LCZ696, Angiotensin II Receptor-Neprilysin Inhibitor, Ameliorates High-Salt-Induced Hypertension and Cardiovascular Injury More Than Valsartan Alone

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BACKGROUND
LCZ696, an angiotensin receptor-neprilysin inhibitor, has recently been demonstrated to exert more beneficial effects on hypertensive or heart failure patients than conventional renin-angiotensin system blockers. However, the mechanism underlying the benefit of LCZ696 remains to be understood. The present study was undertaken to examine the effect of LCZ696 compared with valsartan on hypertension and cardiovascular injury.

METHODS
(i) Using telemetry, we compared the hypotensive effect of LCZ696 and valsartan in spontaneously hypertensive rats (SHR) that were fed a high-salt diet followed by a low-salt diet. (ii) We also examined the comparative effect of LCZ696 and valsartan on salt loaded SHRcp, a model of metabolic syndrome.

RESULTS
(i) LCZ696 exerted a greater blood pressure (BP) lowering effect than valsartan in SHR regardless of high-salt or low-salt intake. Additive dual ARB/NEP inhibitor agent could potentially offer the clinical benefit in treatment of hypertension and cardiovascular diseases without risk of angioedema.

BP reduction by LCZ696 was associated with a significant increase in urinary sodium excretion and sympathetic activity suppression. (ii) LCZ696 significantly ameliorated cardiac hypertrophy and inflammation, coronary arterial remodeling, and vascular endothelial dysfunction in high-salt loaded SHRcp compared with valsartan.

CONCLUSIONS
LCZ696 caused greater BP reduction than valsartan in SHR regardless of the degree of salt intake, which was associated with a significant enhancement in urinary sodium excretion and sympathetic activity suppression. Furthermore, an additive BP lowering effect of LCZ696 led to greater cardiovascular protection in hypertensive rats.

Keywords: angiotensin; blood pressure; cardiovascular protection; dual inhibition; hypertension; neprilysin.

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The renin-angiotensin system and the natriuretic peptide system serve as a counter-regulatory constraint on the activity of the other.1,2 The protective effects of renin-angiotensin system blockade against hypertension and cardiovascular disease are potentially augmented by the increase in natriuretic peptides by inhibition of neutral endopeptidase (NEP), although there is no significant clinical benefit of NEP inhibitor monotherapy.3,4 In fact, omapatrilat, the most extensively studied dual NEP inhibition/angiotensin-converting enzyme (ACE) inhibition agent (vasopeptidase inhibitor), has shown to exert greater blood pressure (BP)-lowering effect and vascular protective effect than ACE inhibitor alone.1,2,5 However, omapatrilat treatment caused the increase in angioedema because of the increase in bradykinin, which hampered the development of omapatrilat as a therapeutic agent.6–8 On the other hand, angiotensin II receptor blockers (ARBs) have a lower risk of angioedema than ACE inhibitors, because of less contribution to metabolism of bradykinin.2,9 Therefore, dual ARB/NEP inhibitor agent could potentially offer the clinical benefit in treatment of hypertension and cardiovascular diseases without risk of angioedema.

LCZ696,10,11 a first-in class dual angiotensin II receptor and neprilysin inhibitor, is a single molecule that is comprised of molecular moieties of valsartan, an ARB, and AHU377, a neprilysin inhibitor (1:1 ratio). Thus, LCZ696 does not only inhibit the AT1 receptor but also slows natriuretic peptide degradation. Natriuretic peptides have potent natriuretic effects, act as vasodilators, inhibit aldosterone secretion and suppress sympathetic activity.12–14

A previous double-blind randomized trial comparing LCZ696 and valsartan with regard to BP lowering indicated that LCZ696 had a greater hypotensive effect than valsartan in hypertensive patients.15 Furthermore, recent findings of Prospective Comparison of ARNI With ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure (PARADIGM-HF), which compared LCZ696 with enalapril in heart failure patients...
with a reduced ejection fraction, demonstrated that LCZ696 was superior to enalapril in reducing the risks of death and of hospitalization after heart failure\(^{16}\) and that LCZ696 prevented the clinical progression of surviving patients with heart failure more effectively than enarapril.\(^{17}\) These clinical findings support the notion that LCZ696 may be a promising therapeutic agent for hypertension or heart failure beyond renin-angiotensin system blockers such as ACE inhibitors or ARB. However, the mechanism underlying the superiority of LCZ696 over conventional RAS blockers in BP reduction and heart failure prevention remains to be determined. Furthermore, to the best of our knowledge, there are only a few reports\(^{8,19}\) regarding LCZ696 preclinical studies using animal models. Therefore, in the present preclinical study, we compared the effect of LCZ696 and valsartan on BP or cardiovascular injury in spontaneously hypertensive rats (SHR) and SHR/NDmcr-cp(+/+) (SHRcp) rats.

**METHODS**

**Ethics statement**

All of the procedures were performed in accordance with the institutional guidelines for animal research and were approved by the Animal Care and Use Committee of Kumamoto University.

**Experimental animals and drugs**

Male SHR/NDmcr-cp(+/+) rats (SHRcp), a rat model of metabolic syndrome,\(^{19,20}\) and male SHRs were purchased from Japan SLC (Shizuoka, Japan). All of the rats were housed in an animal facility with a 12 hours light-dark cycle and were given water ad libitum.

LCZ696 and valsartan were kindly supplied by Novartis Pharma AG. LCZ696 is comprised of molecular moieties of valsartan and AHU377 (a neprilysin inhibitor) in a 1:1 ratio.

**Study protocol**

**Experiment I: effects of LCZ696 and valsartan on BP in SHRs after high-salt intake and low-salt intake.** Miniature telemetry devices were implanted into 12 or 13-week-old SHRs. BP and heart rate variability, low frequency power (LF) of systolic BP, and spontaneous baroreceptor reflex gain (sBRG) were monitored during the dark and light periods. The detailed protocol is shown in Figure 1A. The SHR diet was switched from low-salt (0.3% Na) to high-salt (8% Na) at 14 weeks of age, and the SHRs were divided into 3 groups at 16 weeks of age: (i) vehicle, (ii) LCZ696, and (iii) valsartan. The dose of LCZ696 and valsartan was 20 and 10 mg/kg/day from 16 to 18 weeks of age, respectively, and 60 and 30 mg/kg/day from 18 to 23 weeks of age, respectively (the end of the experiment). At 21 weeks of age, the SHR diet was switched to a low-salt diet from a high-salt diet. LCZ696 or valsartan was orally administered as a powder in gelatin mini-capsules (Torpac, Fairfield, NJ) at the beginning of the dark period once daily. The vehicle group rats were given empty capsules.

**Experiment II: effects of LCZ696 and valsartan on urinary sodium excretion in SHRs.** Fifteen-week-old SHRs fed a low-salt diet were randomly divided into 3 groups and were orally given (i) vehicle, (ii) LCZ696 (60 mg/kg per day), or (iii) valsartan (30 mg/kg per day). The rats were housed in metabolic cages to collect 24-hour urinary samples for 1 week, as described below.

**Experiment III: cardiovascular protection by LCZ696 and valsartan in high-salt loaded SHRcp.** The experimental protocol is demonstrated in Figure 1B. At 9 weeks of age, the SHRcp rat diet was switched from a low-salt (0.3% NaCl) diet to a high-salt (8% NaCl) diet. At 11 weeks of age, SHRcp rats were randomly divided into 3 groups and orally given (i) vehicle, (ii) LCZ696 (60 mg/kg per day), or (iii) valsartan (30 mg/kg per day). After 4 weeks of treatment (15 weeks of age), echocardiography was performed in these rats under isoflurane anesthesia as described below, blood was collected by cardiac puncture, and the heart was rapidly excised from each rat to examine cardiac hypertrophy, inflammation, remodeling, and various biochemical and molecular parameters.

**Statistical analysis**

All of the data are presented as means ± SEM. When data were normally distributed and variances were similar across comparison groups, statistical significance was determined with one-way analysis of variance followed by Fisher’s protected least significant difference test using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). When a normal distribution was not confirmed or similar variances were not obtained among comparison groups, data were analyzed with Kruskal–Wallis test followed by post hoc Dunn multiple comparison test. The time course experiment data were analyzed by one-way analysis of variance with repeated measurement to evaluate the main and interactive effects of time and groups on various parameters. In all of the tests, differences were considered to be statistically significant at a value of $P < 0.05$. 

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**Figure 1.** Protocol of Experiment I in SHRs (A) and Experiment II in SHRcp (B). Low Na, 0.3% Na diet; High Na, 8% Na diet; LCZ, LCZ696; Val, valsartan.
RESULTS

Comparative effects of LCZ696 and valsartan on 24-hour daily average blood pressure in high-sodium or low-sodium diet-fed SHRs

Figure 2A demonstrates daily 24-hour averaged systolic blood pressure (SBP) of high-salt followed by low-salt-fed SHRs that had been orally given vehicle, LCZ696, or valsartan once a day. The twenty-four-hour average SBP was increased with time by the high-salt diet. LCZ696 (20 mg/kg/day) and valsartan treatment (10 mg/kg/day) did not significantly lower the SBP of high-salt diet-fed SHRs throughout 14 days of treatment. However, higher doses (60 mg/kg/day) of LCZ696 significantly reduced SBP of the high-salt diet-fed SHR, while higher doses (30 mg/kg/day) of valsartan only slightly reduced the 24-hour average SBP of high-salt diet-fed SHRs compared with vehicle-treated SHRs. Twenty-four-hour SBP on day 35 was 222 ± 5, 201 ± 4, and 213 ± 2 mm Hg in vehicle, LCZ696, and valsartan-treated groups, respectively (vehicle vs. LCZ696, P < 0.01; LCZ696 vs. valsartan, P > 0.01). The change from high-salt diet to low-salt diet significantly and dramatically reduced the 24-hour average SBP of SHRs in all 3 groups. As in the high-salt diet condition, the SBP of LCZ696-treated SHRs was significantly lower than that of those treated with valsartan under low-salt diet conditions. On day 48, SBP in the LCZ696 group (155 ± 4 mm Hg) was significantly lower than that in the vehicle group (192 ± 3 mm Hg, P < 0.01) and in the valsartan group (172 ± 3 mm Hg, P < 0.01). The 24-hour average diastolic blood pressure (DBP) of SHRs (Figure 2B) was similar to the 24-hour average SBP of SHRs (Figure 2A).

There was no significant difference among the 3 groups regarding heart rate (Supplementary Figure 1A) or locomotor activity (Supplementary Figure 1B) throughout the treatment.

Comparative effects of LCZ696 and valsartan treatment on circadian blood pressure and autonomic function in SHRs fed a high-salt or low-sodium diet

Figure 3A,B demonstrates hourly-averaged SBP and DBP over 24 hours after a high oral dose of each drug (day 31 in Figure 2A) in high-salt diet-fed SHRs. Circadian blood pressure rhythm was similar between LCZ696 and valsartan treatment. LCZ696 administration reduced systolic and diastolic BP of SHRs during both dark and light periods more than valsartan. As demonstrated in Figure 3C,D, a greater reduction of SBP and DBP by LCZ696 than valsartan was also observed in low-salt diet-fed SHRs during both the dark and light periods.

Table 1 indicates the effects of LCZ696 and valsartan on circadian rhythms of autonomic function in high-salt or low-salt-fed SHRs. During the high-salt diet, neither LCZ696 nor valsartan treatment improved LF of SBP or sBRG in the SHRs. Conversely, compared with vehicle, LCZ696 but not valsartan treatment significantly reduced LF of SBP and increased sBRG in low-salt diet-fed SHRs during the dark period.

Effects of LCZ696 and valsartan treatment on urinary sodium excretion in SHRs

Twenty-four-hour excretion, intake, and sodium and water balance were measured in low-salt diet-fed SHRs. There were no differences in 24-hour sodium and water excretion, intake, and balance between vehicle-, LCZ696- and valsartan-treated groups. However, as demonstrated in Figure 4, LCZ696 treatment significantly decreased cumulative sodium balance in SHR compared with vehicle (P < 0.05) or valsartan (P < 0.05). There was no significant difference in urinary cGMP excretion among the vehicle (21.0 ± 1.0 μmol/day), LCZ696 (21.2 ± 3.3 μmol/day), and
Figure 3. Hourly-averaged SBP (A, left panel) or DBP (B, left panel) over 24 hours (12-hour dark period and 12-hour light period) and 12-hour-average SBP (A, right panel) or DBP (B, right panel) in high-salt diet-fed SHRs, and hourly-averaged SBP (C, left panel) or DBP (D, left panel) over 24 hours (12-hour dark period and 12-hour light period) and 12-hour-average SBP (C, right panel) or DBP (D, right panel) in low-salt diet-fed SHRs. Values are the means±SEM (n = 5 in each group). Abbreviations: High Na, 8% Na diet; Low Na, 0.3% Na diet; Veh, vehicle; LCZ, LCZ696; Val, valsartan. *P < 0.05, **P < 0.01 vs. Veh. †P < 0.01 vs. Val.
valsartan (19.2 ± 0.9 μmol/day)-treated groups. Body weight at the end of the treatment (7 days of treatment) did not differ among vehicle (334 ± 7 g), LCZ696 (331 ± 4 g), and valsartan (332 ± 10 g).

**Comparative effects of LCZ696 and valsartan on cardiac hypertrophy in high-salt loaded SHRcp**

The high-salt diet remarkably increased BP of SHRcp (Supplementary Figure 2). LCZ696 treatment significantly reduced BP of high-salt loaded SHRcp compared with vehicle, whereas valsartan treatment did not significantly reduce BP. After 2 weeks of drug treatment, BP was 235 ± 5, 207 ± 12, and 235 ± 2 mm Hg in vehicle-, LCZ696-, and valsartan-treated groups, respectively. LCZ696 but not valsartan treatment significantly reduced left ventricular weight in high-salt loaded SHRcp (Supplementary Table 1). Echocardiographic assessment in Figure 5 and Supplementary Figure 3 indicated that high-salt dramatically increased left ventricular wall thickness and decreased left ventricular cavity in SHRcp compared with low-salt. Interventricular septum and left ventricular posterior wall thickness as well as the left ventricular mass index in salt-loaded SHRcp were significantly decreased by LCZ696 but not by valsartan treatment. Moreover, LCZ696 treatment tended to attenuate the decrease in the left ventricular cavity compared to valsartan treatment.

**Effects of LCZ696 and valsartan treatment on cardiac fibrosis, coronary arterial remodeling, inflammation, and oxidative stress in high-salt loaded SHRcp**

As demonstrated in Figure 6 and Supplementary Figure 4, LCZ696 treatment ameliorated cardiac fibrosis, coronary arterial thickening, and ED-1 positive cell infiltration in high-salt loaded SHRcp to a greater extent than valsartan treatment.

**Effect of LCZ 696 and valsartan treatment on vascular endothelial function in high-salt loaded SHRcp**

As demonstrated in Figure 7, high-salt intake impaired vascular endothelium-dependent relaxation with acetylcholine in SHRcp. LCZ696 treatment significantly ameliorated the impairment of acetylcholine-induced vascular relaxation in high-salt loaded SHRcp, while valsartan treatment failed to ameliorate it. There was no significant difference in vascular endothelial-independent relaxation with sodium nitroprusside among all groups.

**EFFECTS OF LCZ696 AND VALSARTAN TREATMENT ON PLASMA AND SERUM VARIABLES, AND CYCLIC GMP LEVELS IN HIGH-SALT LOADED SHRCp**

As demonstrated in Supplementary Table 2, LCZ696 and valsartan treatments significantly increased plasma renin activity to a similar extent in high-salt loaded SHRcp. Neither...
Figure 5. Effect of LCZ and valsartan treatment on cardiac hypertrophy and function of high-salt loaded SHRcp. Values are the means ± SEM (n = 9 in Low Na, n = 7 in Veh, n = 7 in LCZ, n = 7 in Val). Abbreviations: Low Na, low-salt fed SHRcp; Veh, high-salt loaded SHRcp given vehicle; LCZ, high-salt loaded SHRcp given LCZ696; Val, high-salt loaded SHRcp given valsartan; IVSd, interventricular septum diastolic wall thickness; LVPw, left ventricular posterior diastolic wall thickness; LVM/BM, left ventricular mass per body weight; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; EF, ejection fraction. *P < 0.05, **P < 0.01.

Figure 6. Effect of LCZ696 and valsartan treatment on cardiac fibrosis (A), coronary arterial thickening (B), macrophage infiltration (C), and superoxide (D) levels in high-salt loaded SHRcp. Values are the means ± SEM (n = 9 in Low Na, n = 7 in Veh, n = 7 in LCZ, and n = 7 in Val). Abbreviations: Low Na, low-salt fed SHRcp; Veh, high-salt loaded SHRcp given vehicle; LCZ, high-salt loaded SHRcp given LCZ696; Val, high-salt loaded SHRcp given valsartan; IVSd, interventricular septum diastolic wall thickness; LVPw, left ventricular posterior diastolic wall thickness; LVM/BM, left ventricular mass per body weight; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; EF, ejection fraction. *P < 0.05, **P < 0.01.
LCZ696 or valsartan treatment significantly altered plasma aldosterone levels in high-salt loaded SHRcp. LCZ696 but not valsartan treatment significantly reduced the increase in serum creatinine in high-salt loaded SHRcp. There were no significant differences in total cholesterol, triglyceride, glucose, or insulin levels among vehicle, LCZ696, and valsartan groups. There were no significant differences in serum Na, K, or Cl among all groups.

As shown in Supplementary Table 3, in both plasma and vascular tissues, cyclic GMP levels in LCZ696 group tended to be greater than vehicle or valsartan group, although the difference did not reach statistical significance. Vascular cyclic GMP levels in LCZ696-treated SHRcp fed high-salt diet was significantly greater than low-Na diet fed SHRcp ($P < 0.05$), while no significant difference in vascular cyclic GMP levels was noted between low-Na diet fed SHRcp and vehicle or valsartan group of high-salt fed SHRcp.

**DISCUSSION**

Despite clinical evidence demonstrating the superiority of LCZ696 over conventional RAS blockers for hypertension or heart failure treatment, basic studies investigating the pharmacological effect of LCZ696 have been very much limited. Our present study examined the effects of LCZ696 in detail compared with ARB in SHRs, which is the most popular rat model of hypertension, and in SHRcp, the useful model of metabolic syndrome with hypertension. The major findings of this work were as follows: (i) LCZ696 treatment lowered BP of SHRs more than valsartan treatment regardless of high-salt or low-salt conditions; (ii) greater blood pressure lowering was observed with LCZ696 than valsartan treatment in SHRs, which was associated with significant inhibition of sympathetic activity and significant excretion of urinary sodium; (iii) LCZ696 treatment significantly ameliorated cardiac hypertrophy inflammation and fibrosis, coronary arterial remodeling, and vascular endothelial dysfunction in SHRcp after high-salt diet compared with valsartan treatment.

High-salt intake significantly accelerates the development of hypertension as well as cardiovascular and renal injury. Furthermore, high-salt intake significantly lessens the blood pressure lowering effect of RAS blockers, while low-salt intake significantly enhances their blood pressure lowering effect. Therefore, it is important to determine whether salt intake can affect the BP lowering effect of LCZ696, which encouraged us to examine the effect of LCZ696 compared with valsartan in SHRs under both high- and low-salt diets. In the present study, the dose of LCZ696 given to hypertensive rats was 2-fold larger than that of valsartan alone, since the purpose of this study was to compare the effect of LCZ696 and valsartan alone under the same dose of valsartan (under the same degree of AT1 receptor inhibition). Comparable increase in plasma renin activity between LCZ696 and valsartan alone confirmed similar inhibition of AT1 receptor between the two drugs. Continuous BP monitoring with telemetry revealed that LCZ696 exerted a greater blood pressure lowering effect compared with valsartan regardless of high- or low-salt condition. Furthermore, greater BP reduction by LCZ696 was observed during both dark and light cycles.

It remains to be determined how LCZ696 causes a greater BP reduction than ARB. To elucidate the mechanism
responsible for the greater BP reduction by LCZ696, we examined the effect of LCZ696 on cumulative sodium balance. We demonstrated that cumulative sodium balance was significantly lower in the LCZ696 group than the vehicle or valsartan treatment groups. Therefore, the BP lowering effect of LCZ696 beyond ARB seems to be mediated at least in part by increased urinary sodium excretion. Furthermore, spectral analysis of telemetry suggested that LCZ696 treatment might suppress sympathetic activity more than valsartan treatment as demonstrated by lower LF of SBP in the LCZ696 group compared with the vehicle or valsartan groups. Natriuretic peptides enhance urinary sodium excretion and inhibit sympathetic activity. Collectively, our present work suggests that greater BP reduction by LCZ696 compared with valsartan might be attributed to the increase in natriuretic peptides by neprilysin inhibition. However, further study is required to define the accurate mechanism underlying the additive BP lowering effect of LCZ696 beyond ARB.

There is no clinical or experimental report investigating whether greater BP reduction by LCZ696 than ARB can lead to greater cardiovascular protection. To address this issue, we compared the effect of LCZ696 and valsartan treatment on cardiovascular injury in high-salt loaded SHRcp. Consistent with the observations on SHRs, LCZ696 treatment significantly reduced the BP of high-salt diet-fed SHRcp. The BP reduction of high-salt diet-fed SHRcp by LCZ696 treatment was associated with greater prevention of cardiac hypertrophy than valsartan as demonstrated by cardiac weight and echocardiographic parameters. Furthermore, LCZ696 treatment significantly prevented the progression of cardiac fibrosis and inflammation as well as coronary arterial remodeling in high-salt diet-fed SHRcp compared with valsartan treatment. Furthermore, the impairment of vascular endothelial function in high-salt diet-fed SHRcp was significantly ameliorated by LCZ696 treatment compared to vehicle or valsartan treatment. These findings provided the first experimental evidence that the greater BP lowering effect of LCZ696 compared with ARB better protects against cardiovascular injury. However, very recent in vitro study using cultured cells shows that LCZ696 significantly augments the inhibition of cardiac myocyte hypertrophy and collagen accumulation by valsartan. Accordingly, it cannot be excluded that greater cardioprotection by LCZ696 in the present study might be partially attributed to BP-independent effect of LCZ696.

Study limitation

There are several limitations in this study. First, plasma or vascular cyclic GMP levels were not significantly increased by LCZ696 compared with valsartan, although there was the trend for the increase in cyclic GMP levels by LCZ696. Therefore, the present study did not allow us to determine to what degree natriuretic peptides contributed to the superiority of LCZ696 over valsartan regarding hypotensive effect and cardiovascular protection. Second, neprilysin cleaves not only natriuretic peptides but also other vasoactive peptides. Thus, it cannot be ruled out that greater benefit of LCZ696 than valsartan in hypertensive rats might be partially attributed to other peptide(s) than natriuretic peptides. Finally, in the present study, we found no difference between LCZ696 and valsartan regarding the effects on insulin, glucose, or lipid levels in the blood of high-salt loaded SHRcp. However, further study is needed to compare the metabolic effects of LCZ696 and valsartan.

In conclusion, we compared LCZ696 and ARB treatment on BP and cardiovascular injury in detail in an animal model of hypertension. We obtained evidence that LCZ696 more greatly reduced BP than ARB in genetically hypertensive rats regardless of high-salt or low-salt intake, which was associated with increased urinary sodium excretion or inhibition of sympathetic activity by LCZ696. Furthermore, our present work provided experimental evidence supporting that the additive BP reduction by LCZ696 compared with ARB leads to greater cardiovascular protection. However, further study is required to define the exact mechanism that is responsible for the additive BP lowering effect of LCZ696.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at American Journal of Hypertension (http://ajh.oxfordjournals.org).

DISCLOSURES

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