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ORIGINAL ARTICLE

Alagebrium (ALT-711) improves the anti-hypertensive efficacy of nifedipine in diabetic-hypertensive rats

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Combining drugs with complementary mechanisms of action may contribute to improved hypertension control in diabetic patients. Advanced glycation end-product (AGE) breakers, a new class of candidate drugs targeting aging-related cardiovascular dysfunction, may be useful as novel adjuvant agents to improve the efficacy of diabetic hypertension (DH) treatment. This study evaluated the effects of alagebrium (ALT-711), an AGE breaker, combined with nifedipine, a Ca^{2+} channel blocker, in a rat model of streptozotocin-induced DH. Compared with monotherapy, combination treatment significantly decreased systolic and diastolic blood pressure values, increased the pulse pressure, and decreased the coefficient of variation of the systolic blood pressure. Plasma biochemistry indicated that the concentrations of prostacyclin and nitric oxide were increased. Gene expression analysis showed significantly decreased prepro-endothelin-1expression in the aorta. These results reveal that alagebrium significantly improves the anti-hypertensive actions of nifedipine in a rat model of DH and suggest its potential use in the successful control of clinical DH.
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Keywords: adjuvant agent; AGEs breaker; aging; diabetic hypertension

INTRODUCTION

Studies have demonstrated a higher rate of hypertension in patients suffering from type 1 or type 2 diabetes mellitus (T1DM or T2DM) compared with non-diabetic subjects.[1,2] A population-based study of adolescents showed that T2DM is associated with greater hypertension-related morbidity than T1DM.[3] The coexistence of hypertension and diabetes accelerates the presence and development of microvascular and macrovascular comorbidities.[4] There is strong evidence that blood pressure (BP) control in diabetic patients can help reduce diabetes-related morbidity and mortality.[5,6] However, there is still a large gap between recommendations for BP control and the BP values that have been described in most studies of T1DM[1,7,8] and T2DM.[9]

Because of arterial stiffness and endothelial dysfunction, it is difficult to control hypertension in diabetic patients. Combining drugs with complementary mechanisms of action may improve both BP control and the tolerability of the individual drugs.[10,11] However, none of the existing anti-hypertensive agents specifically targets the pathological process of diabetes. Thus, it is imperative to develop a new combination drug paradigm for the clinical treatment of hypertension in diabetic patients.

Advanced glycation end-product (AGE) breakers, a new class of candidate drugs, target the fundamental pathological processes of aging and diabetes caused by the progressive formation of protein–glucose complexes (namely, AGEs).[12–15] AGEs affect the cardiovascular system and alter cellular responses[16–18] contributing to diabetes-related structural and functional changes in the arteries. AGEs can lead to increased arterial stiffness, endothelial dysfunction, and in some instances, drug resistance in diabetic patients. In clinical studies (that is, SAPPHIRE, SILVER), the first AGE breaker, alagebrium (ALT-711), showed only a slight effect on lowering BP[19] and failed to improve exercise capacity or cardiac function. However, alagebrium significantly decreased pulse pressure (PP) and improved total arterial compliance in aged subjects with vascular stiffness.[20] Additionally, a series of studies have indicated that AGE breakers may be effective in protecting the cardiovascular system by reducing arterial stiffness, enhancing cardiac output and improving left ventricular diastolic distensibility in aging and diabetic animals.[21–27] AGE breakers have demonstrated definite therapeutic potential in aging-related diastolic heart failure.[28]

Although the results from clinical trials with alagebrium have been difficult to interpret, the benefits of AGE breakers on the compliance or stiffness of blood vessels are definitive. The use of AGE breakers as an adjunct therapy for the management of hypertension, in the context of diabetes, is expected to have beneficial effects. Accordingly, we sought to determine whether the combined use of alagebrium with nifedipine, a drug widely used to treat high BP, would enhance the anti-hypertensive action of nifedipine in a rat model of streptozotocin (STZ)-induced diabetic hypertension (DH). We also investigated the potential mechanisms of action of the combination therapy.

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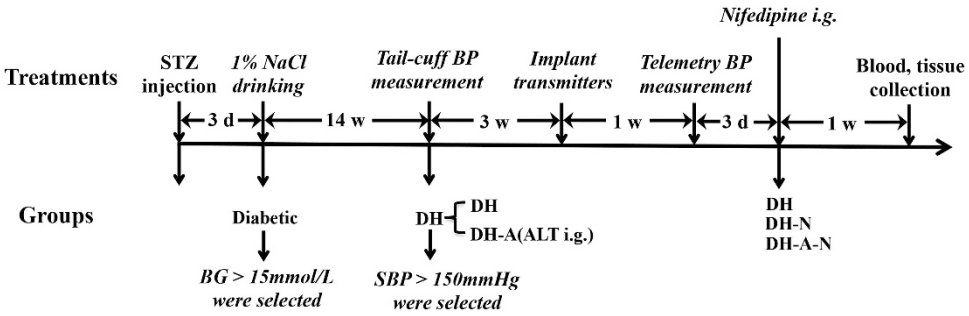

Figure 1 The flow diagram of the establishment and treatment of the diabetic hypertension (DH) rat model.

METHODS

Drugs

Alagebrium was synthesized by the Beijing Institute of Pharmacology and Toxicology as previously described.[29] Purity was determined by ^{1}H-nuclear magnetic resonance spectroscopy, mass spectroscopy and elemental analysis. STZ was purchased from Sigma Chemical Co. (St Louis, MO, USA). Nifedipine was purchased from Zhongan (Tianjin, China).

Animals

Eight-week-old male Wistar rats were obtained from the Laboratory Animal Center of the Beijing Institute of Pharmacology and Toxicology and maintained on a 12-h light-dark cycle. Rats had free access to food and water. All animal experiments and protocols were strictly in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Experiments were performed on 66 rats, 7 of which died during the experiments. Therefore, only the results from 59 rats were included in the final analysis.

Experimental model

As shown in Figure 1, after overnight fasting, diabetes was induced by a single injection of STZ (60 mg kg^{-1}, i.p.) dissolved in citrate buffer (pH 4.5). Control rats were injected with buffer alone. STZ-injected rats were monitored for elevated (>15 mmol l^{-1}) blood glucose levels with OneTouch test strips (Johnson&Johnson, New Brunswick, NJ, USA). Rats with elevated blood glucose levels 72 h after STZ injection were enrolled in the experimental groups.

After diabetes induction, rats were maintained for 19 weeks with free access to chow and water (normal group) or 1% NaCl solution (DH group). Animals with a systolic BP (SBP) exceeding 150 mm Hg at 14 weeks were enrolled in the DH group, which was further divided into two subgroups according to whether rats were given distilled water (DH group) or 12.5 mg kg^{-1} alagebrium (DH-A group) by oral gavage once a day for 5 consecutive weeks. After 3 weeks of treatment, rats were anesthetized with 10% chloral hydrate (4 ml kg^{-1}, i.p.), and a telemetry transmitter (Data Sciences International, St-Paul, MN, USA) was implanted into the abdominal aorta, as previously described.[30] The rats were allowed to recover for 1 week. Thereafter, BP was monitored for 3 consecutive days. At this time, rats in the DH and DH-A groups were further divided into four subgroups: DH only ($n=11$), DH plus nifedipine (DH-N group), DH-A only and DH-A plus nifedipine (DH-A-N group). Nifedipine (0.75 mg kg^{-1}) was administered by oral gavage at 10:00 AM once a day for 1 week. Non-nifedipine groups were given vehicle. Beginning immediately after nifedipine administration, BP was continuously monitored for 10 h every day.

Data collection

SBP, diastolic BP (DBP), and heart rate were continuously monitored by radiotelemetry in conscious and unrestrained rats. PP was calculated as PP = SBP − DBP. The coefficient of variation (CV) of BP was calculated by CV (%) = (standard difference (s.d.) of BP)/(mean BP values (from 1100 to 2000 h)) × 100%.

Table 1 Primer sequences for real-time PCR analysis

	Primer sequence	
Gene	*Upstream*	*Downstream*
ppET-1	5′-TCTTCTCTCTGCTGTTTGTG-3′	5′-TTAGTTTTCTTCCCTCCACC-3′
β-actin	5′-TAAAGACCTCTATGCCAACAC AGT-3′	5′-CACGATGGAGGGGCCGGACTC ATC-3′

Abbreviation: ppET-1, prepro-endothelin-1.

Real-time PCR

Total RNA was isolated using Trizol in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed into cDNA with the ThermoScript RT–PCR system (Gibco BRL, Grand Island, NY, USA). Real-time PCR was performed with the ABI Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's recommendations. The relative mRNA levels were normalized against β-actin and presented as $2^{-\Delta\Delta Ct}$. The primer sequences are listed in Table 1.

Plasma biochemistry

Rats were killed with sodium pentobarbital, blood was collected from the abdominal aorta, and the plasma was separated. Commercially available [^{125}I] 6-keto-progtaglandin F1a (PGF1a) and endothelin-1 (ET-1) radioimmunoassay kits (Puer Weiye, Beijing, China) were used to determine plasma 6-keto-PGF1a and ET-1 concentrations. Plasma nitric oxide (NO) was detected with a commercial Nitric Oxide assay kit (Jiancheng, Nanjing, China) based on the Griess reaction.

Immunohistochemistry

Kidney tissue was fixed in 4% formaldehyde and paraffin embedded. Paraffin sections (4-μm-thick) were prepared and sequentially incubated with 3% H_2O_2 and blocking solution (Pierce, Rockford, IL, USA). They were stained overnight in a humidified box at 4 °C with a biotinylated anti-rat ET-1 monoclonal antibody (1:100, sc-21625, Santa Cruz Biotech, Dallas, TX, USA) followed by a 60-min incubation with a secondary biotin-conjugated anti-rabbit antibody (Zymed, South San Francisco, CA, USA). Finally, the sections were lightly counter stained with hematoxylin and eosin (HE). The slides were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

Statistical analysis

Results are expressed as the mean ± s.d. (standard deviation). For multiple comparisons, statistical analysis was performed by one-way or two-way analysis of variance followed by least-square difference (LSD) multiple comparison tests. A P-value < 0.05 was considered significant.

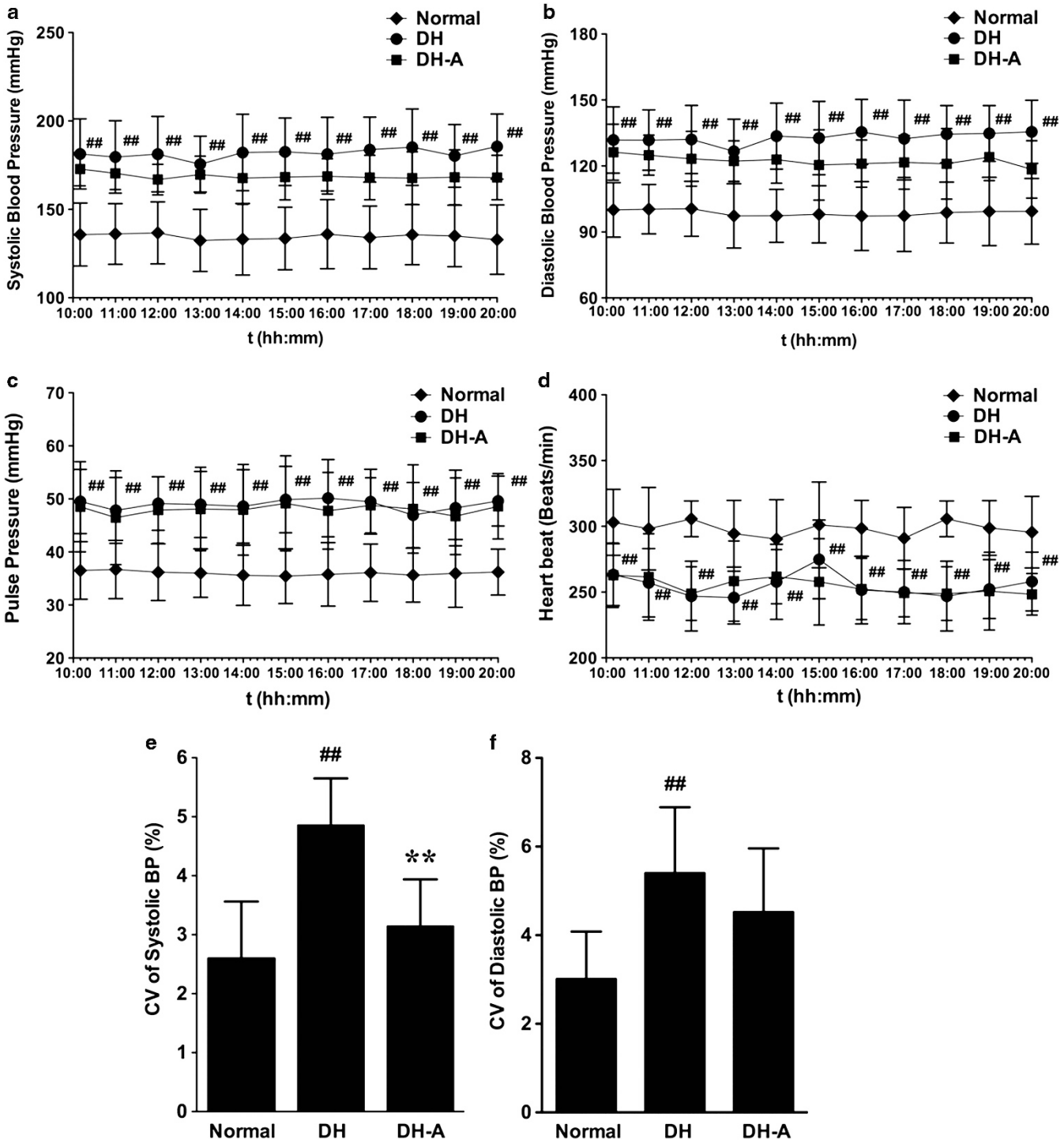

Figure 2 Effects of alagebrium treatment on blood pressure and heart rate in diabetic hypertension (DH) rats. (**a**) Systolic blood pressure. (**b**) Diastolic blood pressure. (**c**) Pulse pressure. (**d**) Heart rate. (**e**) Coefficient of variation (CV) of systolic blood pressure. (**f**) CV of diastolic blood pressure. Values are expressed as the mean ± s.d. $n=12$. $^{\#\#}P<0.01$ *vs.* normal control rats, $^{**}P<0.01$ *vs.* DH rats.

RESULTS

Effects of alagebrium combined with nifedipine on BP

The mean values and CVs of the SBP and DBP in the DH rats were higher compared with those of the control rats (Figure 2, $P<0.01$), indicating that the rat model of DH exhibited clear clinical characteristics of hypertension. Treatment with alagebrium for 4 weeks significantly decreased the CV of SBP but did not affect the mean SBP, DBP, PP or heart rate values. These results suggest that as a monotherapy, alagebrium partially improved the cardiovascular disorder and markedly ameliorated the BP fluctuations in DH rats but had limited efficacy in lowering BP.

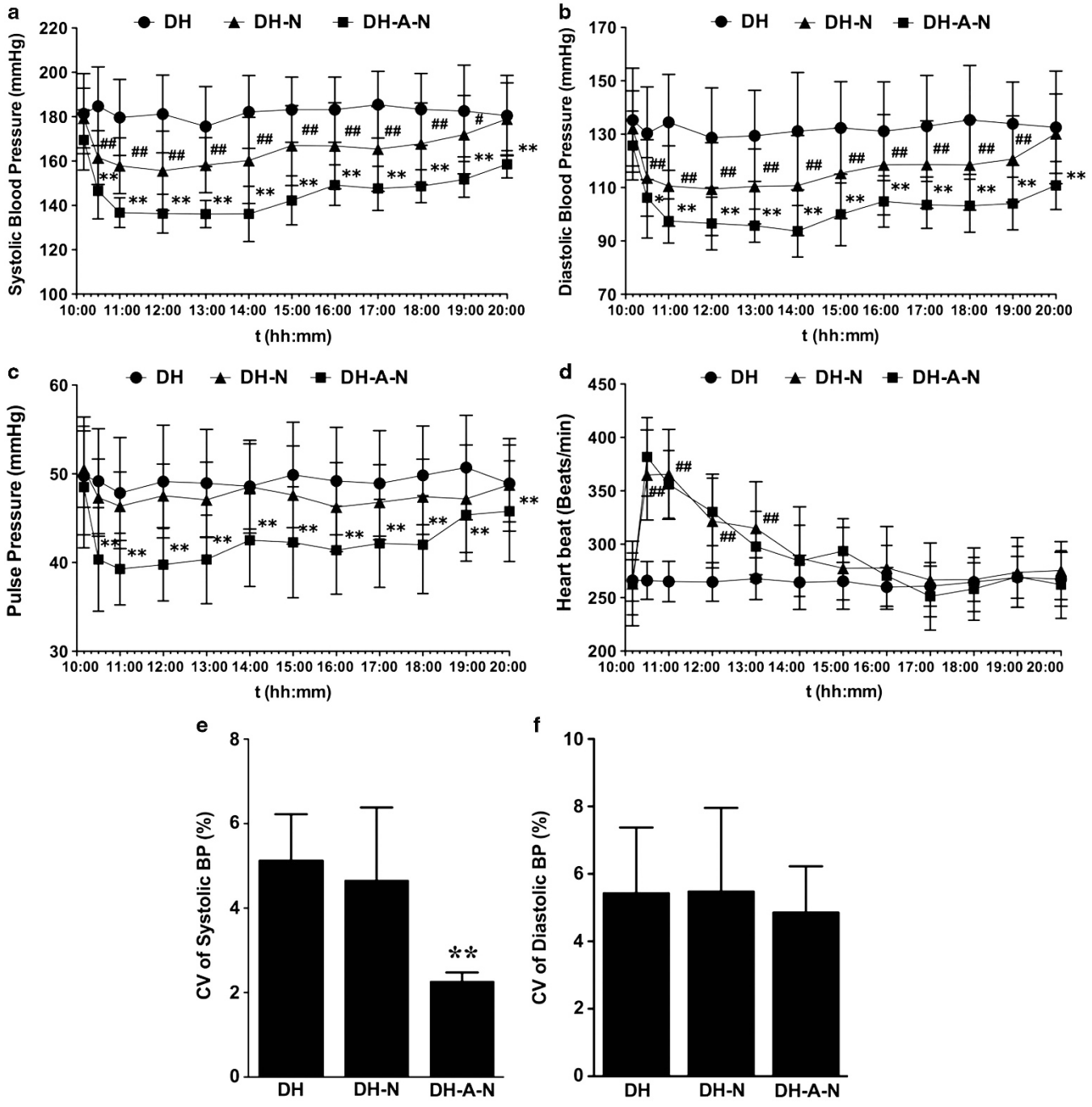

Figure 3 Effects of alagebrium treatment on blood pressure in nifedipine-treated diabetic hypertension (DH) rats. (**a**) Systolic blood pressure. (**b**) Diastolic blood pressure. (**c**) Pulse pressure. (**d**) Heart rate. (**e**) Coefficient of variation (CV) of systolic blood pressure. (**f**) CV of diastolic blood pressure. Values are expressed as the mean ± s.d. $n = 11-12$, $^{\#\#}P<0.01$ *vs.* normal control rats, $^{**}P<0.01$ *vs.* DH rats.

Compared with DH-N rats, DH-A-N animals exhibited significantly lower SBP and DBP values. As shown in Figures 3a and b, the SBP and DBP of DH rats were rapidly lowered immediately after nifedipine treatment. This effect was enhanced in rats treated with alagebrium. The PP and the CV of SBP were significantly decreased (Figures 2e and 3e) in the DH-A-N group compared with the DH-N group. These results indicate that alagebrium enhanced the BP-lowering effect of nifedipine and helped to decrease the PP and the SBP fluctuations in DH rats.

Effects of alagebrium combined with nifedipine on plasma prostacyclin (PGI_2) and NO

PGI_2 and NO are primary vasodilators *in vivo*. As shown in Figure 4, the concentrations of plasma 6-keto-PGF1a (a metabolite of PGI_2) and NO were significantly reduced in the DH rats compared with the normal control rats. Nifedipine alone had no effect on the plasma concentration of PGI_2 or NO. The concentration of the vasodilators, however, was considerably elevated by combination treatment with alagebrium and nifedipine. These results suggest that alagebrium may

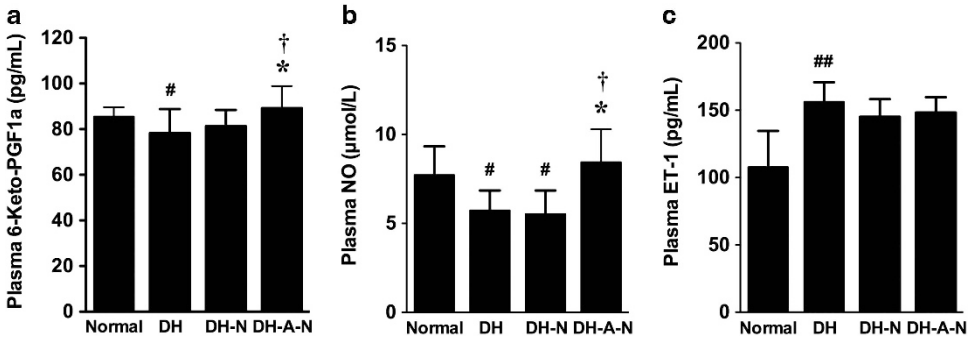

Figure 4 Effects of alagebrium treatment on plasma concentrations of bioactivators in diabetic hypertension (DH) rats. (**a**) 6-keto-PGI1a. (**b**) Nitric oxide (NO). (**c**) Endothelin-1 (ET-1). Values are expressed as the mean ± s.d. $n = 11-12$. $^{\#}P<0.05$,$^{\#\#}P<0.01$ *vs.* normal control rats, $^{\dagger}P<0.05$ *vs.* DH rats, $^{*}P<0.05$.

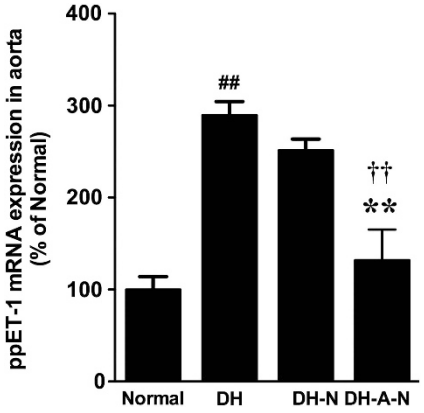

Figure 5 Effects of alagebrium treatment on aortic aortic prepro-Endothelin-1 (ppET-1) gene expression in diabetic hypertension (DH) rats. ppET-1 gene expression was determined by real-time PCR. The results are normalized to β-actin and expressed as the mean fold increase of mRNA ± s.d. Compared with the normal control group. $n = 3$, $^{\#\#}P<0.01$ *vs.* normal control rats, $^{\dagger\dagger}P<0.01$ *vs.* DH rats,$^{**}P<0.01$ *vs.* DH plus nifedipine (DH-N) rats.

promote the synthesis or release of PGI_2 and NO from the endothelium in DH rats, thereby restoring normal function of the vascular endothelium.

Effects of alagebrium combined with nifedipine on ET-1 expression levels

ET-1 is an important vasoconstrictor that is mainly secreted by endothelial cells (ECs). In addition to its systemic effects, ET-1 has a role in normal kidney function. Renal ET-1 is an important regulator of BP under normal physiologic conditions.[31] In the present study, circulating ET-1 content, aortic prepro-ET-1 (ppET-1) mRNA expression (Figure 5), and renal ET-1 protein expression (Figures 6a and b) were significantly increased in the DH rats. Nifedipine alone had no effect on the aortic ppET-1 mRNA expression level and significantly reduced the ET-1 protein expression levels in the cortex and medulla of the kidney (Figure 6c). Combination therapy with alagebrium and nifedipine, however, significantly downregulated aortic ppET-1 mRNA and renal ET-1 protein expression levels, reversing expression to nearly normal levels (Figures 6d and e). Interestingly, neither monotherapy nor combination therapy had any significant effect on the plasma concentration of ET-1 (Figure 4). These results suggest that combination therapy with alagebrium and nifedipinecan suppress the synthesis or release of ET-1 locally but has no effect on systemic ET-1 levels.

DISCUSSION

AGE breakers represent a novel therapeutic strategy for potentially reversing aging-related cardiovascular dysfunction.[32] The representative candidate drug, alagebrium, has ever successfully reached phase 2 in clinical trials.[19,28,32] Our laboratory previously reported a new drug candidate, C36.[33] The beneficial effects of these two AGE breakers on cardiovascular structure and function have been validated in several animal models of aging and diabetes. In this paper, we examined whether alagebrium could act as an adjunctive therapeutic agent for DH management that previously had been proven by us in the same rat model under an anesthetic context (unpublished data). To exclude the possible impact of anesthesia, here we repeated the research in conscious and unrestrained rats. For the first time, we demonstrated that alagebrium improves the anti-hypertensive effects of nifedipine in DH rats. Alagebrium enhanced the anti-hypertensive potency of nifedipine, decreased PP and depressed BP variability, possibly by reversing the dysfunction of the vascular endothelium.

AGEs are markers for the pathophysiological processes of aging and diabetes. STZ-induced diabetic rats are the most commonly used animal model for studying the pharmacodynamic properties and mechanisms of action of AGE breakers.[27] However, single-injection STZ-induced diabetic rats, maintained for up to 8 months, do not exhibit obvious hypertension. Conversely, hypertensive rat models, commonly used to demonstrate pathological conditions of hypertension, often are not associated with increased AGE levels and do not reflect the nature of DH. To evaluate the effects of AGE breakers combined with nifedipine on DH more efficiently, we created an AGE-associated hypertensive animal model. As previously discussed, DH rats exhibited significantly higher and more stable BP (>150 mm Hg). Moreover, this hypertension coincided with hyperglycemia, was AGE-dependent, and was accompanied by increased plasma concentrations of vascular constrictors, such as ET-1.[34]

In this study, 4 weeks of alagebrium treatment slightly and non-significantly lowered the mean SBP and DBP values of DH rats. The most important observation in this study was that the CV of SBP was markedly decreased in DH rats after alagebrium treatment. BP variability, which can be measured through the CV of BP, is an important index in clinical anti-hypertensive therapy.[35] BP variability has been reported to be elevated in patients with (especially severe) hypertension.[36] Increased BP variability can induce damage in organs, independent of high BP.[37–39] Our results demonstrated that

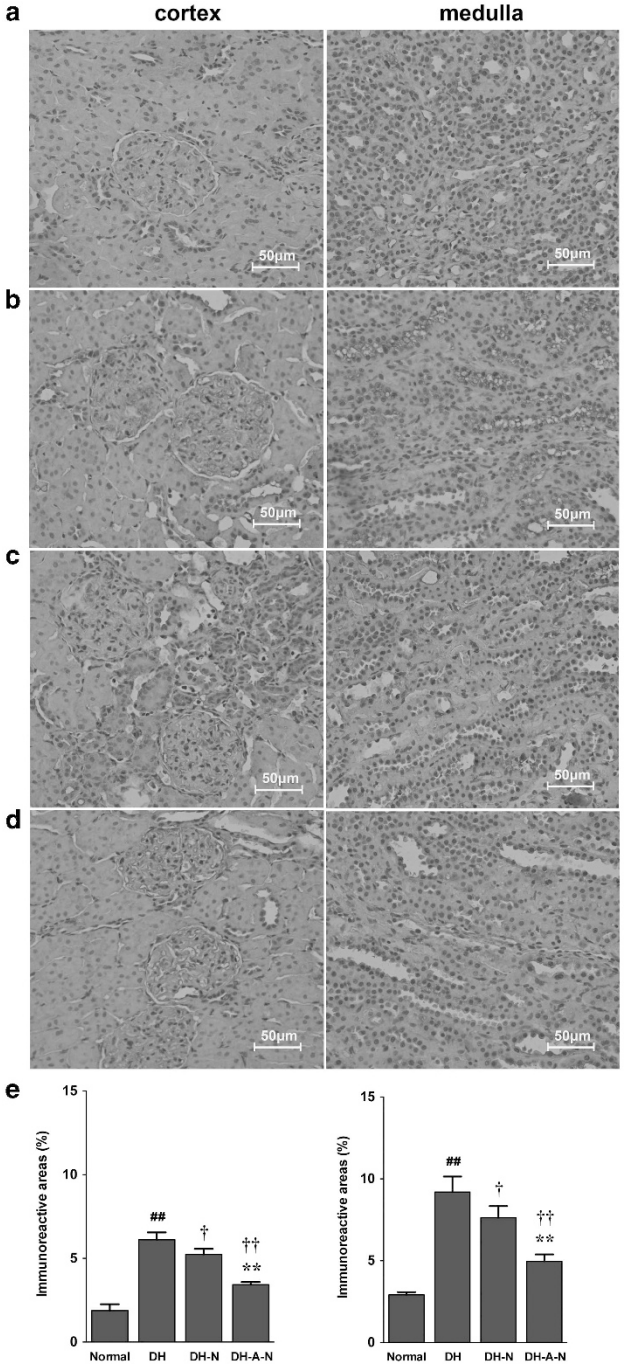

Figure 6 Immunohistochemical analysis of endothelin-1 (ET-1) expression levels in the renal medulla and cortex of diabetic hypertension (DH) rats. (**a**) Normal control. (**b**) DH. (**c**) DH plus nifedipine (DH-N). (**d**) DH plus alagebrium and nifedipine (DH-A-N). (**e**) Immunoreactive areas of ET-1. Sections are counter stained with hematoxylin and eosin. Positive staining is shown as brown. Sections of three kidney tissues from three individual rats in each experimental group were taken for analysis. Immunoreactive area was measured and expressed as a percentage relative to the total tissue area. Values are expressed as the mean ± s.d. $n=3$, $^{\#\#}P<0.01$ *vs.* normal control rats, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *vs.* DH rats, $^{**}P<0.01$ *vs.* DH-N rats. A full color version of this figure is available at the *Hypertension Research* journal online.

alagebrium improves cardiovascular function and reduces the risk factors of diabetes-associated hypertension, which may be beneficial in anti-hypertensive therapy in the elderly.

AGE breakers reverse aging-related structural and functional dysfunctions of the systemic vessels, particularly the arteries.[33] Nifedipine is a selective blocker of voltage-dependent Ca^{2+} channels, suppressing Ca^{2+} influx into the vascular smooth muscle cells and dilating the arterial blood vessels.[40] As the mechanisms of action of the two drugs differ, their synergetic effects were predictable. Our findings suggest that the combination of alagebrium and nifedipine may specifically favor treatment for aging and DH patients. Indeed, nifedipine has been recommended for aging and DH patients because of its effect on improving insulin resistance, which is common in these patients.[41] Therefore, as an adjunct agent for nifedipine, an AGE breaker may have substantial clinical potential.

To clarify whether the improvement of endothelial function by alagebrium benefited the anti-hypertensive effect of nifedipine, we detected the levels of vasoactive substances in DH. In the hypertensive state, vasoconstrictor and vasodilator substances experience increased and decreased secretion, respectively. The vasodilators NO and PGI_2 and the vasoconstrictor ET-1 are mainly secreted by ECs. In this study, the circulating NO and PGI_2 levels were significantly increased, and no change in the ET-1 level was observed in the alagebrium-treated rats when compared with the DH rats. Aortic ppET-1 mRNA and renal ET-1 protein expression levels were decreased in the alagebrium-treated rats. Our results suggest that alagebrium reduces the accumulation of AGEs in blood vessels, thereby benefiting the synthesis or secretion of NO and PGI_2 by vascular ECs and suppressing aortic ppET-1 gene and kidney ET-1 protein expression in DH rats.

The above conclusions are consistent with previous reports that AGEs can inhibit NO and PGI_2 production from ECs through the p38 MAPK and AGE receptor pathways,[42,43] respectively. NO suppresses ET-1 release from ECs and terminates ET-1 signal transduction in a cyclic guanosine monophosphate -independent manner.[44] Although Ca^{2+} channel blockers are able to remove oxygen radicals, increase the endothelial NO synthase activity and thus increase NO release,[45] we believe that in our model, nifedipine promoted increased NO release in the alagebrium-treated DH rats through an improved endothelium. ET-1 levels are primarily associated with BP. Because AGE breakers do not influence BP directly, their effects on the plasma concentration of ET-1 are limited.

The unique pharmacological mechanism of AGE breakers suggests that combination therapy may be a wise choice for the future development of this class of drugs. This study investigated the combination therapy of an AGE breaker with an anti-hypertension drug under the diabetic condition; however, such combinations must be based on complementary mechanisms, or a synergistic effect may not be observed. It was reported that although renin–angiotensin system blockade and AGE inhibition have specific and separate effects on diabetic nephropathy, no further improvement was observed when an AGE breaker and an angiotensin-converting enzyme inhibitor were combined, owing to their many overlapping downstream effects.[46] However, alagebrium and nifedipine have non-overlapping mechanisms of cardiovascular protection and, hence, more potent anti-hypertension effects. Further studies are needed to illuminate the underlying mechanisms of this drug combination before it can be generalized to the clinic.

In conclusion, the AGE breaker alagebriumis were able to augment the anti-hypertensive effect of nifedipine and decrease the CV of

SBP and PP in AGE-associated DH rats. Hypertension is one of the most common comorbidities of diabetes, especially in the aged diabetic population. Hence, the combined use of AGE breakers with anti-hypertensive agents may have great potential in the management of clinical DH.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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