Angiotensin II Receptor–Neprilysin Inhibitor Sacubitril/Valsartan Improves Endothelial Dysfunction in Spontaneously Hypertensive Rats

Takunori Seki, MD, PhD; Kenichi Goto, MD, PhD; Yasuo Kansui, MD, PhD; Toshio Ohtsubo, MD, PhD; Kiyoshi Matsumura, MD, PhD; Takanari Kitazono, MD, PhD

Background—We have previously demonstrated that antihypertensive treatment with renin-angiotensin system inhibitors restores the impaired endothelium-dependent hyperpolarization (EDH)–mediated responses in spontaneously hypertensive rats (SHRs). Herein, we investigated whether the angiotensin II receptor–neprilysin inhibitor sacubitril/valsartan (LCZ696) would improve reduced EDH-mediated responses and whether LCZ696 would exert additional effects on endothelium-dependent and endothelium-independent vasorelaxation compared with an angiotensin II type 1 receptor blocker alone during hypertension.

Methods and Results—SHRs were treated for 3 months with either LCZ696 or valsartan, from the age of 8 to 11 months. Age-matched, untreated SHRs and Wistar-Kyoto rats served as controls. Membrane potentials and contractile responses were recorded from the isolated superior mesenteric arteries. Acetylcholine-induced, EDH-mediated responses were impaired in untreated SHRs compared with Wistar-Kyoto rats. EDH-mediated responses were similarly improved in the LCZ696- and valsartan-treated SHRs. No difference was observed in acetylcholine-induced, nitric oxide–mediated relaxations among the 4 groups. Endothelium-independent relaxations in response to a nitric oxide donor, sodium nitroprusside, and those to levcromakalim, an ATP-sensitive K^+-channel opener, were similar among the 4 groups; however, the sensitivities to levcromakalim were significantly higher in both LCZ696- and valsartan-treated SHRs.

Conclusions—LCZ696 appears to be as effective as valsartan in improving the impaired EDH-mediated responses during hypertension. LCZ696 and valsartan exert similar beneficial effects on endothelium-independent relaxation via enhanced sensitivity of the ATP-sensitive K^+ channel. However, the dual blockade of renin-angiotensin system and neutral endopeptidase with LCZ696 does not appear to provide additional benefit over valsartan alone on vasomotor function in mesenteric arteries of SHRs. (***J Am Heart Assoc***. 2017;6:e006617. DOI: 10.1161/JAHA.117.006617.)

Key Words: endothelial function • endothelial-derived relaxant factor • hypertension • renin angiotensin system

H ypertension is one of the most prevalent and important risk factors for cardiovascular disease across the world.[1] In hypertension, endothelial dysfunction precedes the development of arterial atherosclerosis. It appears to be associated with an increased risk of cardiovascular disease, including cerebrovascular disease, ischemic heart disease,

renal failure, and heart failure.[2–4] Thus, it is of clinical importance to find effective treatments for endothelial dysfunction in hypertension.

In addition to the release of several vasoactive factors, such as nitric oxide (NO) and prostacyclin, vascular endothelial cells regulate arterial tone through the generation of endothelium-dependent hyperpolarization (EDH).[5–8] Although the mechanisms of endothelial dysfunction in hypertension seem to vary,[2,3] we have demonstrated that the impaired EDH generation accounts for the decreased endothelium-dependent relaxation of mesenteric arteries of spontaneously hypertensive rats (SHRs),[9–12] the most commonly used animal model for human essential hypertension.[13] Reduced EDH-mediated responses have also been reported in the mesenteric arteries of rats with chronic heart failure.[14]

Sacubitril/valsartan (LCZ696) is a new class of cardiovascular drug that is composed of the angiotensin (Ang) type 1 receptor blocker (ARB) valsartan and the neutral

Clinical Perspective

What Is New?

- The present study demonstrated that sacubitril/valsartan is effective in improving the impaired endothelium-dependent hyperpolarization–mediated responses in mesenteric arteries of spontaneously hypertensive rats.

What Are the Clinical Implications?

- Sacubitril/valsartan may serve as a novel tool with which to prevent cardiovascular diseases associated with hypertension.

endopeptidase (NEP) inhibitor sacubitril, in the form of a sodium salt complex.[15,16] Sacubitril is a prodrug that is converted by enzymatic cleavage of the ethyl ester into its active form, sacubitrilat. It has been reported that LCZ696 showed significantly greater reduction in blood pressure compared with valsartan in patients with hypertension.[17] Moreover, in the PARADIGM-HF (Prospective Comparison of ARNI With ACEi to Determine Impact on Global Mortality and Morbidity in Heart Failure) trial, which was a double-blind randomized study comparing LCZ696 and the Ang-converting enzyme inhibitor enalapril in patients with heart failure with reduced ejection fraction, LCZ696 was superior to enalapril at reducing the risks of death and hospitalization for heart failure.[18] However, the mechanisms underlying the superiority of LCZ696 over renin-Ang system (RAS) inhibitors in hypertension and heart failure are not known.

Along with its blockade of Ang type 1 receptor, LCZ696 reduces the degradation of several vasoactive peptides, including adrenomedullin, bradykinin, calcitonin gene-related peptide, natriuretic peptides, substance P, Ang II, and endothelin-1 (ET-1), through the inhibition of an NEP.[19,20] Because adrenomedullin, bradykinin, calcitonin gene-related peptide, substance P, and C-type natriuretic peptide act as potent vasodilators,[19,20] it is tempting to speculate that the superiority of LCZ696 over RAS inhibitors in hypertension and heart failure is related to the beneficial actions of some of these peptides on the cardiovascular system.

We have demonstrated that antihypertensive treatment with RAS inhibitors restores the impaired EDH-mediated hyperpolarization and relaxation in mesenteric arteries of SHRs.[10,11] Interestingly, Kusaka et al recently reported that LCZ696 was more effective at improving acetylcholine (ACh)–induced relaxation compared with the ARB valsartan alone in high-salt diet–fed SHR/NDmcr-cp(+/+) rats; however, the underlying mechanism remained unresolved.[21]

Although controversial,[22] it was reported that C-type natriuretic peptide released from the endothelial cells on stimulation with ACh could produce hyperpolarization of rat mesenteric arteries.[23] Bradykinin has also induced EDH-mediated relaxation in several vascular beds, including human coronary arteries.[24] Some vasoactive substances, such as adrenomedullin and calcitonin gene-related peptide, have been reported to elicit endothelium-independent relaxation in rat mesenteric arteries.[25–27] Thus, it is intriguing to hypothesize that an accumulation of vasoactive substances via NEP inhibition would exert beneficial influences on vascular endothelial and/or smooth muscle function. Indeed, NEP inhibition with thiorphan was reported to augment the C-type natriuretic peptide–induced relaxation in porcine coronary arteries[28] and to potentiate the bradykinin-induced relaxation in small human resistance arteries.[29]

We, thus, conducted the present study to determine the following: (1) whether treatment with the dual blockade of RAS and NEP with LCZ696 would improve endothelial dysfunction, particularly that attributable to EDH; and (2) whether LCZ696 would exert additive effects on endothelium-dependent and endothelium-independent relaxations compared with the ARB valsartan alone in mesenteric arteries of SHRs. Because previous studies have reported that a high-salt diet upregulates EDH to compensate for the loss of NO in mesenteric arteries of the rats,[30,31] SHRs were fed a normal-salt diet in the present study to rule out the confounding effect of salt on endothelial function.

Methods

Handling of Animals

This study was approved by the Committee on the Ethics of Animal Experimentation of Kyushu University (Fukuoka, Japan). Male SHR/Izm and age-matched male Wistar-Kyoto (WKY)/Izm rats (n=8) were fed standard rat chow and had free access to tap water. At the age of 8 months, SHRs were assigned to 1 control group (SHR-C; n=12) and 2 treatment groups: treated with either LCZ696, 60 mg/kg per day (SHR-L; n=11), or valsartan, 20 mg/kg per day (SHR-V; n=12), for 3 months. The 60-mg LCZ696 contains 35% more valsartan than 20-mg valsartan alone (ie, 27 versus 20 mg).

LCZ696 or valsartan dissolved in corn oil was orally administered via gastric gavage once daily. The dose of each agent was based on preliminary experiments (2 weeks of treatment) in which the blood pressure of 8-month-old SHRs was lowered to comparable extents. Untreated WKY rats served as normotensive controls. There were 8 to 12 rats in each group. LCZ696 and valsartan were supplied by a manufacturer.

The systolic blood pressure (SBP) and heart rate were measured in conscious rats by the tail-cuff method before, during, and at the end of the treatment. The rats were euthanized by deep anesthesia with isoflurane and inferior

vena cava exsanguination. The superior mesenteric artery was excised and bathed in cold Krebs solution with the following composition (in mmol/L): Na^+ 137.4, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134, and glucose 11.5. The artery was then cleaned of adherent connective tissues and cut into 3- and 1.2-mm rings for the electrophysiological experiment and the tension experiment, respectively.

Isometric Tension Recording

Rings (1.2 mm) with intact endothelium were placed in 5-mL organ chambers filled with 36°C Krebs solution that had been aerated with 93% O_2 to 7% CO_2 (pH 7.4). Isometric contractile responses were recorded, as described.[9–12] The rings were then allocated to 1 of the following treatments: (1) control, (2) indomethacin (10^{-5} mol/L), and (3) indomethacin (10^{-5} mol/L) and N^ω-nitro-L-arginine (L-NAME; 10^{-4} mol/L).

The rings were contracted with phenylephrine (PE; 10^{-5} mol/L), and relaxations in response to ACh were determined in a cumulative manner. In this vascular bed, relaxation in response to ACh under these conditions has been abolished by the removal of the endothelium.[9] In some preparations, the rings were contracted with 77 mmol/L KCl solution in the presence of indomethacin (10^{-5} mol/L), and the relaxation in response to ACh was observed for the evaluation of the NO-mediated relaxation.[9] Relaxations in response to levcromakalim, a direct activator of ATP-sensitive K^+-channel (K_{ATP}), and sodium nitroprusside (SNP) were studied in rings contracted with PE (10^{-5} mol/L) in the presence of indomethacin (10^{-5} mol/L) and L-NAME (10^{-4} mol/L). The extent of the relaxation was expressed as the percentage of the initial contraction.

Electrophysiological Experiments

Transverse strips cut along the longitudinal axis of the 3-mm rings were placed in the experimental chamber (2 mL) with the endothelial layer up, and the strips were then superfused with 36°C Krebs solution aerated with 95% O_2 to 5% CO_2 (pH 7.3–7.4) at the rate of 3 mL/min. After equilibration for at least 60 minutes, the membrane potentials of the vascular smooth muscle cells were recorded using a conventional microelectrode technique, as described.[9] Thereafter, hyperpolarizations in response to ACh and levcromakalim were recorded. Indomethacin (10^{-5} mol/L) and L-NAME (10^{-4} mol/L) were present in all experiments to inhibit the influence of prostanoids and NO, respectively.

Drugs and Solutions

The following drugs were used: ACh chloride, PE hydrochloride, indomethacin, L-NAME, levcromakalim, SNP, LCZ696, and valsartan. Indomethacin was dissolved in dimethyl sulfoxide. Levcromakalim was dissolved in ethanol. LCZ696 and valsartan were dissolved in cone oil. All other drugs were dissolved in distilled water. All drugs were further diluted 1000-fold in Krebs solution to give the final chamber concentrations.

Statistical Analysis

Results are presented as mean±SEM. All statistical analyses were performed using GraphPad Prism version 5 for Windows. We analyzed the concentration-response curves of hyperpolarization and relaxation by performing a 2-way repeated-measures ANOVA, where the 2 independent variables were agonist and group, followed by a Bonferroni test for multiple comparisons. The mean value of the concentration-response data was fit to a sigmoid curve with a variable slope factor using nonlinear regression analysis (GraphPad Prism), which was used to calculate the concentration of agonist causing half-maximal response (EC_{50} value) for hyperpolarization and relaxation. The EC_{50} values are expressed as the negative logarithm of the molar concentration. Changes in SBP and heart rate were analyzed by 2-way repeated-measures

Table 1. Systolic Blood Pressure, Heart Rate, and Body Weight Before and After 3 Months of Treatment in the 4 Groups

| Group	Blood Pressure, mm Hg		Heart Rate, bpm		Body Weight, g		No. of Animals
	Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment	
SHR-C	219±3*	225±4*	441±7	439±10	380±2*	464±5*†	12
SHR-L	225±5*	158±7†‡§	449±14	413±16	385±8*	454±7*†	11
SHR-V	214±4*	173±2†‡	440±9	416±18	366±2*‡	455±5*†	12
WKY	140±4‡	150±4‡	416±18	443±9	418±3‡	514±6†‡	8

Values are mean±SEM. bpm indicates beats per minute; SHR-C, spontaneously hypertensive rats in the control group; SHR-L, SHRs treated with LCZ696, 60 mg/kg per day; SHR-V, SHRs treated with valsartan, 20 mg/kg per day; and WKY, Wistar-Kyoto.
*$P<0.05$ vs WKY.
†$P<0.05$ vs before treatment.
‡$P<0.05$ vs SHR-C.
§$P<0.05$ vs SHR-V.
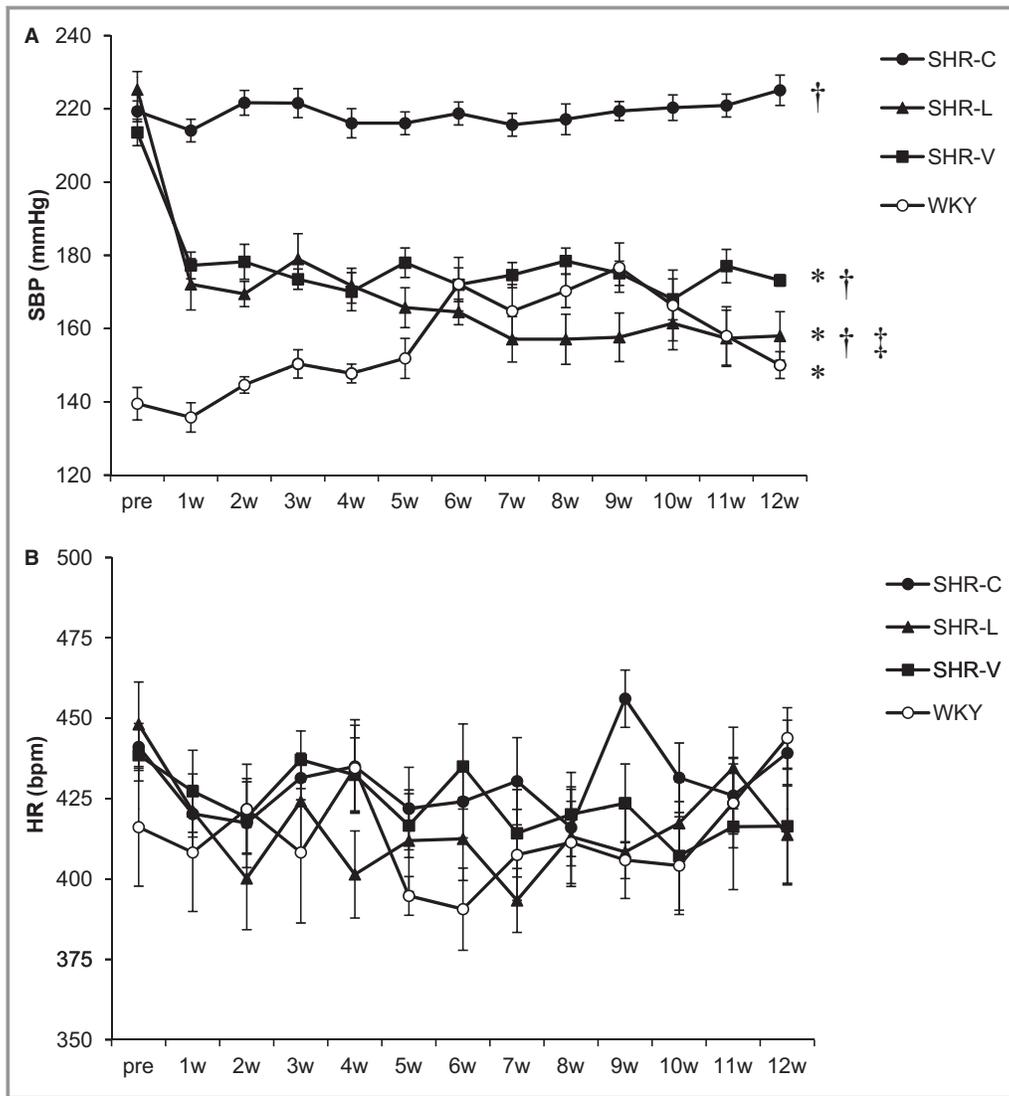
Figure 1. Time course of systolic blood pressure (SBP; A) and heart rate (HR; B) in the spontaneously hypertensive rats in the control group (SHR-C), SHRs treated with sacubitril/valsartan, 60 mg/kg per day (SHR-L), SHRs treated with valsartan, 20 mg/kg per day (SHR-V), and Wistar-Kyoto (WKY) rats. Values are mean±SEM (n=8–12 rats per group). *$P<0.05$ vs SHR-C, †$P<0.05$ vs WKY, ‡$P<0.05$ vs SHR-V. bpm indicates beats per minute.

ANOVA, where the 2 independent variables were time and group, followed by a Bonferroni test for multiple comparisons. Other variables were analyzed by 1-way ANOVA, followed by a Bonferroni test for multiple comparisons, and by the paired Student *t* test. $P<0.05$ was considered significant.

Results

SBP, Heart Rate, and Body Weight

Both LCZ696 and valsartan administered alone significantly lowered the SBP in the SHRs (Table 1, Figure 1A). Although these 2 treatments showed a similar reduction in the SBP for the first 4 weeks after the treatments were initiated, LCZ696 achieved a significantly greater SBP reduction than valsartan at 7, 8, 9, 11, and 12 weeks. When examining group differences, the blood pressures for SHR-L and SHR-V were significantly different from each other (Figure 1A). There was no significant difference in heart rate between the WKY and SHR groups before or after treatment (Table 1, Figure 1B). The body weights were significantly smaller in the SHRs compared with the WKY rats both before and after treatment (Table 1).

Resting Membrane Potential in Mesenteric Arteries

The resting membrane potential of the mesenteric artery was significantly less negative in the SHR-C group
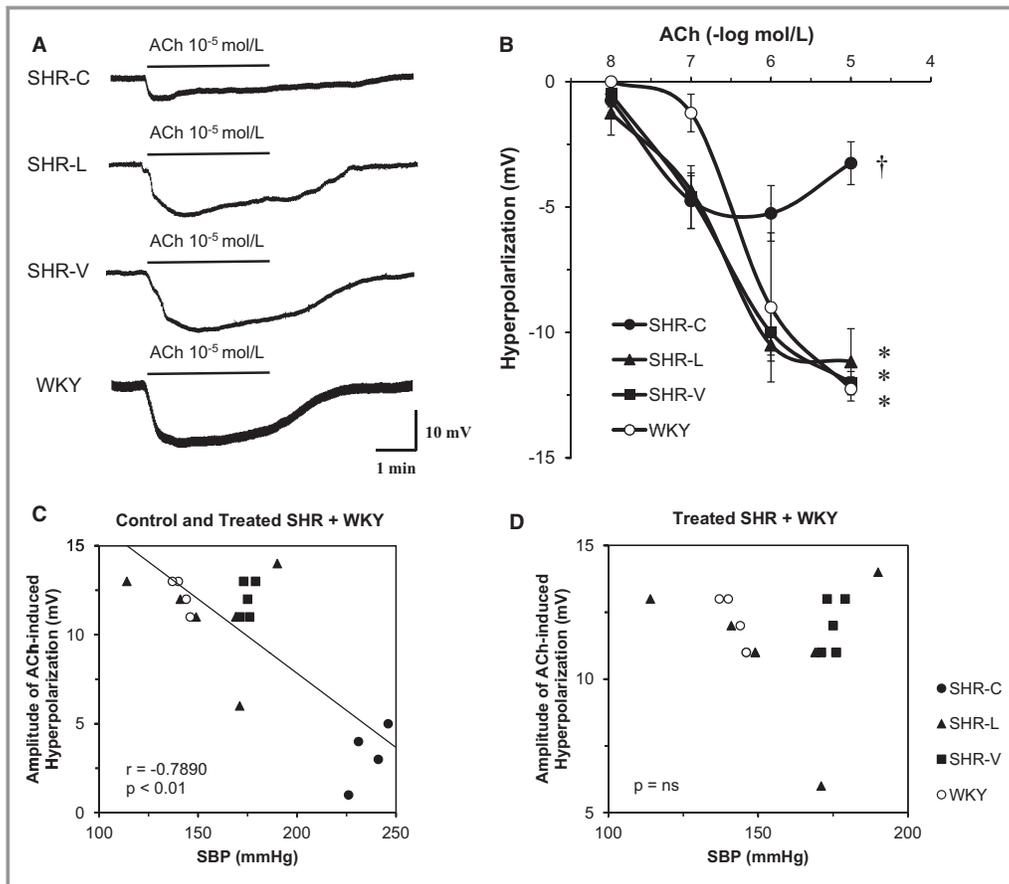
Figure 2. A, Representative tracings showing hyperpolarization in response to acetylcholine (ACh; 10^{-5} mol/L) in the resting state of the membrane in endothelium-intact mesenteric arteries of spontaneously hypertensive rats in the control group (SHR-C), SHRs treated with sacubitril/valsartan, 60 mg/kg per day (SHR-L), SHRs treated with valsartan, 20 mg/kg per day (SHR-V), and Wistar-Kyoto (WKY) rats. B, Concentration-response curves of hyperpolarization in response to ACh on the basis of A. Relationship between the amplitude of ACh (10^{-5} mol/L)–induced hyperpolarization in endothelium-intact mesenteric arteries and systolic blood pressure (SBP) in all groups (C) and in treated SHR and WKY rats (D). Indomethacin (10^{-5} mol/L) and N^{ω}-nitro-L-arginine (10^{-4} mol/L) were present throughout. Values are mean±SEM (n=4–6 rats per group). NS indicates not significant. *$P<0.05$ vs SHR-C, †$P<0.05$ vs WKY.

(-39.9 ± 1.0 mV) compared with the WKY group (-47.5 ± 1.4 mV; $P<0.05$). In addition, the resting membrane potential was significantly more negative in the treated SHRs (SHR-L, -47.0 ± 1.4 mV; SHR-V, -45.3 ± 0.8 mV) compared with the SHR-C group ($P<0.05$). There was no significant difference in the resting membrane potential between the SHR-L and SHR-V groups.

EDH in Mesenteric Arteries

Representative tracings of ACh-induced EDH-mediated hyperpolarization in the resting state of the membrane are shown in Figure 2. The negative logarithm of the molar concentration values are as follows: SHR-C, not determined; SHR-L, 6.8 ± 0.2; SHR-V, 6.8 ± 0.2; and WKY, 6.3 ± 0.3 (n=4–6 per group; not significant. The maximal hyperpolarizations were as

follows: SHR-C, -5.3 ± 1.1 mV; SHR-L, -11.2 ± 1.3 mV; SHR-V, -12.0 ± 0.4 mV; and WKY, -12.3 ± 0.5 mV (n=4–6 per group; $P<0.05$ SHR-C versus WKY).

The ACh-induced EDH was significantly less in the SHR-C group compared with the WKY group (Figure 2A and 2B). Both the LCZ696 (SHR-L) and valsartan (SHR-V) treatments significantly improved the ACh-induced EDH, to a similar extent for WKY rats (Figure 2A and 2B). There was no significant difference in ACh-induced EDH between the SHR-L and SHR-V groups (Figure 2A and 2B). There was a significant negative relationship between the amplitude of the ACh (10^{-5} mol/L)–induced EDH and SBP when all groups were included in the analysis (Figure 2C). However, no significant relationship was observed between these 2 parameters in the subgroup of treated SHR and WKY rats (Figure 2D).
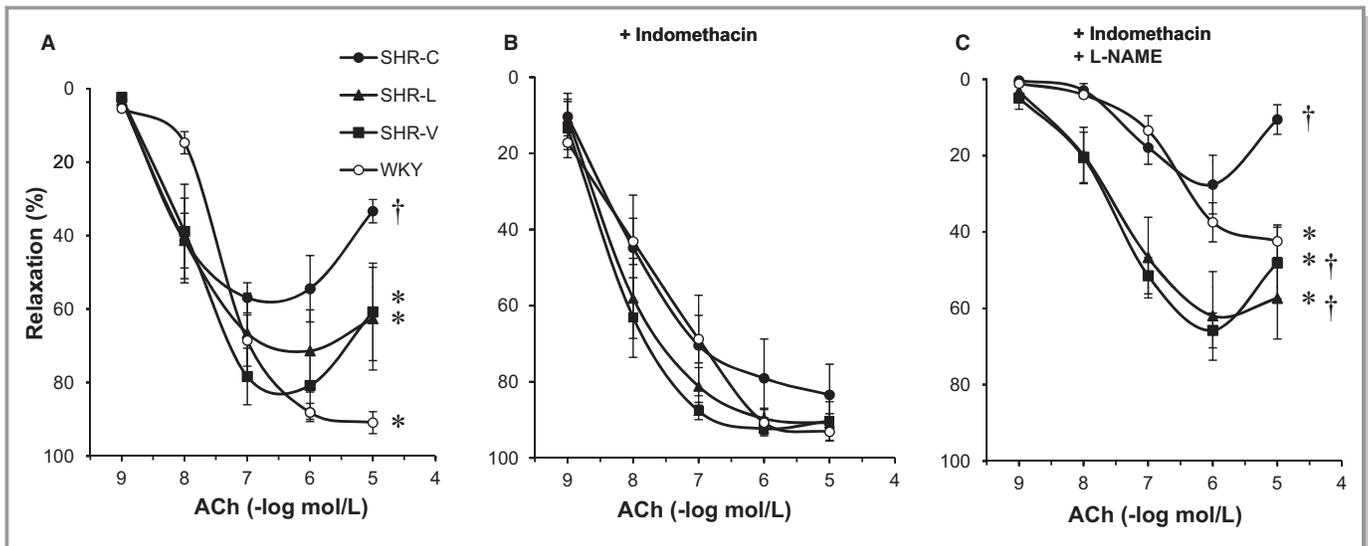
Figure 3. Concentration-response curves of relaxation in response to acetylcholine (ACh) in endothelium-intact mesenteric arterial rings precontracted with phenylephrine (10^{-5} mol/L) in the spontaneously hypertensive rats in the control group (SHR-C), SHRs treated with sacubitril/valsartan, 60 mg/kg per day (SHR-L), SHRs treated with valsartan, 20 mg/kg per day (SHR-V), and Wistar-Kyoto (WKY) rats. A, Without indomethacin and N^{ω}-nitro-L-arginine (L-NAME). B, Effects of indomethacin (10^{-5} mol/L). C, Effects of indomethacin (10^{-5} mol/L) and L-NAME (10^{-4} mol/L). Values are mean±SEM (n=6 rats per group). *$P<0.05$ vs SHR-C, †$P<0.05$ vs WKY.

Endothelium-Dependent Relaxation in Mesenteric Arteries

In mesenteric arterial rings precontracted with 10^{-5} mol/L PE in the absence of indomethacin (10^{-5} mol/L), ACh induced a dose-dependent relaxation in all groups, but it produced small relaxation in SHR-C (Figure 3A, Table 2). There were no significant differences in the maximal relaxations among the treated SHR and WKY rats, but the sensitivities to ACh were significantly higher in the treated SHRs compared with the WKY group (Figure 3A, Table 2). Indomethacin markedly augmented relaxation in the SHR-C group to a similar extent for treated SHR and WKY rats, but

the sensitivities to ACh were still significantly higher in the treated SHRs compared with the WKY group (Figure 3B, Table 2).
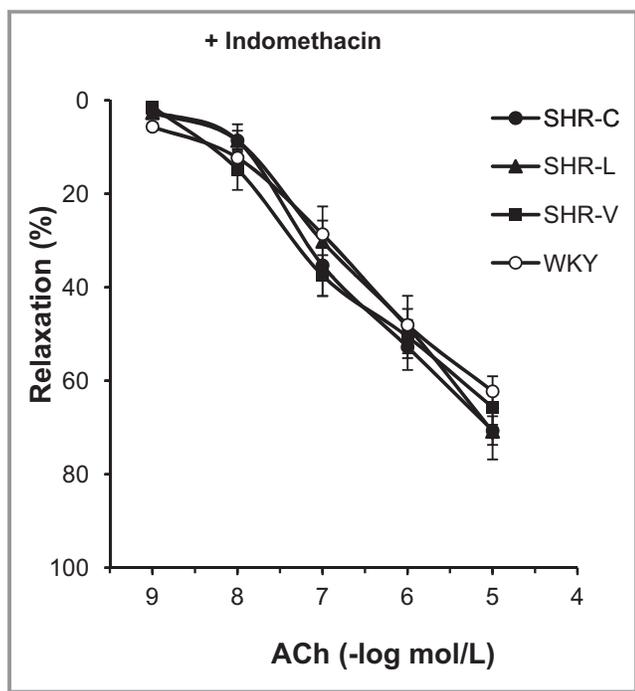
In the presence of indomethacin and L-NAME (10^{-4} mol/L), the ACh-induced EDH-mediated relaxation was significantly less in the SHR-C group than in the WKY group (Figure 3C, Table 2). Both the LCZ696 (SHR-L) and valsartan (SHR-V) treatments markedly restored the ACh-induced EDH-mediated relaxation, and the relaxation in the treated SHR preparations was even more pronounced than that in the WKY preparations (Figure 3C, Table 2). There was no significant difference in ACh-induced EDH-mediated relaxation between the SHR-L and SHR-V groups (Figure 3C, Table 2).

Table 2. Relaxation to Acetylcholine, Sodium Nitroprusside, and Levcromakalim in the Mesenteric Arteries of SHR and WKY Rats

| | Acetylcholine | | | | | | | | | |
| | Alone | | Plus Indomethacin | | Plus Indomethacin and L-NAME | | Sodium Nitroprusside | | Levcromakalim | |
	pD2	Max, %	pD2	Max, %	pD2	Max, %	pD2	Max, %	pD2	Max, %
SHR-C	ND	56.9±4.1*	8.0±0.3	83.4±8.1	7.7±0.6*	27.6±7.7	8.8±0.2	99.8±0.2	6.6±0.1	95.7±0.5
SHR-L	8.3±0.4*	69.1±11.2	8.3±0.2*	90.9±2.5	7.6±0.4*	62.0±11.6†	8.2±0.2	98.7±0.5	7.0±0.1*†	96.4±0.9
SHR-V	8.2±0.3*	80.8±9.2	8.4±0.2*	92.3±1.5	7.8±0.3*	65.8±4.6*†	8.7±0.3	99.3±0.5	7.0±0.1†,*	96.5±0.6
WKY	7.4±0.1	90.9±3.0†	7.6±0.2	93.1±2.4	6.6±0.2†	42.4±4.3	8.6±0.1	99.7±0.2	6.6±0.2	93.6±1.8

Values are mean±SEM. There were 6 rats per group. L-NAME indicates N^{ω}-nitro-L-arginine; Max, maximal relaxation to drugs; ND, not determined; pD2, negative logarithm of molar concentration of the drug causing half-maximal relaxation in the phenylephrine ($10-5$ mol/L)–precontracted arterial rings; SHR, spontaneously hypertensive rat; SHR-C, SHRs in the control group; SHR-L, SHRs treated with LCZ696, 60 mg/kg per day; SHR-V, SHRs treated with valsartan, 20 mg/kg per day; and WKY, Wistar-Kyoto.
*$P<0.05$ vs WKY.
†$P<0.05$ vs SHR-C.

Figure 4. Concentration-response curves of relaxation in response to acetylcholine (ACh) in endothelium-intact mesenteric arterial rings precontracted with 77 mmol/L KCl in the presence of indomethacin (10^{-5} mol/L) in spontaneously hypertensive rats in the control group (SHR-C), SHRs treated with sacubitril/valsartan, 60 mg/kg per day (SHR-L), SHRs treated with valsartan, 20 mg/kg per day (SHR-V), and Wistar-Kyoto (WKY) rats. Values are mean±SEM (n=6 rats per group).

When rings pretreated with indomethacin were contracted with 77 mmol/L KCl, no difference was found in ACh-induced NO-mediated relaxation among the 4 groups (Figure 4). The negative logarithm of the molar concentration values were as follows: SHR-C, 7.0±0.1; SHR-L, 6.7±0.2; SHR-V, 7.2±0.2; and WKY, 6.8±0.1 (n=6 per group; not significant. The maximal relaxation values were as follows: SHR-C, 70.6±3.1%; SHR-L, 70.8±6.0%; SHR-V, 65.7±3.7%; and WKY, 62.2±3.2% (n=6 per group; not significant).

Endothelium-Independent Hyperpolarization and Relaxation in Mesenteric Arteries

The relaxations in response to SNP, an NO donor, in rings precontracted with 10^{-5} mol/L PE did not differ among the 4 groups (Figure 5A, Table 2). Levcromakalim (10^{-5} mol/L), a direct activator of K_{ATP}, produced a comparable degree of hyperpolarization in the mesenteric arteries in all groups: SHR-C, −24.8±0.9 mV; SHR-L, −24.1±1.3 mV; SHR-V, −22.3±1.3 mV; WKY, −21.6±1.5 mV (n=4–5 per group; not significant). Although the maximal relaxations to levcromakalim in rings precontracted with 10^{-5} mol/L PE did not differ among the 4 groups, the sensitivities to levcromakalim

were significantly higher in the treated SHRs compared with the other groups (Figure 5B, Table 2).

Discussion

Our findings demonstrated that treatment with the Ang receptor neprilysin inhibitor LCZ696 and the ARB valsartan improved the impaired EDH and EDH-mediated relaxation in mesenteric arteries of SHRs. However, the dual blockade of RAS and NEP with LCZ696 did not exert any additional benefit over valsartan alone in ameliorating the impairment of endothelial function in this model.

In this study, SHRs were treated with either LCZ696 or valsartan for 3 months. Although the dose of each drug was determined on the basis of the results of preliminary experiments (2 weeks of treatment) in which the blood pressure of 8-month-old SHRs was lowered to comparable extents, there was a significant difference in SBP between LCZ696- and valsartan-treated SHRs when the length of treatment was extended to 3 months. The underlying mechanism of additive reduction in blood pressure in LCZ696-treated SHRs is not clear from the present study, but might simply relate to the higher valsartan dose (ie, 27 versus 20 mg). The greater blood pressure–lowering effect of LCZ696 might also be attributable to the increase in natriuretic peptides, leading to enhanced urinary sodium excretion and suppressed sympathetic activity.[21] Alternatively, because Ang II–Ang type 2 receptor and Ang-converting enzyme 2–Ang-(1–7) pathways generally oppose the actions of the Ang II–AT1R axis,[32] increases in plasma Ang II and/or Ang-(1–7) levels, if any, would be associated with significant reduction of blood pressure during treatment with LCZ696. This was evidenced by the treatment with vasopeptidase inhibitor omapatrilat in SHRs.[33] The mechanisms underlying the additive reduction in SBP in LCZ696-treated SHRs remain to be elucidated.

Herein, the ARB valsartan ameliorated the impaired EDH and EDH-mediated relaxation in mesenteric arteries of SHRs. These data are consistent with our previous study showing that another ARB, candesartan, improved impaired EDH-mediated responses in this vascular bed.[10] LCZ696 also improved EDH and EDH-mediated relaxation in mesenteric arteries of SHRs in the present study; however, the beneficial effects of LCZ696 on EDH-mediated responses appeared to be similar to those of valsartan alone. These findings suggest that vasoactive peptides, such as C-type natriuretic peptide, bradykinin, and substance P, which can be accumulated by NEP inhibition,[19,20] may not play a crucial role in the improvement of EDH-mediated responses during LCZ696 treatment in this vascular bed. Nevertheless, caution should be exercised in extrapolating the present results to other peripheral arteries, because the nature of EDH may be
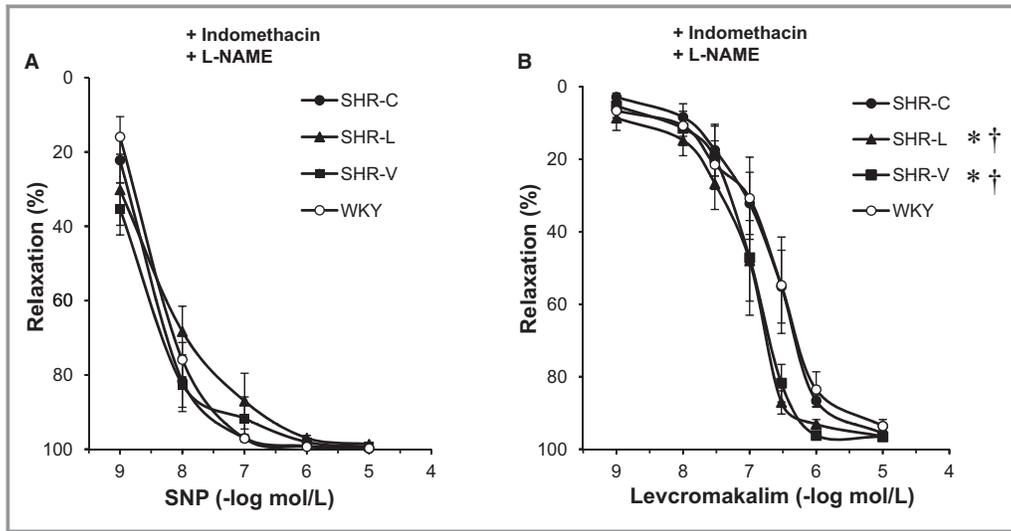
Figure 5. Concentration-response curves of relaxation in response to sodium nitroprusside (SNP; A) and levcromakalim (B) in endothelium-intact mesenteric arterial rings precontracted with phenylephrine (10^{-5} mol/L) in the presence of indomethacin (10^{-5} mol/L) and N$^{\omega}$-nitro-L-arginine (L-NAME; 10^{-4} mol/L) in the spontaneously hypertensive rats in the control group (SHR-C), SHRs treated with sacubitril/valsartan, 60 mg/kg per day (SHR-L), SHRs treated with valsartan, 20 mg/kg per day (SHR-V), and Wistar-Kyoto (WKY) rats. Values are mean±SEM (n=6 rats per group). *P<0.05 vs SHR-C, †P<0.05 vs WKY.

heterogeneous depending on the animal species and vascular bed studied.[34]

Although LCZ696 did not exert an additional benefit over valsartan alone in restoring EDH-mediated responses, despite the significant reduction in SBP, this does not imply that LCZ696 is less effective in improving endothelial function associated with hypertension. Our previous findings showed that there was a significant negative relationship between the amplitude of EDH and SBP in mesenteric arteries of SHRs; however, this negative relationship disappeared when the blood pressure was lowered within normotensive range.[10] In addition, blood pressure reduction alone did not improve EDH in mesenteric arteries of WKY rats.[35]

Indeed, in the present study, there was no significant relationship between the amplitude of EDH and SBP in the treated SHRs and WKY rats within the normotensive range. Thus, it seems likely that the further reduction in blood pressure within the normotensive range achieved with LCZ696 treatment in this study did not lead to a greater restoration of EDH in mesenteric arteries of SHRs. Similar to our present findings, Pu et al demonstrated that the treatment of stroke-prone SHRs with either valsartan or valsartan plus the NEP inhibitor CGS25354 improved ACh-induced relaxations comparably, despite the significant reduction in blood pressure in the valsartan+NEP inhibitor CGS25354–treated group.[36]

In contrast to our present findings, Kusaka et al reported that LCZ696 is more efficacious in improving ACh-induced relaxations than valsartan alone in SHR/NDmcr-cp(+/+) rats

fed a high-salt diet.[21] Because blood pressure lowering per se improves endothelial dysfunction in hypertension,[2,10] the superiority of LCZ696 over valsartan alone on ACh-induced relaxations may simply be attributable to the greater blood pressure reduction by LCZ696 than valsartan in SHR/NDmcr-cp(+/+) rats fed a high-salt diet.[21] Another possible explanation is that LCZ696 exerts beneficial effects on vasodilator function independently of blood pressure change under the experimental conditions used in the study by Kusaka et al.[21] Indeed, blood pressure–independent favorable effects of LCZ696 on cardiac and renal fibrosis have been reported.[37,38] The mechanisms by which LCZ696 improves vasodilator function more effectively than valsartan alone in high-salt diet conditions remain to be determined.

In addition to increasing several vasodilator substances, NEP inhibition may also elevate the level of a potent vasoconstrictor, ET-1.[19,20,39] In mice overexpressing human prepro–ET-1 restricted to the endothelium, an exaggerated production of ET-1 reduced endothelium-dependent, NO-mediated relaxation through the generation of reactive oxygen species.[40] However, in the present study, ACh-induced, NO-mediated relaxations in mesenteric arteries did not differ between LCZ696- and valsartan-treated SHR groups. Thus, it appears likely that ET-1 produced via NEP inhibition, if any, did not exert deleterious effects on endothelial function in our experimental conditions.

Consistent with our previous study,[10] the endothelium-independent relaxations in response to the NO donor SNP and those to the K_{ATP} channel opener levcromakalim were

preserved in mesenteric arteries of SHRs compared with those of WKY rats. In addition, the relaxations in response to SNP or levcromakalim treated with either LCZ696 or valsartan alone were, for the most part, similar to those of the nontreated SHR and WKY rats. However, the sensitivity to levcromakalim (as shown by the leftward shift in the dose-response curves) was significantly higher in both LCZ696- and valsartan-treated SHRs.

The reason for the increased sensitivity to levcromakalim in treated SHRs is not clear, but it might result from the blockade of Ang II with valsartan. This possibility is on the basis of the report that Ang II inhibited K_{ATP} channels through the activation of protein kinase C in freshly isolated vascular smooth muscle cells from rat mesenteric arteries.[41] Interestingly, pretreatment with fosinopril or valsartan reduced myocardial no reflow after acute myocardial infarction and reperfusion attributable to the activation of K_{ATP} channels in miniswine.[42]

Because vasoactive substances, such as adrenomedullin and calcitonin gene-related peptide, have produced membrane hyperpolarization through the opening of K_{ATP} channels in smooth muscle cells of rat mesenteric arteries,[26,27] the net result of such hyperpolarizing changes in smooth muscle cells would produce endothelium-independent vasodilation, thereby contributing to the blood pressure–lowering effect of LCZ696. However, this notion seems not to be the case in the present study because there was no difference in the resting membrane potential of mesenteric arteries between the LCZ696- and valsartan-treated groups. Thus, NEP inhibition per se appears to have had little effect on the vasodilator function of the mesenteric artery smooth muscle cells of SHRs in this study.

In conclusion, EDH and EDH-mediated relaxation were impaired in the mesenteric arteries of SHRs. Treatment with LCZ696 or the ARB valsartan improved EDH-mediated responses to similar extents. In addition, both treatments partially improved endothelium-independent relaxation. These findings suggest that LCZ696 exerts beneficial effects on endothelium-dependent relaxation and on endothelium-independent relaxation (although this effect was small). Our findings also indicate that an accumulation of vasoactive substances through the inhibition of NEP with LCZ696 does not appear to exert an additional benefit over valsartan alone on the endothelium-dependent and endothelium-independent vasodilator function in mesenteric arteries of SHRs. The clinical relevance of these findings warrants further investigation.

Sources of Funding

Disclosures

None.

References

1. Lawers CM, Hoorn SV, Rodgers A. Global burden of blood-pressure-related disease. *Lancet*. 2008;371:1513–1518.

2. Shimokawa H. Endothelial dysfunction in hypertension. *J Atheroscler Thromb*. 1998;4:118–127.

3. Schiffrin EL. Beyond blood pressure: the endothelium and atherosclerosis progression. *Am J Hypertens*. 2002;15:115S–122S.

4. Cameron AC, Lang NN, Touyz RM. Drug treatment of hypertension: focus on vascular health. *Drugs*. 2016;76:1529–1550.

5. Hill CE, Phillips JK, Sandow SL. Heterogeneous control of blood flow amongst different vascular beds. *Med Res Rev*. 2001;21:1–60.

6. Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br J Pharmacol*. 1988;93:515–524.

7. Chen G, Suzuki H, Weston AH. Acetylcholine releases endothelium-derived hyperpolarizing factors and EDRF from rat blood vessels. *Br J Pharmacol*. 1988;95:1165–1174.

8. Garland CJ, McPherson GA. Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br J Pharmacol*. 1992;105:429–435.

9. Fujii K, Tominaga M, Ohmori S, Kobayashi K, Koga T, Takata Y, Fujishima M. Decreased endothelium-dependent hyperpolarization to acetylcholine in smooth muscle of the mesenteric artery of spontaneously hypertensive rats. *Circ Res*. 1992;70:660–669.

10. Goto K, Fujii K, Onaka U, Abe I, Fujishima M. Renin-angiotensin system blockade improves endothelial dysfunction in hypertension. *Hypertension*. 2000;36:575–580.

11. Goto K, Fujii K, Kansui Y, Iida M. Changes in endothelium-derived hyperpolarizing factor in hypertension and ageing: response to chronic treatment with renin-angiotensin system inhibitors. *Clin Exp Pharmacol Physiol*. 2004;31:650–655.

12. Seki T, Goto K, Kiyohara K, Kansui Y, Murakami K, Haga Y, Ohtsubo T, Matsumura K, Kitazono T. Downregulation of endothelial transient receptor potential vanilloid type 4 channel and small-conductance of Ca2+-activated K+ channels underpins impaired endothelium-dependent hyperpolarization in hypertension. *Hypertension*. 2017;69:143–153.

13. Nabika T, Nara Y, Ikeda K, Endo J, Yamori Y. Genetic heterogeneity of the spontaneously hypertensive rat. *Hypertension*. 1991;18:12–16.

14. Xu Y, Henning RH, Lipsic E, van Buiten A, van Gilst WH, Buikema H. Acetylcholine stimulated dilatation and stretch induced myogenic constriction in mesenteric artery of rats with chronic heart failure. *Eur J Heart Fail*. 2007;9:144–151.

15. Gu J, Noe A, Chandra P, Al-Fayoumi S, Ligueros-Saylan M, Sarangapani R, Maahs S, Ksander G, Rigel DF, Jeng AY, Lin TH, Zheng W, Dole WP. Pharmacokinetics and pharmacodynamics of LCZ696, a novel dual-acting angiotensin receptor-neprilysin inhibitor (ARNI). *J Clin Pharmacol*. 2010;50:401–414.

16. Vardeny O, Tacheny T, Solomon SD. First-in-class angiotensin receptor neprilysin inhibitor in heart failure. *Clin Pharmacol Ther*. 2013;94:445–448.

17. Ruilope LM, Dukat A, Böhm M, Lacourcière Y, Gong J, Lefkowitz MP. Blood-pressure reduction with LCZ696, a novel dual-acting inhibitor of the angiotensin II receptor and neprilysin: a randomised, double-blind, placebo-controlled, active comparator study. *Lancet*. 2010;375:1255–1266.

18. McMurray JJ, Packer M, Desai AS, Gong J, Lefkowitz MP, Rizkala AR, Rouleau JL, Shi VC, Solomon SD, Swedberg K, Zile MR; PARADIGM-HF Investigators and Committees. Angiotensin-neprilysin inhibition versus enalapril in heart failure. *N Engl J Med*. 2014;371:993–1004.

19. Bavishi C, Messerli FH, Kadosh B, Ruilope LM, Kario K. Role of neprilysin inhibitor combinations in hypertension: insights from hypertension and heart failure trials. *Eur Heart J*. 2015;36:1967–1973.

20. Braunwald E. The path to an angiotensin receptor antagonist-neprilysin inhibitor in the treatment of heart failure. *J Am Coll Cardiol*. 2015;65:1029–1041.

21. Kusaka H, Sueta D, Koibuchi N, Hasegawa Y, Nakagawa T, Lin B, Ogawa H, Kim-Mitsuyama S. LCZ696, angiotensin II receptor-neprilysin inhibitor, ameliorates high-salt-induced hypertension and cardiovascular injury more than valsartan alone. *Am J Hypertens*. 2015;28:1409–1417.

22. Sandow SL, Tare M. C-type natriuretic peptide: a new endothelium-derived hyperpolarizing factor? *Trends Pharmacol Sci*. 2007;28:61–67.

23. Chauhan SD, Nilsson H, Ahluwalia A, Hobbs AJ. Release of C-type natriuretic peptide accounts for the biological activity of endothelium-derived hyperpolarizing factor. *Proc Natl Acad Sci U S A*. 2003;100:1426–1431.

24. Batenburg WW, Garrelds IM, van Kats JP, Saxena PR, Danser AH. Mediators of bradykinin-induced vasorelaxation in human coronary microarteries. *Hypertension*. 2004;43:488–492.

25. Ishizaka Y, Ishizaka Y, Tanaka M, Kitamura K, Kangawa K, Minamino N, Matsuo H, Eto T. Adrenomedullin stimulates cyclic AMP formation in rat vascular smooth muscle cells. *Biochem Biophys Res Commun*. 1994;200: 642–646.

26. Goto K, Fujii K, Onaka U, Abe I, Fujishima M. Effects of adrenomedullin and PAMP on membrane potential and neurotransmission. *Peptides*. 2000;21:257–263.

27. Dunn WR, Hardy TA, Brock JA. Electrophysiological effects of activating the peptidergic primary afferent innervation of rat mesenteric arteries. *Br J Pharmacol*. 2003;140:231–238.

28. Márton Z, Pataricza J, Krassói I, Varró A, Papp JG. NEP inhibitors enhance C-type natriuretic peptide–induced relaxation in porcine isolated coronary artery. *Vascul Pharmacol*. 2005;43:207–212.

29. Dalzell JR, Seed A, Berry C, Whelan CJ, Petrie MC, Padmanabhan N, Clarke A, Biggerstaff F, Hillier C, McMurray JJ. Effects of neutral endopeptidase (neprilysin) inhibition on the response to other vasoactive peptides in small human resistance arteries: studies with thiorphan and omapatrilat. *Cardiovasc Ther*. 2014;32:13–18.

30. Sofola OA, Knill A, Hainsworth R, Drinkhill M. Change in endothelial function in mesenteric arteries of Sprague-Dawley rats fed a high salt diet. *J Physiol*. 2002;543:255–260.

31. Goto K, Kansui Y, Oniki H, Ohtsubo T, Matsumura K, Kitazono T. Upregulation of endothelium-derived hyperpolarizing factor compensates for the loss of nitric oxide in mesenteric arteries of dahl salt-sensitive hypertensive rats. *Hypertens Res*. 2012;35:849–854.

32. Chappell MC. Biochemical evaluation of the renin-angiotensin system: the good, bad, and absolute? *Am J Physiol Heart Circ Physiol*. 2016;310:H137–H152.

33. Ferrario CM, Averill DB, Brosnihan KB, Chappell MC, Iskandar SS, Dean RH, Diz DI. Vasopeptidase inhibition and Ang-(1-7) in the spontaneously hypertensive rat. *Kidney Int*. 2002;62:1349–1357.

34. Sandow SL. Factors, fiction and endothelium-derived hyperpolarizing factor. *Clin Exp Pharmacol Physiol*. 2004;31:563–570.

35. Goto K, Fujii K, Onaka U, Abe I, Fujishima M. Angiotensin-converting enzyme inhibitor prevents age-related endothelial dysfunction. *Hypertension*. 2000;36:581–587.

36. Pu Q, Brassard P, Javeshghani DM, Iglarz M, Webb RL, Amiri F, Schiffrin EL. Effects of combined AT1 receptor antagonist/NEP inhibitor on vascular remodeling and cardiac fibrosis in SHRSP. *J Hypertens*. 2008;26:322–333.

37. von Lueder TG, Wang BH, Kompa AR, Huang L, Webb R, Jordaan P, Atar D, Krum H. Angiotensin receptor neprilysin inhibitor LCZ696 attenuates cardiac remodeling and dysfunction after myocardial infarction by reducing cardiac fibrosis and hypertrophy. *Circ Heart Fail*. 2015;8:71–78.

38. Wang BH, von Lueder TG, Kompa AR, Huang L, Webb R, Jordaan P, Atar D, Krum H. Combined angiotensin receptor blockade and neprilysin inhibition attenuates angiotensin-II mediated renal cellular collagen synthesis. *Int J Cardiol*. 2015;186:104–105.

39. Roksnoer LC, van Veghel R, de Vries R, Garrelds IM, Bhaggoe UM, Friesema EC, Leijten FP, Poglitsch M, Domenig O, Clahsen-van Groningen MC, Hoorn EJ, Jan Danser AH, Batenburg WW. Optimum AT1 receptor-neprilysin inhibition has superior cardioprotective effects compared with AT1 receptor blockade alone in hypertensive rats. *Kidney Int*. 2015;88:109–120.

40. Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, Schiffrin EL. Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation*. 2004;110:2233–2240.

41. Kubo M, Quayle JM, Standen NB. Angiotensin II inhibition of ATP-sensitive K+ currents in rat arterial smooth muscle cells through protein kinase C. *J Physiol*. 1997;503:489–496.

42. Zhao JL, Yang YJ, You SJ, Jing ZC, Wu YJ, Cheng JL, Gao RL. Pretreatment with fosinopril or valsartan reduces myocardial no-reflow after acute myocardial infarction and reperfusion. *Coron Artery Dis*. 2006;17:463–469.