

学位論文

Cardioprotective Effects of LCZ696 (sacubitril/valsartan)
After Experimental Acute Myocardial Infarction
(LCZ696(サクビトリル/バルサルタン)の急性心筋梗塞後の心保護効果)

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PRECLINICAL RESEARCH

Cardioprotective Effects of LCZ696 (Sacubitril/Valsartan) After Experimental Acute Myocardial Infarction



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CME/MOC Objective for This Article: Upon completion of this activity, the learner should be able to: 1) identify the potential cardioprotective mechanisms of LCZ696 in a mice model undergoing an experimental myocardial infarction; 2) recognize the current guidelines of medical therapy in the post-myocardial infarction population; and 3) review the findings of PARADIGM-HF and the use of LCZ696 in the heart failure population.

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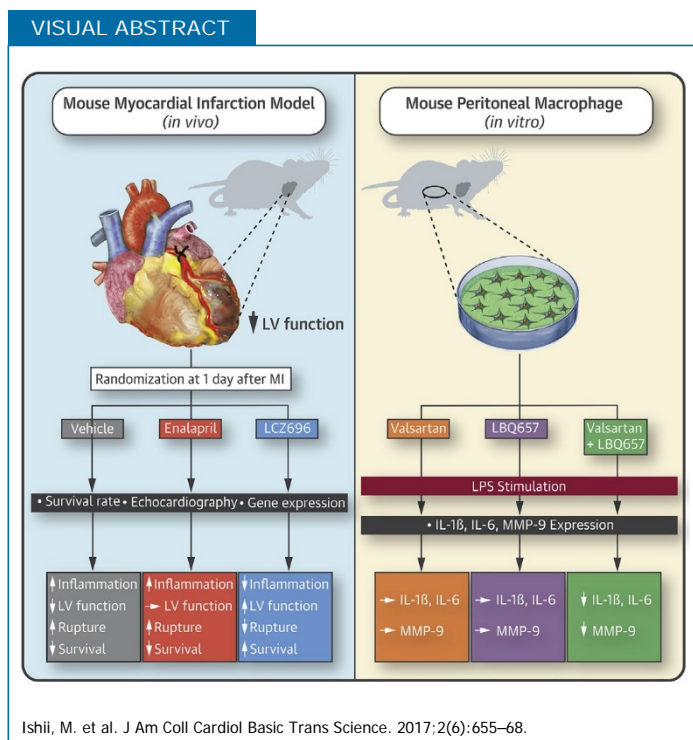
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HIGHLIGHTS

- Although the long-term effects of LCZ696 (sacubitril/valsartan) on cardiac dysfunction have been previously

elucidated, the short-term effects on acute phase after acute MI is unknown.

- Wild-type mice subjected to ligation of left anterior descending artery were randomly allocated to the vehicle, enalapril, or LCZ696 group 1 day after MI, resulting in the belief that LCZ696 could prevent cardiac rupture compared to the vehicle, whereas there was no significant difference in rate of cardiac rupture-free survival in the enalapril compared to the vehicle.
- At 3 days after MI, the expression of interleukin-1 β , interleukin-6, matrix MMP-9 mRNA and MMP-9 activity in the infarcted myocardium were significantly lower, plasma aldosterone levels were significantly lower, and cGMP levels were significantly higher, in the LCZ696 than the other groups.
- In vitro assay using peritoneal macrophages found that the combination of valsartan plus LBQ657 (active form of sacubitril) reduced the LPS-induced gelatinolytic activity and MMP-9 mRNA expression of macrophages.
- LCZ696 could suppress pro-inflammatory cytokines and extracellular matrix degradation in macrophages, and provides protection against post-MI cardiac rupture in mice.

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All authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* [author instructions page](#).

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SUMMARY

LCZ696 (sacubitril/valsartan) can lower the risk of cardiovascular events in chronic heart failure. However, it is unclear whether LCZ696 can improve prognosis in patients with acute myocardial infarction (MI). The present study shows that LCZ696 can prevent cardiac rupture after MI, probably due to the suppression of pro-inflammatory cytokines, matrix metalloproteinase-9 activity and aldosterone production, and enhancement of natriuretic peptides in mice. These findings suggest the mechanistic insight of cardioprotective effects of LCZ696 against acute MI, resulting in the belief that LCZ696 might be useful clinically to improve survival after acute MI. (J Am Coll Cardiol Basic Trans Science 2017;2:655–68) © 2017 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

ABBREVIATIONS
AND ACRONYMS

ARB = angiotensin receptor blocker
RAAS = renin-angiotensin-aldosterone system
NP = natriuretic peptide
HF = heart failure
MI = myocardial infarction
%FS = percent fractional shortening

LCZ696 (sacubitril/valsartan) is comprised of the neprilysin inhibitor sacubitril and the angiotensin receptor blocker (ARB) valsartan, the so-called angiotensin receptor–neprilysin inhibitor (1). Although persistent overdrive of the renin-angiotensin-aldosterone system (RAAS) leads to the development of heart failure (HF), the natriuretic peptide (NP) system, which is degraded by neprilysin, is also activated as counter-regulatory of the RAAS in HF pathology, and has beneficial effects, such as vasodilation, natriuresis, and anti-cardiac remodeling (2–4). By simultaneously inhibiting the angiotensin receptor and neprilysin, LCZ696 improves cardiac dysfunction, hypertension, cardiovascular injury, and ischemic brain damage in experimental and clinical studies (5–10). A previous randomized clinical trial showed that LCZ696 significantly reduces the risks of cardiovascular death and hospitalization for HF with reduced ejection fraction than enalapril, resulting in a new treatment option for chronic HF beyond the RAAS blockade (5). In experimental HF with reduced ejection fraction models, LCZ696 ameliorates cardiac remodeling compared to vehicle, probably due to superior suppression of cardiac fibrosis and hypertrophy compared with either stand-alone neprilysin inhibitor or ARB (6), and improves cardiac function with the reduction of fibrosis by suppressing transforming growth factor (TGF)- β (9).

Although the long-term benefits of LCZ696 on cardiac function and prognosis have been explained, it remains to be elucidated whether it can also ameliorate cardiac dysfunction in the short-term. The aim of the present study was to determine the effects of LCZ696 on acute phase of experimental myocardial infarction (MI) in mice, and was to clarify whether LCZ696 has the cardioprotective effects beyond the RAAS blockade by comparison with enalapril that had been selected as a control arm in the PARADIGM-HF (Prospective comparison of angiotensin receptor–neprilysin inhibitor with ACEI [angiotensin-converting enzyme inhibitor]

to Determine Impact on Global Mortality and morbidity in Heart Failure) trial (5).

METHODS

ANIMALS. All animal procedures were approved by the Animal Care and Use Committee of Kumamoto University, and conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (11). Male C57BL/6J (wild-type) mice were purchased from CLEA Japan Inc. (Tokyo, Japan). All mice were housed under a 12-h light-dark cycle and provided with regular chow diet and water ad libitum, and were used for experiments between 10 and 12 weeks of age.

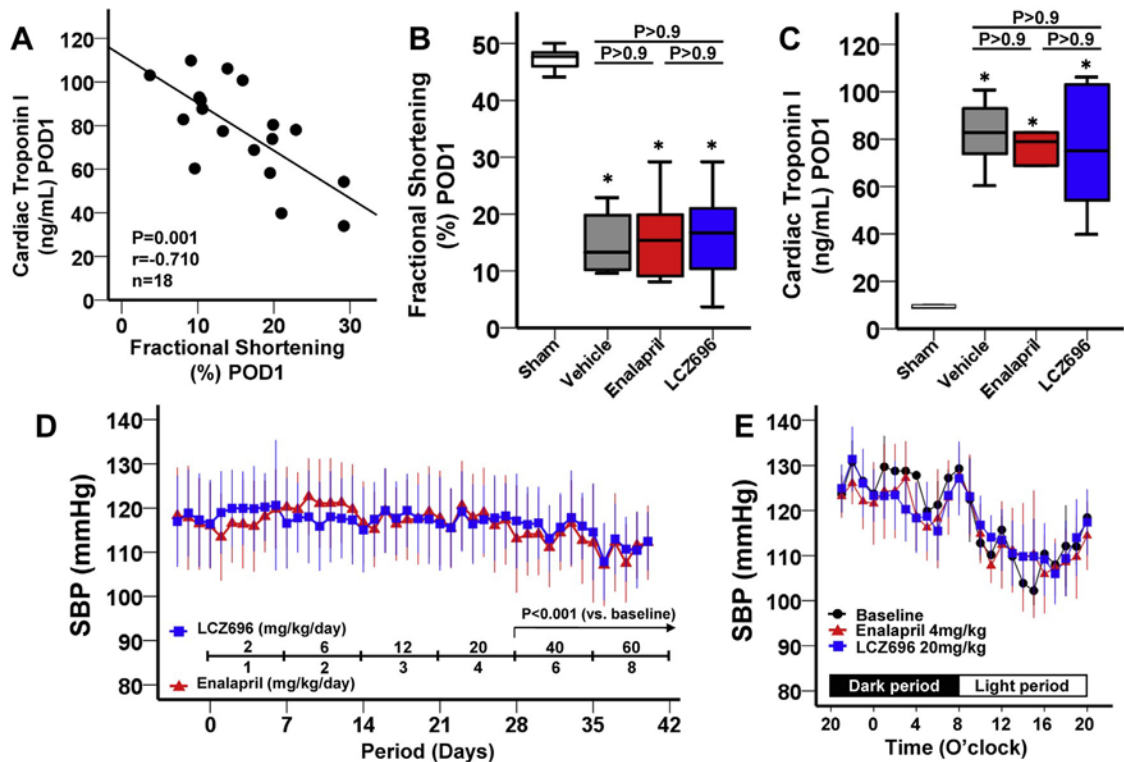
MATERIALS. LCZ696 was kindly provided by Novartis Pharma AG (Basel, Switzerland). Enalapril was purchased from Wako Pure Chemical Industries (Osaka, Japan). Both drugs were formulated in corn oil, and administered orally by gastric gavage at the beginning of the dark period once daily. Valsartan was purchased from Sigma-Aldrich (St. Louis, Missouri), and LBQ657 (an active form of sacubitril) was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada), which were used in an *in vitro* assay.

PRELIMINARY DOSE-RANGING STUDY USING RADIOTELEMETRY.

At 10 weeks of age, each mouse was implanted surgically with a telemetry device (Data Sciences International, St. Paul, Minnesota) for recording arterial pressure, as described in detail previously (12). After a recovery period of at least 2 weeks, baseline blood pressure (BP) and heart rate were recorded for 3 days. After the baseline recording, mice were orally administered LCZ696 and enalapril in incremental doses every week (LCZ696: 2, 6, 12, 20, 40, and 60 mg/kg body weight [BW]/day; enalapril: 1, 2, 3, 4, 6, and 8 mg/kg BW/day) to examine the antihypertensive effect.

EXPERIMENTAL MOUSE MODEL, ENROLLMENT CRITERIA, AND RANDOMIZATION. Mice were anesthetized with 2.0% isoflurane, and MI was induced by permanent

FIGURE 1 Preliminary Study Using Echocardiography, Biomarker Measurement, and Radiotelemetry



(A) Correlation curve for serum troponin I level and fractional shortening obtained from 18 mice 1 day after myocardial infarction (MI), with a correlation coefficient of 0.710 ($p < 0.001$). (B, C) At 1 day after MI, fractional shortening in infarcted mice was significantly lower, and the serum troponin I level in infarcted mice was significantly higher, compared with sham-operated mice ($p < 0.05$ and $p < 0.05$, respectively). There were no differences in these parameters between MI mice treated with vehicle, enalapril, and LCZ696. Results were mean \pm SD. $n = 5$ to 6 per group. * $p < 0.05$ vs. sham. (D) 24-h average SBP in mice treated with enalapril and LCZ696. Each drug was administered in an incremental manner every week by gastric gavage once daily. The dose of 20 mg/kg BW/day LCZ696 and 4 mg/kg BW/day enalapril were the maximum that did not lower baseline blood pressure. Results were mean \pm SD. $n = 4$ per group. (E) Circadian rhythm of SBP in mice treated with LCZ696, 4 mg/kg BW/day enalapril, and baseline during the 12-h dark and 12-h light periods. There was no interaction between group and period ($p = 0.182$ for interaction). Results were mean \pm SD. BW = body weight; POD = post-operative day; SBP = systolic blood pressure.

ligation of the left anterior descending coronary artery at the level of the left atrium with 8-0 silk suture under mechanical ventilation (tidal volume, 0.6 ml; rate, 110 breaths per min), as described in detail previously (13). Significant ischemic changes on electrocardiography and color changes in the cardiac ischemic area indicated successful coronary occlusion. Within 24 h after coronary ligation, we evaluated M-mode percent fractional shortening (%FS) in the surviving mice.

A pilot study performed to determine the correlation between %FS and serum troponin I level in 18 MI mice at 24 h after MI showed a correlation coefficient of 0.710 ($p = 0.001$) (Figure 1A). To minimize the effect of variability in infarct size, mice with %FS $\geq 30\%$ were excluded due to the small infarct size.

Thus, the study included only mice with %FS of $< 30\%$. These mice were divided at random into 3 treatment groups: the LCZ696 (20 mg/kg BW/day, $n = 75$), enalapril (4 mg/kg BW/day, $n = 79$), and vehicle (corn oil only, $n = 77$) groups. The treatment was started 1 day after MI by oral gavage. There were no differences in infarct size at postoperative day 1 among the vehicle, enalapril, and LCZ696 groups allocated by the %FS and serum troponin level (Figures 1B and 1C, respectively), indicating successful randomization. To investigate the cardioprotective effect, which is independent of the antihypertensive effect, we selected the maximum dose of each drug that did not lower the baseline BP measured by telemetry (Figures 1D and 1E). In the sham-operated mice, the same surgery was conducted without

coronary ligation. Cardiac rupture was defined as blood clot in the chest cavity and left ventricular wall tear.

Detailed methods are provided in the [Supplemental Appendix](#).

SAMPLE POWER ANALYSIS. Before the start of the study, statistical power was performed using the IBM

SPSS SamplePower (IBM Corporation, Armonk, New York) to estimate the required sample size. The sample size for survival analysis between 2 groups (vehicle group and LCZ696 group) was based on a 2-tailed Kaplan-Meier survival analysis by the log-rank test with a significant level set at 0.0166, which was calculated by Bonferroni correction, a power level of 0.80, median survival time of the control group of 11.55 days, and hazard ratio of the control group relative to treatment group of 0.33, as reported previously (14). The required sample size was 74 in each group with a total of 222 mice. The sample size for echocardiography assessment (%FS) between 2 groups (vehicle group and LCZ696 group) was based on a 2-tailed unpaired Student's *t*-test with a significant level set at 0.0166, which was calculated by Bonferroni correction, a power level of 0.80, the mean difference in those groups of 15%, and the within-group standard deviation of the control group of 19.3%, as reported previously (15). The required sample size was 36 in each group with a total of 108 mice.

STATISTICAL ANALYSIS. Data of normally distributed continuous variables are expressed as mean \pm SD, whereas those with skewed distribution are presented as median values (interquartile range [IQR]). To determine change from baseline (defined as the average of 2 days before administration) within group, the data of 24-h systolic blood pressure (SBP) measured by telemetry was analyzed by 1-way analysis of variance (ANOVA) with repeated measures followed by a Bonferroni multiple comparison adjustment. The data of circadian rhythm of SBP measured by telemetry were analyzed by 2-way ANOVA with repeated measures including the interaction between group and period, followed by multiple comparisons with the Bonferroni method. The data of echocardiography were analyzed by 2-way ANOVA with repeated measures followed by multiple comparisons with the Bonferroni method. The Kaplan-Meier method with the log-rank test followed by a Bonferroni multiple comparison adjustment was used to compare survival curves among the groups. Two-group comparisons were analyzed by the Mann-Whitney *U* test, whereas multiple groups' comparisons were analyzed by the ANOVA or Kruskal-Wallis test for continuous variables followed by multiple

comparisons with the Bonferroni method, as appropriate. A 2-tailed *p* value of <0.05 denoted the presence of a statistically significant difference. All statistical analyses were performed with The Statistical Package for Social Sciences software version 23.0 (IBM Corporation).

RESULTS

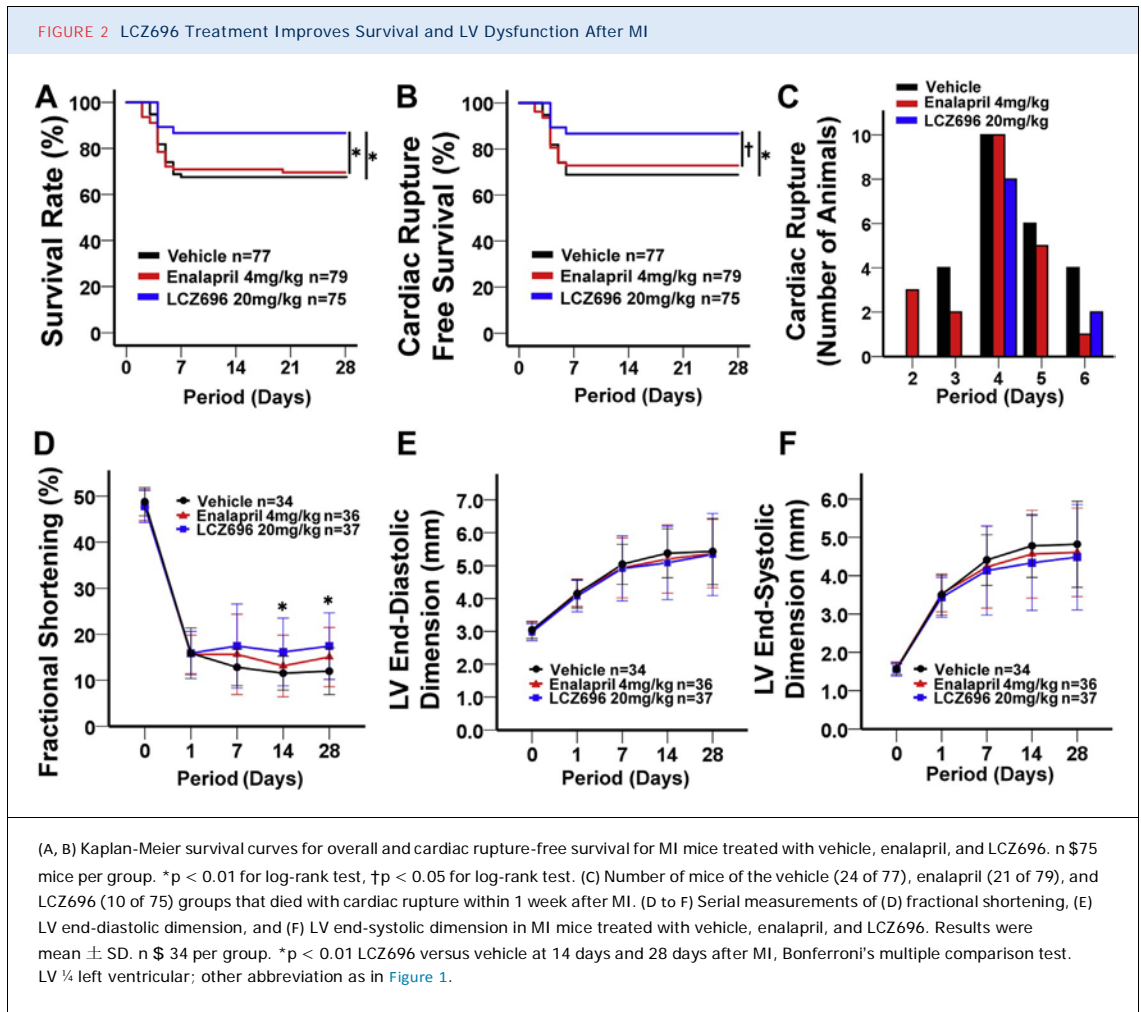
SURVIVAL AND CARDIAC RUPTURE AFTER MI. The post-MI survival rate was significantly higher in the LCZ696 group compared with the vehicle ($p < 0.01$) and enalapril ($p < 0.01$) group (Figure 2A). Interestingly, the most frequent cause of death (94.8%) in the groups was left ventricular (LV) rupture, which was confirmed by blood coagulation around the pericardial sac and small slits commonly observed in the LV wall. Figure 2B shows the LV rupture-free survival curves, indicating that the LCZ696 group had a significantly lower rate of death due to LV rupture compared with the vehicle ($p < 0.01$) and enalapril ($p < 0.05$) groups. Figure 2C shows the number of the mice that died of LV rupture, which occurred within 6 days after MI. The remaining 5.2% deaths were due to HF. Between 8 days and 28 days after MI, only 1 mouse (from the enalapril group) died due to HF.

PHYSIOLOGIC AND ECHOCARDIOGRAPHIC PARAMETERS.

Figures 2D to 2F show changes in echocardiographic parameters, including FS, left ventricular end-diastolic dimension (LVDd), and left ventricular end-systolic dimension (LVDs). There were no significant differences in LVDd, LVDs, and %FS before and 1 day after MI among the groups. The %FS was significantly improved 14 days and 28 days after MI in the LCZ696 group compared with the vehicle group ($p < 0.05$, mean difference; 4.60, 95% confidence interval: 0.03 to 9.18, and $p < 0.05$, mean difference; 5.45, 95% confidence interval: 0.78 to 10.10, respectively), and tended to improve compared with enalapril (Figure 2D), whereas there were no differences in LVDd and LVDs between the groups (Figures 2E and 2F). Enalapril tended to improve %FS after MI compared with the vehicle group, but had not reached statistical significance.

HISTOMORPHOMETRIC AND IMMUNOHISTOCHEMICAL ANALYSIS.

To determine the effect of LCZ696 on the acute pathophysiological response to MI, we evaluated the histological and immunohistochemical changes in the infarcted region 3 days after MI. Masson's trichrome and immunohistochemical-stained tissues showed equal accumulation of collagen fibers and inflammatory cells, such as FA-11-positive macrophages and Gr-1-positive granulocytes, into the



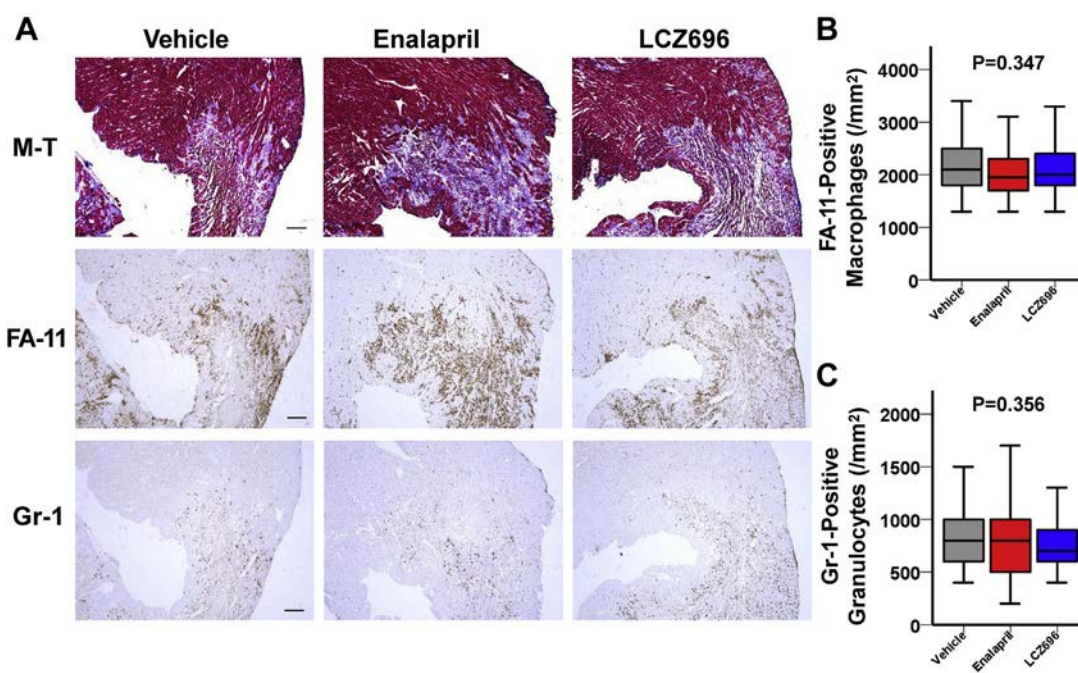
infarcted regions of the vehicle, enalapril, and LCZ696 groups (Figures 3A to 3C).

EXPRESSION OF mRNA IN INFARCTED AND NON-INFARCTED REGIONS. To investigate the mechanism of suppression of cardiac rupture by LCZ696, the mRNA expression levels of cytokines involved in cardiac inflammation and fibrosis were measured in the infarcted region 3 days after MI. The interleukin (IL)-1 β mRNA expression was significantly lower in LCZ696 compared to the vehicle (p ¼ 0.028) and enalapril (p ¼ 0.033) groups, and the IL-6 mRNA expression was significantly lower in LCZ696 compared to enalapril (p ¼ 0.036) (Figures 4B and 4C), whereas there were no differences in tumor necrosis factor (TNF)- α and monocyte chemoattractant protein-1 (MCP-1) mRNA expression levels among the 3 groups (Figures 4A and 4D). The mRNA expression levels of pro-fibrotic and fibrotic cytokines, such as TGF- β 1,

collagen type I α 1, and collagen type 3 α 1 in the infarcted region were not different among the groups (Figures 4E to 4G). Also, in the noninfarcted region, there were no significant differences in the expression levels of those mRNA expressions.

Next, we evaluated the mRNA expression of matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP), which are involved in cardiac rupture after MI, including MMP-2 (16,17), MMP-9 (18,19), and TIMP-1 (20), in the infarcted region 3 days after MI. There were no significant differences in MMP-2 mRNA expression among the 3 groups (Figure 4H), whereas MMP-9 mRNA expression was significantly lower in the infarcted region of LCZ696-treated mice compared to the vehicle (p ¼ 0.015) and enalapril (p ¼ 0.003) groups (Figure 4I). The mRNA expression of TIMP-1, which counteracts the activity of MMP-9, was significantly lower in the LCZ696 group compared to the vehicle

FIGURE 3 Characterization of Inflammatory Response in the Border Area of the Infarcted Region 3 Days After MI



(A) Representative images of LV cross-sections with Masson's trichrome staining (M-T) and immunohistochemistry (FA-11 and Gr-1) in the border of infarcted region 3 days after MI in the vehicle, enalapril, and LCZ696 groups. Scale bar $\frac{1}{4}$ 200 mm (B and C). Immunohistochemical staining showed macrophages (FA-11), and granulocytes (Gr-1) in the infarcted region 3 days after MI between the vehicle, enalapril, and LCZ696 groups. Results were mean \pm SD. n $\frac{1}{4}$ 7 per group.

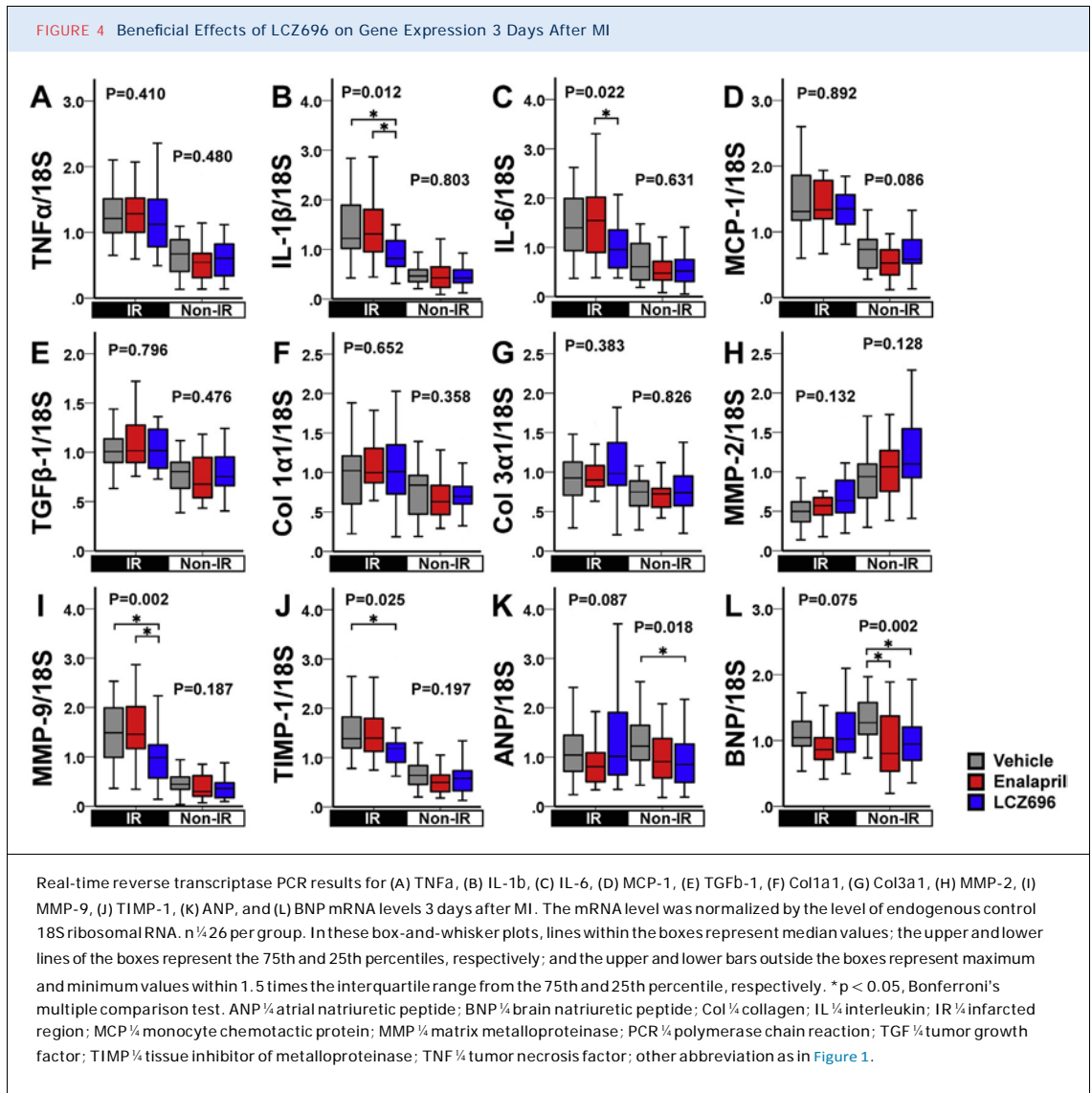
(p $\frac{1}{4}$ 0.045) (Figure 4J). On the other hand, in the noninfarcted region, there were no significant differences in the expression levels of these mRNA.

We measured atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA expression levels in the infarcted and noninfarcted regions 3 days after MI. ANP mRNA expression level in the noninfarcted region was significantly lower in the LCZ696 group (p $\frac{1}{4}$ 0.023) compared with the vehicle (Figure 4K). A similar trend was noted for BNP mRNA expression (LCZ696, p $\frac{1}{4}$ 0.025; enalapril, p $\frac{1}{4}$ 0.002; compared with the vehicle group) (Figure 4L). In the infarcted region, however, there were no significant differences in the expression levels of these mRNA.

GELATINOLYTIC ACTIVITY AND MACROPHAGE-DERIVED MMP LOCALIZATION IN INFARCTED REGION. To determine gelatinolytic activity derived from the enhanced MMP-9 mRNA expression in the infarcted region, we performed in situ zymography and MMP-9 activity assay. Figure 5A shows representative in situ zymographic images of the infarcted region 3 days after MI. Analysis of these images showed significantly lower gelatinolytic activity in LCZ696 than the vehicle

(p < 0.001) and enalapril (p $\frac{1}{4}$ 0.001) groups (Figure 5B). Activity assay showed that MMP-9 activity was significantly lower in LCZ696 compared with the vehicle (p $\frac{1}{4}$ 0.014) and enalapril (p $\frac{1}{4}$ 0.007) groups, whereas there were no differences in total MMP-9 among the three groups (Figures 5C and 5D). Double immunofluorescence images showed the localization of MMP-9 on F4/80-positive macrophages, indicating that macrophages might be 1 of the main sources of MMP-9 in the infarcted myocardium 3 days after MI (Figure 5E).

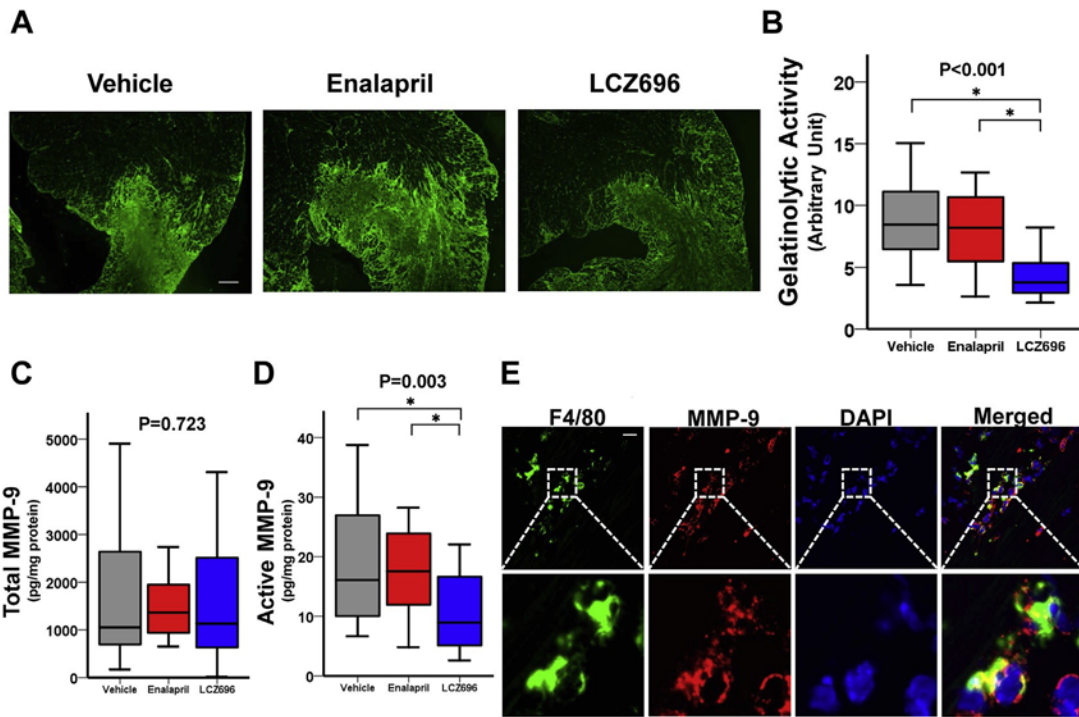
IN VITRO ASSAY WITH PERITONEAL MACROPHAGES. To determine whether LCZ696 could suppress MMP-9 activity in macrophages as double immunofluorescence staining showed the colocalization of MMP-9 on F4/80-positive macrophages, we conducted an in vitro assay using peritoneal macrophages. As shown in Figures 6A and 6B, lipopolysaccharide (LPS)-induced MMP-9 activity detected by gelatin zymography was significantly lower in the supernatant pre-treated with valsartan β LBQ657 compared to those with valsartan alone, and LBQ657 alone (p $\frac{1}{4}$ 0.007 and p $\frac{1}{4}$ 0.001, respectively). On the other



hand, there was no detectable MMP-2 activity in the 3 groups (Figure 6A). Figures 6C and 6D showed the protein levels of IL-1 β and IL-6 in the supernatants of LPS-stimulated peritoneal macrophages. Both IL-1 β and IL-6 concentrations were significantly decreased in the group pre-treated with valsartan + LBQ657 compared to those with valsartan alone, or LBQ657 alone (IL-1 β : p = 0.015, p = 0.007; and IL-6: p = 0.021, p = 0.005, respectively). As shown in Figure 6E, the LPS-induced MMP-9 mRNA expression was significantly lower in peritoneal macrophages pre-treated with valsartan + LBQ657 compared to those with valsartan alone, and LBQ657 alone (p = 0.028, and p = 0.002, respectively). On the other hand, there were no significant differences in the LPS-induced mRNA expression of IL-1 β and IL-6 among the groups (Figures 6F and 6G).

PLASMA ALDOSTERONE AND CYCLIC GUANOSINE MONOPHOSPHATE LEVELS AFTER MI. To evaluate effects of LCZ696 on plasma biomarkers, plasma aldosterone and cyclic guanosine monophosphate (cGMP) were measured 3 days after MI. As shown in Figure 7A, plasma aldosterone levels were significantly lower in the LCZ696 group compared to the vehicle (p = 0.014), but not different from those in the enalapril group (p = 0.482). Plasma levels of cGMP, which is the second messenger of natriuretic peptides, were significantly higher in the LCZ696 than the enalapril (p < 0.001), and vehicle (p = 0.033) groups (Figure 7B). To evaluate the balance between both systems of renin-angiotensin-aldosterone and natriuretic peptide, plasma aldosterone/cGMP ratio was calculated. As shown in Figure 7C, the aldosterone/cGMP ratio was significantly lower in the LCZ696 than

FIGURE 5 LCZ696 Suppresses MMP-9 Activity in the Infarcted Region 3 Days After MI



(A) Representative images of LV cross-sections with in situ zymography in the border area of the infarcted region 3 days after MI of the vehicle, enalapril, and LCZ696 groups. Scale bar $\frac{1}{4}$ 200 μ m (B). Semi-quantitative analysis of gelatinolytic activity showed significantly lower activity in the LCZ696 compared to the vehicle and enalapril groups. $n = 7$ per group. $*p < 0.05$, Bonferroni's multiple comparison test. (C, D) Specific MMP-9 activity assay in the infarcted region 3 days after MI, showing no differences in (C) total MMP-9 among the vehicle, enalapril, and LCZ696 groups. However, (D) active MMP-9 was significantly lower in the LCZ696 compared to the vehicle and enalapril group. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; and the upper and lower bars outside the boxes represent maximum and minimum values within 1.5 times the interquartile range from the 75th and 25th percentile, respectively. $n = 24$ per group. $*p < 0.05$, Bonferroni's multiple comparison test. (E) Double immunofluorescence images showing co-localization of MMP-9-positive cells with F4/80-positive cells in the border area of the infarcted region 3 days after MI. Scale bar $\frac{1}{4}$ 20 μ m. DAPI $\frac{1}{4}$ 4', 6-diamidino-2-phenylindole; other abbreviations as in Figure 4.

the enalapril ($p = 0.002$) and vehicle ($p = 0.001$) groups.

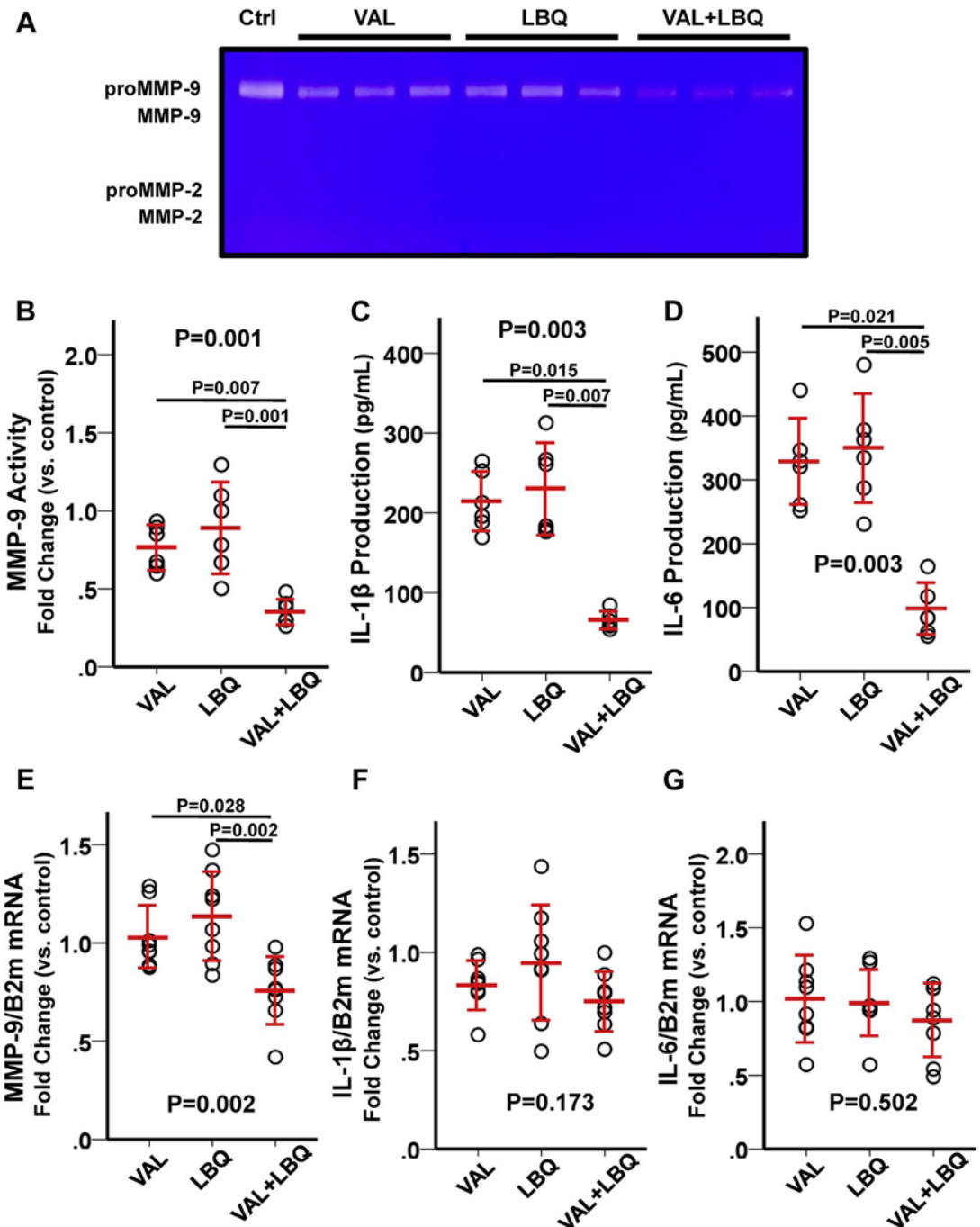
DISCUSSION

The present study showed that LCZ696 improved the imbalance between the renin-angiotensin-aldosterone and natriuretic peptide systems, and prevented cardiac rupture after MI, probably due to the inhibition of inflammation and degradation response of macrophages (Figure 8). To the best of our knowledge, this is the first study to show that early treatment with LCZ696 after MI might have a cardioprotective effect and improve survival through the inhibition of acute phase of post-MI inflammatory and degradation response.

The increase in the incidence of ischemic heart disease-related HF has become a global health and

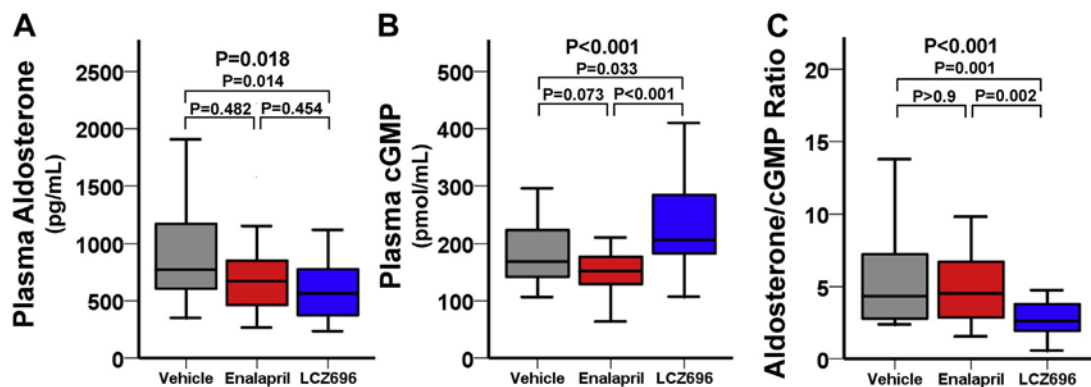
economic problem (21,22). Although HF is an important complication in the acute and chronic phases after acute MI, cardiac rupture is also a major lethal complication, even in the percutaneous coronary intervention era (23,24). To reduce cardiovascular events and all-cause mortality, early administration of angiotensin-converting enzyme inhibitors (ACEi), ARBs, and β -blockers after MI is recommended by the current American and European guidelines (25,26). The present study indicated that treatment with the maximum dose of LCZ696, which did not lower baseline BP, improved survival during the acute phase of MI. The rates of overall survival and cardiac rupture-free survival were significantly improved in the LCZ696-treated group when compared to the untreated control animals, whereas there was no significant difference in the overall survival and cardiac rupture-free survival in the enalapril-treated

FIGURE 6 Combined Effects of Valsartan With LBQ657 in In Vitro Assay Using Peritoneal Macrophages



(A) Gelatin zymography on the supernatants of peritoneal macrophages stimulated with LPS (10 ng/ml) for 48 h. (B) Semi-quantitative analysis of gelatinolytic activity showed significantly lower activity in VAL + LBQ compared to the VAL-alone and LBQ-alone groups. n = 6 per group. Quantification of (C) IL-1 β , and (D) IL-6 concentrations in the supernatants of peritoneal macrophages stimulated with LPS (10 ng/ml) for 48 h. n = 6 per group. (E to G) Real-time reverse transcriptase PCR result for (E) MMP-9, (F) IL-1 β , and (G) IL-6 mRNA level in peritoneal macrophages stimulated with LPS (10 ng/ml) for 48 h. The mRNA level was normalized by the level of endogenous control beta-2-microglobulin RNA. n = 8 per group. Results were mean \pm SD. Ctrl = control; LBQ = LBQ657; LPS = lipopolysaccharide; VAL = valsartan; other abbreviations as in Figure 4.

FIGURE 7 Beneficial Effects of the Dual Action of Neurohumoral System in LCZ696 Treatment

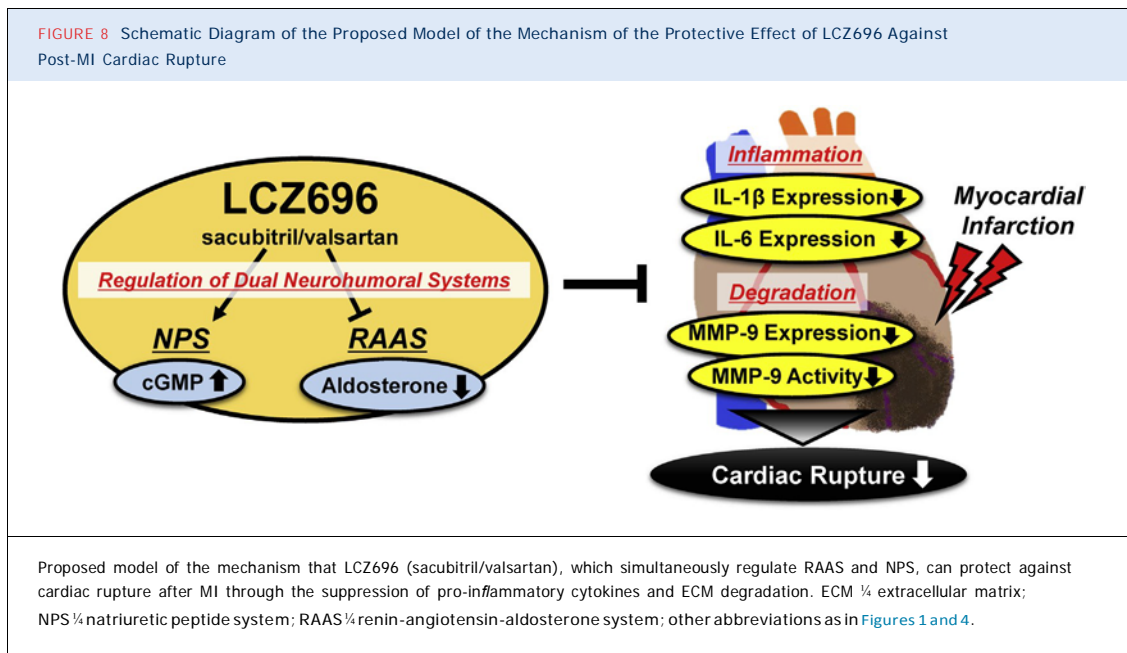


Quantification of plasma (A) aldosterone and (B) cGMP levels by ELISA 3 days after MI. (C) The ratio of plasma aldosterone to plasma cGMP. n = 28 per group. In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; and the upper and lower bars outside the boxes represent maximum and minimum values within 1.5 times the interquartile range from the 75th and 25th percentile, respectively. The inter-group comparisons were corrected by the Bonferroni's multiple comparison test. cGMP = cyclic guanosine monophosphate; ELISA = enzyme-linked immunosorbent assay; other abbreviation as in Figure 1.

group when compared to untreated control animals. Although the %FS after MI was improved significantly in the LCZ696 animals, it was not statistically different from values in the enalapril-treated group. Although ACEi has been established as a standard drug against chronic HF and cardiac remodeling after acute MI, enalapril did not improve the survival rate after MI compared to the vehicle group in the present study. However, because we chose the dose of 4 mg/kg of enalapril based on the absence of hemodynamic effects on BP, we cannot exclude that higher doses of enalapril would have had a beneficial effect on overall survival and cardiac rupture-free survival. The discrepancy between the previous evidences and present findings might be due to the specific dose of enalapril which was a maximum dose that did not significantly lower baseline blood pressure, as measured by telemetry method. We aimed to investigate the nonhypotensive effects of LCZ696 because BP tends to be decreased on acute phase after severe MI in the clinical setting, and it is difficult for those patients to receive the dose that lowers blood pressure. The present study provides the novel finding that LCZ696 could improve survival and remodeling after MI compared to enalapril even at the nonhypotensive dose. Although the effects of neprilysin inhibitor alone could not be assessed, previous studies showed that neprilysin inhibitor alone could not reduce cardiac hypertrophy in a post-MI rat model (27,28). Based on these observations, it is

possible that the effects of LCZ696 after MI may be derived from the effects of combined RAAS blockade and neprilysin inhibition rather than RAAS blockade or neprilysin inhibition alone in the setting of nonhypotensive dose. Further clinical investigation is needed to confirm whether the nonhypotensive doses of LCZ696 have a beneficial effect for survival and cardiac remodeling during the acute phase of MI.

Although the molecular mechanisms of the signaling pathways involved in cardiac rupture after MI are not yet completely understood, previous studies reported that MMP-9, which degrades extracellular matrix (ECM), is involved in tissue repair, remodeling, and development of cardiac rupture after MI (20,29), whereas inhibition of MMPs, including MMP-9, prevents cardiac rupture (19,20). The main sources of MMP-9 during the acute phase of MI are thought to be neutrophils, macrophages, and the myocardium (13,18,20,30). The present study showed that LCZ696 significantly decreased MMP-9 mRNA expression and activity, which was associated with a significant reduction in the rate of cardiac rupture compared to the vehicle and enalapril groups. Furthermore, in situ zymography showed that the extent of the gelatinolytic activity paralleled the extent of infiltration of macrophages (Figures 3A and 5A), a finding similar to that reported in our previous studies (13,31). In addition, double immunofluorescence images identified MMP-positive macrophages in the infarcted myocardium 3 days after MI



(Figure 5E). In vitro assay using peritoneal macrophages showed that the combination of valsartan plus LBQ657 reduced the LPS-induced gelatinolytic activity and MMP-9 mRNA expression of macrophages. Thus, it is possible that LCZ696 may inhibit MMP-9 secretion by macrophages in infarcted lesions in the acute phase of MI.

Although the molecular mechanism by which LCZ696 inhibits MMP-9 in the infarcted myocardium is poorly understood, we believe that LCZ696 protected against cardiac rupture by inhibiting MMP-9 activation. Measurement of plasma biomarkers showed that LCZ696 enhanced plasma cGMP levels and decreased plasma aldosterone levels. LCZ696 is a novel compound comprised of a neprilysin inhibitor (sacubitril) and an ARB (valsartan), whereby sacubitril suppresses the breakdown of natriuretic peptides, such as ANP and BNP, leading to the activation of natriuretic peptide receptor A and an increase in cGMP. On the other hand, valsartan blocks the actions of angiotensin II, leading to the suppression of aldosterone production (1). These dual actions of LCZ696 might inhibit the activation of MMP-9 in the infarcted myocardium during the acute phase of MI based on the following evidences. Previous studies showed that IL-1b and IL-6 play important roles in the regulation of MMP-9 produced by macrophages (32–34). Saren *et al.* (32) showed that IL-1b induces the expression of MMP-9 by human-monocyte-derived macrophages, and Bhaskar *et al.* (33) showed that monoclonal antibodies targeting IL-1b inhibit macrophage-induced secretion of MMP-9 in vitro.

Another study reported that IL-6 induces MMP-9 expression by modulating Janus kinase (JAK)-dependent induction of IL-10 (34). Moreover, therapeutic doses of valsartan result in strong suppression of production of various pro-inflammatory cytokines, such as IL-1b, IL-6, and TNF-a by macrophages (35). Furthermore, natriuretic peptide acts directly to decrease the secretion of IL-6 and TNF-a by macrophage (36). In the present study, LCZ696 significantly decreased the mRNA expression levels of IL-1b and IL-6 in the infarcted region 3 days after MI, and in vitro study showed that LPS-induced mRNA expression of IL-1b and IL-6 tended to be lower, and the IL-1b and IL-6 protein production in the supernatant were significantly lower in peritoneal macrophages pre-treated with valsartan þ LBQ657 compared to those with valsartan alone and LBQ657 alone. It is possible that these pro-inflammatory cytokine signaling pathways seem to regulate the MMP-9 expression of macrophages in the infarcted region after MI. These findings in in vitro assay using peritoneal macrophages suggest that simultaneous regulation of 2 neurohumoral systems (i.e., the inhibition of RAAS by ARB and the enhancement of NP system by neprilysin inhibitor) may suppress the inflammatory cytokines such as IL-1b and IL-6, and MMP-9 activity, and may have beneficial effects on the cardiac remodeling after MI.

STUDY LIMITATIONS. First, this is an experimental study to examine the effects of only the maximum dose of LCZ696 or enalapril that did not lower the

SBP in an MI model by permanent ligation of the left coronary artery in mice. Therefore, it is possible that the other doses of LCZ696 or enalapril might have different effects on the cardiac rupture and remodeling after MI. Second, another MI model such as myocardial ischemia/reperfusion might also have the different effects on the infarct size or cardiac remodeling post-MI. Third, although we conducted an *in vitro* assay using peritoneal macrophages to confirm the beneficial effects of LCZ696 on cardiac remodeling after an MI, we should examine whether *in vitro* studies using the other cell types such as endothelial cells, fibroblasts, and cardiomyocytes also might show the same effects as peritoneal macrophages. Fourth, randomized clinical trials will be needed to confirm the cardioprotective effects of LCZ696 in the acute phase of an MI in humans.

CONCLUSIONS

We showed that LCZ696, despite non-antihypertensive dose, protected against cardiac rupture and improved the survival rate after MI, probably due to the suppression of pro-inflammatory cytokines and ECM degradation in macrophages, by dual regulation of RAAS and NP systems. LCZ696 is potentially useful clinically to improve survival after acute MI.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: LCZ696 (sacubitril/valsartan), an angiotensin receptor-neprilysin inhibitor, is 1 of the standard treatments for chronic HF since a previous trial showed that LCZ696 reduced cardiovascular events in patients with HF with reduced ejection fraction compared to enalapril. Administration of LCZ696 on acute phase of acute MI could suppress the expression of pro-inflammatory cytokines and tissue degradation, and protect against cardiac rupture.

TRANSLATIONAL OUTLOOK: Future clinical studies should explore whether treatment of LCZ696 for patients with acute MI can improve clinical outcomes.

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KEY WORDS myocardial infarction, natriuretic peptide, renin-angiotensin-aldosterone

APPENDIX For supplemental text and references, please see the online version of this paper.



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