs, advanced glycation end-products; ANOVA, analysis of variance; ANP, atrial natriuretic peptide; ARB, angiotensin receptor blocker; BP, blood pressure; cGMP, cyclic guanosine monophosphate; CVD, cardiovascular disease; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; L-NAME, N-nitro-L-arginine methyl ester; MGO, methylglyoxal; NO, nitric oxide; NP, natriuretic peptide; RAAS, renin-angiotensin-aldosterone system; SNP, sodium nitroprusside; STZ, streptozotocin.

Introduction

Endothelial dysfunction is fundamental to the development of cardiovascular disease[1-3]. Shear stress, hyperglycemia, and increased oxidative stress on the endothelium are known to trigger the inappropriate activation of the renin-angiotensin-aldosterone system (RAAS), accompanied by suppression of the

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natriuretic peptide system, thus leading to an impaired endothelial function. Nitric oxide (NO), which is predominantly synthesized in the endothelium, is not only a well-known vasodilator, but it is also recognized as a crucial antiplatelet, antithrombotic, and anti-inflammatory vasoprotective agent[4-6].

Over the past few decades, RAAS blocking agents, including ACE inhibitors, ARBs, and combination treatment with ACE and neprilysin inhibitors (such as omapatrilat) have been extensively used for the treatment of hypertension and cardiovascular disease[7]. While omapatrilat has shown superior protective effects compared to ACE inhibitors, its use has been limited because of the occurrence of severe angioedema caused by suppressed bradykinin degeneration[8, 9].

LCZ696, also known as sacubtril/valsartan, is a first-in-class dual-inhibiting drug comprising valsartan, an angiotensin II receptor blocker, and sacubitril, a neprilysin inhibitor responsible for the degradation of natriuretic peptides (NPs), in a 1:1 molar ratio. LCZ696 increases not only atrial natriuretic peptide (ANP) and plasma and urinary cGMP but also the angiotensin II level, although the functions of angiotensin II are blocked by valsartan[10]. In the Global Mortality and Morbidity in Heart Failure (PARADIGM-HF) study, LCZ696 has shown superiority to the ACE inhibitor enalapril, and the results were consistent in diabetic subgroups[11, 12]. In addition, the systolic and diastolic blood pressure (BP) reduction was significantly greater in the LCZ696 200 and 400 mg daily treated groups than in the valsartan 160 and 320 mg daily treated groups, as demonstrated in a double-blind randomized (PARAMOUNT) trial[13].

Pre-clinical studies have demonstrated LCZ696 to have a protective effect on hypertension[14], cardiac fibrosis[15], hypertrophy[16], inflammation[17], and vascular dysfunction[14, 18]. The administration of LCZ696 improved endothelium-dependent vascular relaxation in response to Ach in spontaneously hypertensive rats fed a high-salt diet, while valsartan treatment failed to ameliorate it. Interestingly, LCZ696 did not affect endothelium-independent relaxation[14]. Furthermore, LCZ696 demonstrated superiority to valsartan in promoting vascular relaxation by increasing NO bioavailability in heart failure-induced rats after 8 weeks of treatment[18]. These studies suggest that LCZ696 increased NP bioavailability, which might result in a better endothelial function and cardioprotective effects through NO bioavailability at least partially[19-21].

However, there is a notable gap in the existing literature regarding the specific influence of LCZ696 on the endothelium-dependent function, specifically in the context of endothelial NO synthesis and its underlying mechanisms during hyperglycemia. Therefore, the present study aimed to test the hypothesis that treatment with LCZ696 ameliorates diabetes-induced endothelial dysfunction by promoting eNOS activity.

2. Methods

2.1. Animals and Drug Administration

C57BL/6J wild-type mice were purchased from Japan SLC, Inc (Japan). LCZ696 and valsartan were supplied by Novartis Pharma AG (Basel, Switzerland). Eight-week-old male mice were intraperitoneally injected with a single dose of either streptozotocin (STZ, 180 mg/kg, Santa Cruz) or vehicle to induce diabetes. Three days after the injection, diabetic mice were randomly divided into LCZ696 (100 mg/kg/day), valsartan (50 mg/kg/day), and vehicle groups, and the drugs were added to the drinking water for three weeks. These doses were chosen based on previous studies, in which the same doses of LCZ and valsartan were used without observing any severe adverse effects in mice[22, 23]. Aortic segments obtained from 8-week-old male C57BL/6J mice were used for an *ex vivo* vascular reactivity assay. Mice were maintained under a controlled temperature (23 ± 1℃) with a 12-h artificial light and dark cycle. All experimental procedures conformed to the guidelines for animal experimentation at Tokushima University. The Animal Care and Use Committee of Tokushima University reviewed and approved the study protocol under #T2020-127.

2.2. Measurement of the Blood Pressure and Metabolic Parameters

The BP was measured using a tail-cuff system in conscious mice (Softron). At the time of sacrifice, blood was collected from the heart into EDTA-containing tubes, and plasma was separated by centrifugation (9000 rpm for 15 min) at 4℃ and stored until further analyses at −80℃. Plasma total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were measured at Sanritsu Zelkova Examination Center (Japan).

2.3. Vascular Reactivity Assay

Vascular reactivity was analyzed as previously documented[24]. After three weeks of LCZ696 and valsartan administration, the descending thoracic aorta was isolated and cut into 2-mm rings with special care taken to preserve the endothelium and mounted in organ baths filled with modified Krebs-Henseleit buffer (118.4 mM NaCl, 4.7 mM KCl, 2.5

Table 1. Metabolic parameters after 3 weeks of treatment

	CTRL	STZ	LCZ	VAL
Blood glucose, mg/dl	144.3 ± 4.7***	723.3 ± 23.5	654.8 ± 26.5	691.9 ± 48.3
Heart rate, bpm	691 ± 11***	474 ± 23	536 ± 19*	479 ± 15
Systolic blood pressure, mmHg	103.3 ± 1.8	103.3 ± 4.2	91.0 ± 2.4*	93.0 ± 3.0*
Diastolic blood pressure, mmHg	69.4 ±2.0**	54.6 ± 3.2	45.1 ± 2.9	48.8 ± 4.6
Total-cholesterol, mg/dl	110.9 ± 2.4***	354.8 ± 20.5	383.4 ± 46.0	392.5 ± 34.7
Triglycerides, mg/dl	122.7 ± 11.0*	320.2 ± 57.7	415.9 ± 92.5	384.0 ± 117.2
HDL-cholesterol, mg/dl	60.4 ± 1.5***	128.8 ± 7.4	98.5 ± 12.2*	127.5 ± 10.7

CTRL, non-diabetic control; STZ, non-treated diabetic group; LCZ, LCZ696-treated group. VAL, valsartan-treated group; HDL, high-density lipoprotein. *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$ vs. STZ group. All values are presented as mean ± SEM.

mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose) that was aerated (95% O_2 and 5% CO_2) and warmed (37℃). The changes in isometric tension were recorded using a polygraph (LabChart). Aortic rings were primed with 31.4 mM KCl and then pre-contracted with phenylephrine, producing a submaximal (60% of maximum) contraction. After a plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach; 10^{-9} to 10^{-4} M) and sodium nitroprusside (SNP; 10^{-9} to 10^{-4} M) to obtain cumulative concentration–response curves. The endothelium-dependent and endothelium-independent vascular reactivity was analyzed in response to Ach and SNP, respectively. In our *ex vivo* experiment, aortic segments were incubated with 1mM methylglyoxal (MGO) with or without 100 nM ANP and 100 μM N-nitro-l-arginine methyl ester (l-NAME) for 1 h before examining vascular reactivity.

2.4. Cell Culture Experiments

Human umbilical vein endothelial cells (HUVEC) were purchased from Life Technologies and cultured in EGM-2 medium (Lonza). HUVEC (passages 4–6) were stimulated with 500 μM MGO for 30 min in the presence or absence of 10 nM ANP in EBM-2 (Lonza) containing 2% FBS (Cytiva).

2.5. Western Blot Analysis

Radioimmunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail and phosphatase inhibitors was used to prepare the protein lysates of HUVEC or aortic tissue. 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 for 1 h at room temperature, the membranes were incubated overnight at 4℃ with primary antibodies against phosphorylated-eNOS^{Ser1177}, phosphorylated-eNOS^{Thr495}, eNOS (BD Biosciences), phosphorylated-Akt^{Ser473}, Akt (Cell Signaling Technology), or β-actin (Sigma). After five washes with TBS-T buffer, each membrane was incubated with horseradish peroxidase-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).

2.6. Statistical Analysis

All numerical values are expressed as the mean ± SEM. The unpaired Student's *t*-test was used to compare the parameters between the two groups. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple comparisons test. Two-way ANOVA followed by Dunnett's multiple comparison test was used to compare dose-response curves. Statistical significance was set at $p<0.05$.

3. Results

3-1. Effect of LCZ696 on the Metabolic Parameters

The blood glucose and lipid levels significantly increased in STZ-induced diabetic mice. After 3 weeks of administration, systolic BP was lower in the LCZ696 and valsartan groups than in the STZ group, whereas there was no difference between the LCZ696 and valsartan groups **(Table 1)**.

3.2. LCZ696 Attenuated Endothelial Dysfunction in Diabetic Mice

The induction of diabetes by STZ impaired endothelial relaxation in response to Ach compared to that in the non-diabetic control group ($P<0.001$). However, the administration of LCZ696 ($P<0.001$) and valsartan ($P<0.01$) ameliorated endothelium-dependent vascular dysfunction **(Fig. 1A)**, whereas endothelium-independent vasodilation in response to

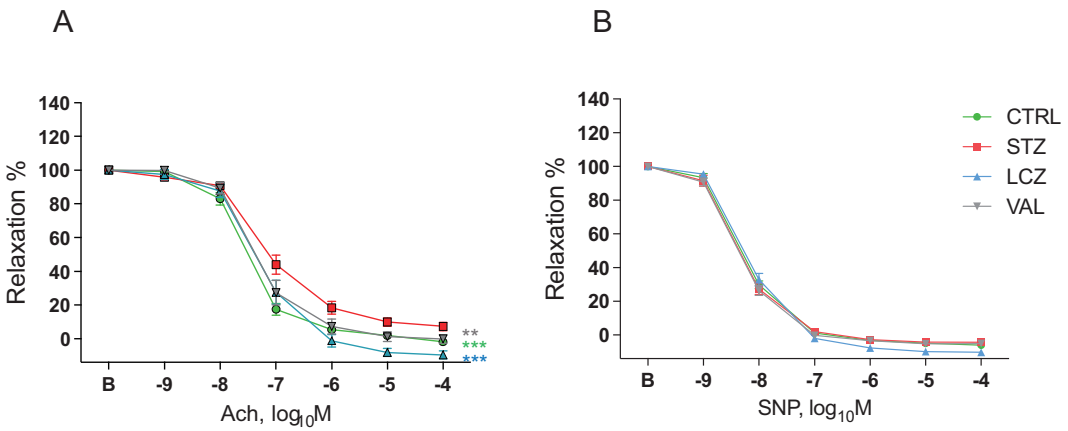

Fig. 1. LCZ696 administration ameliorated endothelial dysfunction in diabetic mice

Endothelium-dependent or -independent vascular relaxation in response to Ach (A) and SNP (B), respectively, was determined in the aortic segments of non-diabetic mice and diabetic mice treated with LCZ696, valsartan, or vehicle. (A) The induction of diabetes by STZ injection impaired endothelium-dependent vascular relaxation compared to non-diabetic mice. Treatment with LCZ696 and valsartan ameliorated this response. (B) There was no difference in the endothelium-independent vascular response among the four groups (n = 12-19, per group). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. STZ group. CTRL: non-diabetic control. STZ: untreated diabetic group. LCZ: LCZ696-treated group. VAL: Valsartan-treated group. All values are presented as the mean ± SEM.

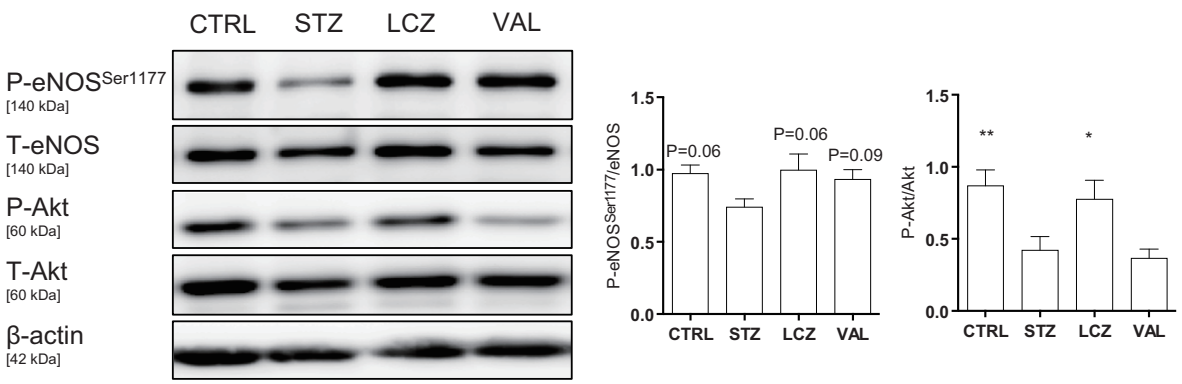

Fig. 2. LCZ696 administration restored eNOS^{Ser1177} phosphorylation in diabetic mice

A western blot analysis demonstrated that the induction of diabetes by STZ decreased eNOS^{Ser1177} and Akt phosphorylation in the aorta, whereas LCZ696 treatment restored such phosphorylation. (n = 12-18, per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. STZ group. CTRL: non-diabetic control. STZ: untreated diabetic group. LCZ: LCZ696-treated group. VAL: Valsartan-treated group. All values are presented as the mean ± SEM.

SNP did not differ among the groups (Fig. 1B). As shown in Fig. 2, Akt phosphorylation in the aorta was reduced in diabetic mice ($P < 0.01$). The administration of LCZ696 restored Akt phosphorylation ($P < 0.05$); however, valsartan did not. A similar tendency was observed for the phosphorylation of eNOS^{Ser1177}; however, no significant difference was observed.

3.3. ANP Improved the Phosphorylation of eNOS and Akt in MGO-Treated HUVEC

Based on the findings of the *in vivo* experiments, we treated HUVEC with ANP and MGO, a major cell-permeant precursor of AGEs, to investigate the mechanism by which LCZ696 ameliorated endothelial dysfunction. We next focused on the effect of the increased bioavailability of ANP that is expected by LCZ696 treatment. Incubation with MGO for 30 min resulted in a reduced phosphorylation of eNOS^{Ser1177} ($P < 0.05$) and Akt ($P < 0.01$), while the phosphorylation of eNOS^{Thr495} increased ($P < 0.001$). Therefore, the eNOS^{Ser1177}/eNOS^{Thr495} ratio decreased in the MGO-treated group. The presence of ANP inhibited MGO-induced elevation of eNOS^{Thr495} ($P < 0.01$) and a decrease in eNOS^{Ser1177} ($P = 0.06$). As a result, the ratio of eNOS^{Ser1177}/eNOS^{Thr495} increased in the ANP-treated group in the presence of MGO ($P < 0.05$), as shown in Fig. 3.

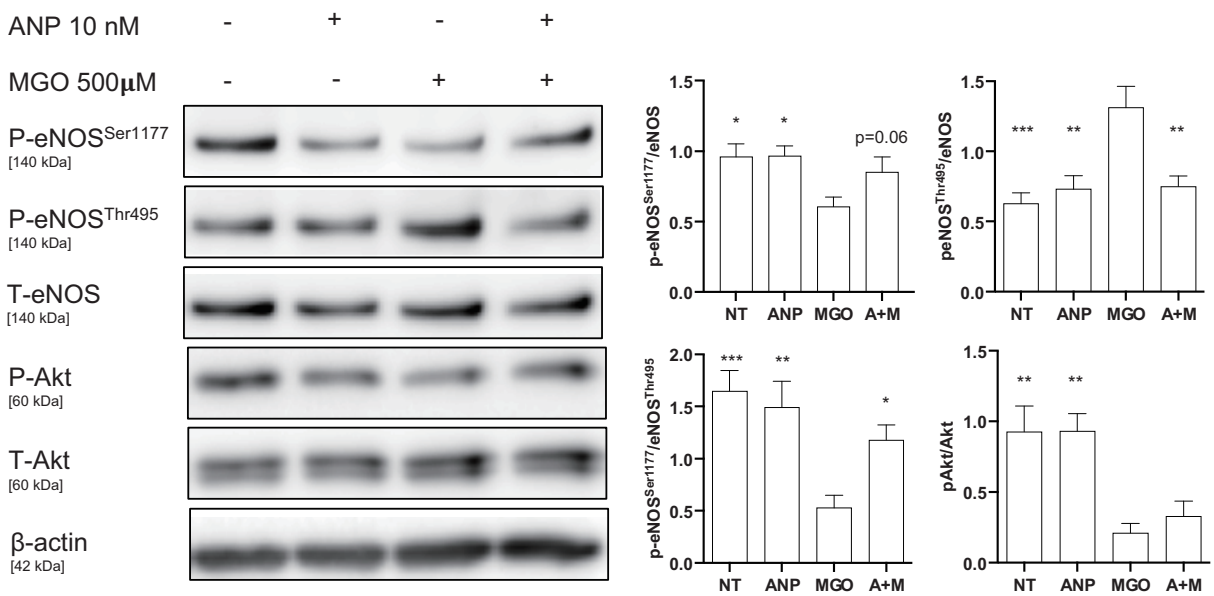

Fig. 3. ANP ameliorated eNOS phosphorylation in MGO-treated HUVEC

Incubation with MGO attenuated eNOS^{Ser1177} and Akt phosphorylation and promoted eNOS^{Thr495} phosphorylation in HUVEC. Therefore, the eNOS^{Ser1177}/eNOS^{Thr495} phosphorylation ratio decreased by MGO treatment ($n=7$ per group). However, ANP tended to increase eNOS^{Ser1177} phosphorylation and significantly decrease the eNOS^{Thr495} phosphorylation induced by MGO. Therefore, the eNOS^{Ser1177}/eNOS^{Thr495} phosphorylation ratio was significantly recovered in the presence of ANP ($n=7$ per group). *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$ vs. MGO-treated group. ANP, ANP-treated group; MGO, MGO-treated group; A+M, combination treatment group with ANP and MGO. All values are presented as the mean ± SEM.

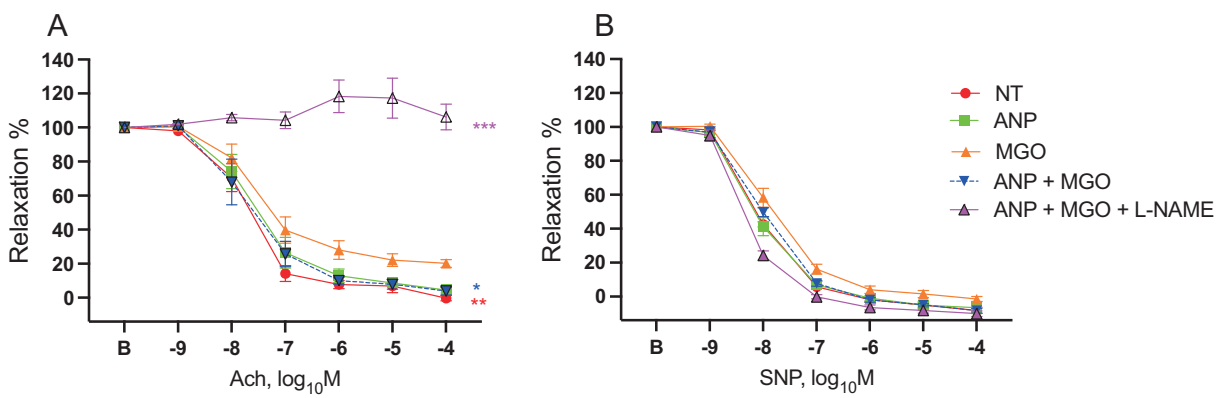

Fig. 4. ANP ameliorated endothelium-dependent vascular relaxation impaired by MGO

(A) MGO significantly impaired the endothelium-dependent vascular relaxation in the aortic segments obtained from C57BL/6 mice. Incubation with ANP ameliorated MGO-induced endothelial dysfunction. However, in the presence of L-NAME, the effect of ANP was abrogated ($n=7$, per group). (B) Both ANP and MGO did not affect the vascular response to SNP ($n=7$, per group). *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$ vs. MGO group. NT: non-treated group, ANP: ANP-treated group, MGO: MGO-treated group, ANP + MGO: combination treatment of ANP and MGO, ANP + MGO + L-NAME: combination treatment of ANP, MGO, and L-NAME. All values are presented as the mean ± SEM.

3.4. ANP Improved Endothelium-Dependent Relaxation in the MGO-Treated Aortic Segments

To assess the impact of ANP on the vascular function, aortic segments were stimulated with MGO in the presence or absence of ANP and examined for vascular reactivity. MGO impaired endothelium-dependent relaxation, as evidenced by the reduced response to Ach compared with the non-treated group ($P<0.01$). However, this impairment was reversed by the presence of ANP ($P<0.05$). In addition, the beneficial effect of ANP on endothelium-dependent relaxation was completely abrogated in the presence of L-NAME ($P<0.001$), as shown in **Fig. 4A**. Nonetheless, ANP, MGO, and L-NAME did not

affect the endothelium-independent vascular function, which was examined in response to SNP, as shown in **Fig. 4B**.

4. Discussion

It is well established that endothelial dysfunction is a primary contributor to the development of cardiovascular disease and it also affects the prognosis of diabetic patients. In this study, the induction of diabetes by STZ injection impaired endothelium-dependent vascular relaxation in response to Ach, along with a decreased phosphorylation of eNOS^{Ser1177} and Akt in the aorta compared to the non-diabetic control group. Although both LCZ696 and valsartan treatment improved endothelial relaxation in response to Ach, LCZ696 showed a greater effect than valsartan. The observed blood pressure reduction was similar for both LCZ696 and valsartan. There was no difference in the response to SNP among the groups. In addition, LCZ696 treatment tended to ameliorate eNOS^{Ser1177} phosphorylation and significantly ameliorated Akt phosphorylation in the aorta of diabetic mice compared to the STZ group, whereas valsartan failed to achieve this. Consistent with our findings, a previous study demonstrated that four weeks of treatment with LCZ696 attenuated high-salt-diet-induced endothelial dysfunction in spontaneously hypertensive rats, whereas valsartan alone failed to ameliorate it. That study also demonstrated no difference in endothelium-independent relaxation with SNP[14]. Moreover, Trivedi *et al.* demonstrated that LCZ696 showed a time-dependent superiority over valsartan in the vascular relaxation responses to both Ach and SNP, which correlated with increased NO bioavailability in both the circulation and myocardium[18]. Thus, these findings suggest that the elevated bioavailability of NPs by neprilysin inhibition during treatment with LCZ696 may have a beneficial impact on NO synthesis and exert superior effects on preventing cardiovascular complications compared with stand-alone ARBs. One clinical study also demonstrated that LCZ696 improved the endothelial function in patients with chronic heart failure with a reduced ejection fraction[25]. In addition, *ex vivo* and *in vitro* experiments that had been conducted in normoglycemic conditions strengthened the concept that the combination of neprilysin inhibitor and ARB treatment has potential benefits in enhancing endothelial NO synthesis as a result of reducing oxidative stress, inflammation, or inhibiting vasoconstrictors such as endothelin-1 and angiotensin II[14-21, 26, 27]. The results of these studies suggested that LCZ696 may have a beneficial effect on the endothelial function not only in diabetic conditions, but also in non-diabetic conditions.

In addition to its role in the degradation of NPs, neprilysin is also responsible for the inactivation of other circulating vasodilating mediators, such as substance P and bradykinin[28]. It is well documented that bradykinin and substance P can increase the activity of eNOS[29, 30]. Moreover, NPs (ANP, BNP, and CNP) and their receptors (NPR-A, NPR-B, and NPR-C) mediate various benefits on blood pressure homeostasis, cardiac hypertrophy and remodeling, vascular relaxation, and RAAS. Most of these effects are mediated by increased cGMP via the interaction of NPs with the NPR-A and NPR-B receptors[31]. Specifically, chronic ANP treatment almost completely restored endothelium-dependent relaxation in response to Ach, whereas no difference was observed among the groups in response to SNP in a rabbit model[19].

To clarify the underlying mechanism, *in vitro* and *ex vivo* experiments were performed using ANP, MGO, and L-NAME. MGO is a highly reactive a-dicarbonyl compound that is generated as an end-product of glycolysis; therefore, MGO is elevated in both type 1 and type 2 diabetic patients[32]. Our *in vitro* experiment showed that MGO decreases the phosphorylation of eNOS^{Ser1177} and Akt, and, on the other hand, enhances the phosphorylation of eNOS^{Thr495} in HUVEC[33, 34]. However, incubation with ANP tended to increase the phosphorylation of eNOS^{Ser1177} and Akt, and significantly decreased eNOS^{Thr495} phosphorylation. These results suggested that ANP promoted eNOS activation in the presence of MGO. To confirm this effect from the point of view of vascular relaxation, we performed an *ex vivo* experiment. The findings clearly demonstrated that ANP ameliorates the endothelium-dependent vascular relaxation induced by MGO. L-NAME, a well-known inhibitor of NO synthesis, completely abolished the effects of ANP. These results suggest that ANP may have beneficial effects on the endothelial function by enhancing eNOS activity under diabetic conditions, at least partially. These results are consistent with those of previous studies demonstrating that ANP promotes NOS activity in the endothelium of L-NAME-injected rats[35].

In conclusion, our findings demonstrate that LCZ696 ameliorates diabetes-induced endothelial dysfunction at least partially by enhancing the eNOS activity. Furthermore, ANP ameliorated MGO-induced vascular dysfunction, and this effect correlated with an increase in eNOS phosphorylation. These results provide insight into the superior effects

of LCZ696 over valsartan on the endothelial function, which is partially due to the increased bioavailability of NPs and NO. This comparative analysis not only provides valuable insights into the pharmacological actions of LCZ696 but it also suggests its potential advantages over traditional ARBs in preserving endothelial health. The findings of this study may have implications in optimizing the therapeutic strategies targeting endothelial dysfunction and various types of cardiovascular intervention. However, future studies should aim to investigate the mechanisms by which NPs improve the eNOS activity and endothelial function under diabetic conditions.

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Competing Interests

The authors declare that they have no conflict of interest.

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