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ORIGINAL ARTICLE

# Carperitide induces coronary vasodilation and limits infarct size in canine ischemic hearts: role of NO

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**Carperitide is effective for heart failure (HF) owing to its diuretic and vasodilatory effects. This recombinant peptide may also have direct cardioprotective effects because carperitide reduces the severity of heart failure and limits infarct size. Because coronary vasodilation is an important cardioprotective treatment modality, we investigated whether carperitide increased coronary blood flow (CBF) and improved myocardial metabolic and contractile dysfunction during ischemia in canine hearts. We also tested whether carperitide is directly responsible for limiting the infarct size. We infused carperitide at 0.025–0.2 μg kg$^{-1}$ min$^{-1}$ into the canine coronary artery. A minimum dose of 0.1 μg kg$^{-1}$ min$^{-1}$ was required to obtain maximal vasodilation. To test the effects of carperitide on ischemic hearts, we reduced perfusion pressure in the left anterior descending coronary artery such that CBF decreased to one-third of the baseline value. At 10 min after carperitide was infused at a dose of 0.1 μg kg$^{-1}$ min$^{-1}$, we observed increases in CBF, fractional shortening (FS) and pH levels in coronary venous blood without concomitant increases in cardiac nitric oxide (NO) levels; these changes were attenuated using either the atrial natriuretic peptide receptor antagonist HS-142-1 or the NO synthase inhibitor L$^{\omega}$-nitroarginine methyl ester (L-NAME). Cyclic guanosine monophosphate (GMP) levels in the coronary artery were elevated in response to carperitide that also limited the infarct size after 90 min of ischemia and subsequent reperfusion. Again, these effects were blunted by L-NAME. Carperitide increases CBF, reduces myocardial contractile and metabolic dysfunction and limits infarct size. In addition, NO is necessary for carperitide-induced vasodilation and cardioprotection in ischemic hearts.**
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**Keywords:** atrial natriuretic peptide; coronary blood flow; cyclic GMP levels; ischemic hearts; nitric oxide

## INTRODUCTION

Despite effective medical therapies, heart failure (HF) remains a major cause of morbidity and mortality worldwide.[1–3] Importantly, ischemic heart disease is a major cause of HF.[4] Significant clinical efforts, therefore, are directed at preventing acute myocardial infarction (AMI) via coronary vasodilation and, in individuals experiencing AMI, reducing the size of the infarct and ischemia/reperfusion injury.[5] The endogenous protein hormone atrial natriuretic peptide (ANP) is mainly released from atrial tissue, and carperitide—recombinant human ANP—is widely used in patients with HF.[6–8] The beneficial effects of carperitide have been attributed to various cardiovascular-protective activities, including diuresis, natriuresis, vasodilatation and reduced activity in the sympathetic nervous system and renin–angiotensin–aldosterone system.[9] Recently, we showed that carperitide limits infarct size and improves cardiac function in patients with AMI (J-WIND study).[10] Among the many cardioprotective effects of carperitide, the most prominent in ischemic heart disease is coronary vasodilation. Because carperitide shares a signaling pathway with nitric oxide (NO),[9,11] and the two molecules are known to interact,[12–14] we hypothesized that carperitide increases coronary blood flow (CBF) in ischemic hearts and that inhibiting endogenous NO signaling would blunt the observed coronary vasodilation.

To test these hypotheses, we determined whether carperitide mediates vasodilation and attenuates the severity of metabolic and contractile dysfunction in ischemic canine hearts. We also examined the role of endogenous NO in these effects. Finally, we investigated carperitide-mediated limitations of the infarct size and whether NO contributes to this cardioprotective activity.

## METHODS

### Instrumentation

Female beagle dogs weighing 10–14 kg were anesthetized with intravenous pentobarbital sodium (30 mg kg$^{-1}$). Preparative methods are detailed in a previous study.[15] After the chest was opened, coronary perfusion pressure (CPP) and CBF to the perfused myocardium were measured. A pair

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of ultrasonic crystals was inserted ~1 cm apart in the inner one-third of the myocardium to measure the myocardial segment length with an ultrasonic dimension gauge.

All procedures complied with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, 1996 revision) and were approved by the National Cerebral and Cardiovascular Center Committee for Laboratory Animal Use.

## Experimental protocols

*Protocol I: effects of carperitide on coronary vasodilation in nonischemic hearts.* Five dogs were used in this protocol. Coronary arterial and venous blood was sampled for blood gas analysis. Myocardial oxygen consumption (ml per 100 g per min) is calculated by CBF (ml per 100 g per min) × the oxygen difference between coronary arterial and venous blood (ml $dl^{-1}$). We measured CPP and CBF after dogs were randomly administered carperitide at a dose of 0.025, 0.05, 0.1 or 0.2 $\mu g\,kg^{-1}\,min^{-1}$ (Daiichi-Sankyo KK, Tokyo, Japan) into the left anterior descending coronary artery (LAD). A preliminary study showed that CBF stabilized 5–10 min after a change in the carperitide dose.

*Protocol II: effects of carperitide on myocardial ischemia produced by coronary hypoperfusion with or without an ANP receptor antagonist.* Twelve dogs were used in this protocol. Coronary arterial and venous blood was sampled for analysis of blood gas and the levels of lactate and plasma NO metabolites (nitrate and nitrite). The following hemodynamic parameters were measured: left ventricular pressure, dP/dt and the segmental length of the perfused myocardium. After hemodynamic parameters were stabilized, we infused either saline ($n=7$) or the ANP receptor antagonist HS-142-1 (40 $\mu g\,kg^{-1}\,min^{-1}$, $n=5$). At 5 min after infusion onset, an occluder attached to the extracorporeal bypass tube was used to reduce CPP such that CBF decreased to one-third of the control value. Thereafter, the occluder was adjusted to maintain CPP at this level. We confirmed that 10 min was required to obtain a stable state in the hypoperfused myocardium. After 10 min, carperitide (0.1 $\mu g\,kg^{-1}\,min^{-1}$) was infused into the LAD and all hemodynamic and metabolic parameters were measured again after 20 min. After acquiring the data, carperitide infusion was discontinued and all hemodynamic and metabolic parameters were measured after 20 min.

We injected microspheres before (10 min after the onset of coronary hypoperfusion), during (30 min) and after (50 min) carperitide infusion.

*Protocol III: effects of carperitide on myocardial ischemia produced by coronary hypoperfusion with or without a NO synthase inhibitor.* Twelve dogs were used in this protocol. We measured the same hemodynamic and metabolic parameters described in protocol II. We infused either saline ($n=7$) or $L^{\omega}$-nitroarginine methyl ester (L-NAME), an inhibitor of NO synthase (NOS; 10 $\mu g\,kg^{-1}\,min^{-1}$; $n=5$). At 5 min after infusion onset, CPP was reduced such that CBF decreased to one-third of the control value. Thereafter, the occluder was adjusted to maintain CPP at this level. After 10 min, carperitide (0.1 $\mu g\,kg^{-1}\,min^{-1}$) was infused into the LAD and all hemodynamic and metabolic parameters were measured again after 20 min. After acquiring the data, carperitide infusion was discontinued and all hemodynamic and metabolic parameters were measured after 20 min.

*Protocol IV: effects of carperitide on cyclic GMP levels in epicardial coronary arteries of ischemic hearts.* We determined whether carperitide increases coronary arterial cyclic guanosine monophosphate (GMP) levels in ischemic myocardium. An occluder attached at the extracorporeal bypass tube was used to reduce CPP such that CBF decreased to one-third of control CBF. Thereafter, the occluder was adjusted to maintain CPP at the low level. After 10 min, carperitide (0.1 $\mu g\,kg^{-1}\,min^{-1}$) was infused into the LAD for 10 min and the epicardial LAD (ischemic region) and left circumflex (nonischemic control region) coronary arteries were rapidly removed in the presence ($n=5$) or absence ($n=7$) of L-NAME using precooled stainless steel scissors and tongs. Samples were quickly stored in liquid nitrogen.

*Protocol V: effects of carperitide on myocardial infarct size following coronary occlusion and reperfusion.* In 36 dogs, the bypass tube to the LAD was occluded for 90 min, followed by reperfusion for 6 h as saline, carperitide (0.1 $\mu g\,kg^{-1}\,min^{-1}$), L-NAME (10 $\mu g\,kg^{-1}\,min^{-1}$) with carperitide (0.1 $\mu g\,kg^{-1}\,min^{-1}$) and L-NAME (10 $\mu g\,kg^{-1}\,min^{-1}$) were administered ($n=9$ for each group) from 10 min before occlusion until 1 h after reperfusion onset, except at the time of coronary occlusion. In all groups, infarct size was assessed 6 h after reperfusion onset.

The area affected by myocardial necrosis and the area at risk were measured in the dogs after protocol completion by an individual who had no knowledge of the specific treatment given to each animal. Infarct size is expressed as a percentage of the area at risk. Regional myocardial blood flow was determined as described previously.[16] Microspheres were administered 80 min after the onset of coronary occlusion.

## Assays

Lactate was assessed using an enzymatic assay, and the lactate extraction ratio was obtained as the coronary arteriovenous difference in the lactate concentration multiplied by 100 and divided by the arterial lactate concentration. Levels of plasma NO metabolites (nitrate and nitrite) were analyzed using an automated procedure based on the Griess reaction.[17] Nitrate and nitrite concentration differences between coronary venous and arterial blood were used to quantify cardiac NO levels.

The method used to measure cyclic GMP levels has been described previously.[18] After removing adventitial connective tissues from the coronary arteries (20–40 mg), frozen tissue was powdered, homogenized at 4 °C in 1 ml of ice-cold 6% trichloroacetic acid and centrifuged at 2500 × *g* for 20 min. The supernatant was removed, extracted three times with 3 ml of diethyl ether saturated with water and stored at −80 °C. Cyclic GMP concentrations in the supernatant were measured within 7 days using a radioimmunoassay. Briefly, 100 µl of dioxane–triethylamine mixture containing succinic acid anhydride was used to succinylate cyclic GMP in 100 µl of supernatant. After a 10-min incubation, the reaction mixture was combined with 800 µl of 0.3 M imidazole buffer (pH 6.5). Then, 100 µl of succinyl cyclic GMP tyrosine methyl ester iodinated with $^{125}I$ (15 000–20 000 counts per min) was added to the assay mixture containing 100 µl of the supernatant and 100 µl of diluted anti-sera in the presence of chloramines. The mixture was kept at 4 °C for 24 h. A cold solution of dextran-coated charcoal (500 µl) was added to the mixture in an ice-cold water bath. The charcoal was spun down, and 0.5 ml of the supernatant was assessed for radioactivity in a gamma spectrometer. Cyclic GMP levels were normalized based on protein content in the coronary artery that was assayed using the Lowry method.[19]

## Measurements of regional CBF

Regional myocardial blood flow was determined using a microsphere-based technique as previously reported.[20] Nonradioactive microspheres (Sekisui Plastic, Tokyo, Japan) are made of inert plastic labeled with different stable heavy elements as described in detail in previous studies.[16,20] Microspheres were suspended in isotonic saline with 0.01% Tween-80 to prevent aggregation. Microspheres were ultrasonicated for 5 min followed by 5 min of vortexing immediately before injection. Approximately 1 ml of the microsphere suspension ($2–4 \times 10^5$ spheres per ml) was injected into the left atrium followed by several warm (37 °C) saline flushes (5 ml).

X-ray fluorescence of stable heavy elements was measured using a wavelength dispersive spectrometer (PW 1480; Phillips Co. Ltd., Almelo, Netherlands). This X-ray fluorescence spectrometer has been previously described in detail. In brief, when microspheres are irradiated by the primary X-ray beam, electrons fall back to a lower orbit and emit measurable energy as characteristic fluorescence depending on the element. Therefore, X-ray fluorescence from several differently labeled microspheres in the mixture can be assessed. In protocol II, myocardial blood flow in the endocardium versus that in the epicardium (End/Epi flow ratio) was calculated and normalized based on the wet weight of the sampled myocardium. In protocol V, regional myocardial blood flow was calculated according to the following formula: time flow = tissue count × reference flow/reference count. The results are expressed in ml $min^{-1}$ per g of wet sample.

### Statistical analysis

Statistical analysis was performed using two-way analysis of variance[21,22] to compare data among the groups. When analysis of variance reached significance, paired data were compared using Bonferroni's test. Changes in the hemodynamic and metabolic parameters over time were compared by analysis of variance for repeated measures. Analysis of covariance, by endocardial collateral blood flow in the inner half of left ventricle wall as the covariate, was used to account for the effect of endocardial collateral blood flow on infarct size. All results are expressed as mean ± s.e.m., and $P<0.05$ was considered significant.

## RESULTS

Mean blood pressure (103 ± 2 mm Hg) and heart rate (139 ± 2 beats per min) did not differ significantly among the groups. These systemic hemodynamic parameters did not change significantly before, during or after coronary hypoperfusion or complete coronary occlusion with or without administration of a pharmacologic agent.

### Effects of carperitide on CBF in nonischemic hearts

Figure 1 shows CBF during infusions of carperitide. CBF increased based on the dose of carperitide, with saturation of coronary vasodilation observed at a dose of 0.1 $\mu g\,kg^{-1}\,min^{-1}$ (Figure 1a) despite no changes in CPP (102 ± 2 mm Hg) or myocardial oxygen consumption (Figure 1b).

### Effects of carperitide on CBF and the severity of myocardial ischemia

Figure 2 shows CBF (Figure 2b) and fractional shortening (FS) (Figure 2c) whereas CPP was reduced (Figure 2a) with or without the denoted pharmacologic agents. Carperitide increased both CBF and FS whereas CPP was held constant, effects that were blunted by the ANP receptor antagonist HS-142-1. The myocardial End/Epi flow ratio was also augmented by carperitide (Figure 3). Carperitide increased both lactate extraction ratio (Figure 4a) and pH levels (Figure 4b) in coronary venous blood from the ischemic area; HS-142-1 inhibited these effects without increasing cardiac NOx levels (differences in nitrate and nitrite levels between coronary venous and arterial blood; Figure 4c).

Figure 5 shows CBF (Figure 5b) and FS (Figure 5c) whereas CPP was reduced (Figure 5a) with or without L-NAME. Carperitide increased both CBF and FS without changes in CPP, the effects that were inhibited by an inhibitor of NOS (L-NAME). Carperitide increased both lactate extraction ratio (Figure 6a) and pH levels (Figure 6b) in coronary venous blood from the ischemic area; L-NAME blunted these effects without increasing cardiac NOx levels (Figure 6c).

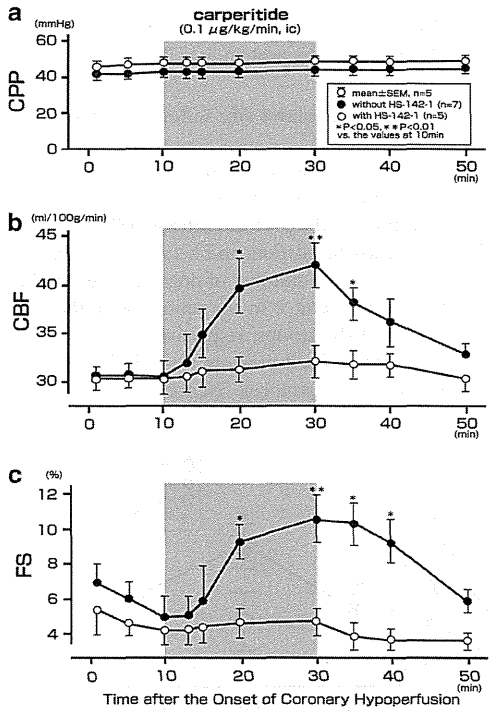


**Figure 2** Effects of carperitide on coronary blood flow (CBF) and fractional shortening (FS) in ischemic hearts with or without HS-142-1. Carperitide increased both CBF (**b**) and FS (**c**) whereas coronary perfusion pressure (CPP; **a**) was held constant, effects that were blunted by the atrial natriuretic peptide (ANP) receptor antagonist HS-142-1. $*P<0.05$, $**P<0.01$ *vs.* the values at 10 minutes.

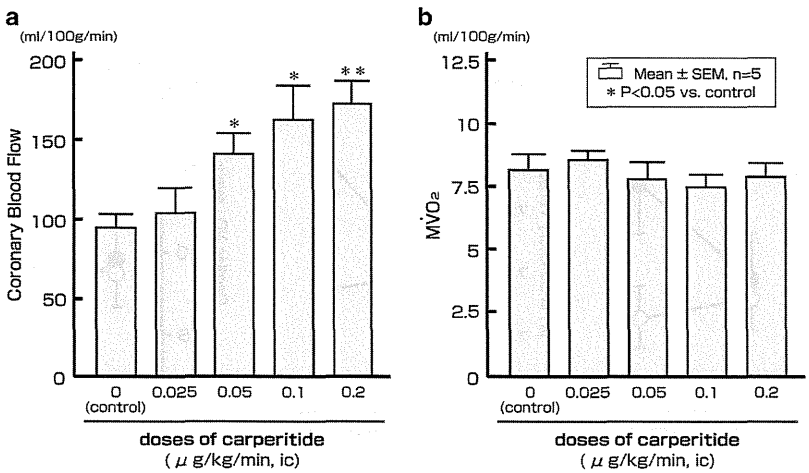


**Figure 1** Effects of carperitide on coronary blood flow (CBF) and myocardial oxygen consumption ($MVO_2$) in nonischemic hearts. CBF increased as the carperitide dose increased (**a**) despite no changes in myocardial oxygen consumption (**b**). $*P<0.05$, $**P<0.01$ *vs.* the control.

We also investigated cyclic GMP levels during carperitide-induced coronary vasodilation. Without carperitide treatment, myocardial ischemia (CPP: 105 ± 4 to 42 ± 2 mm Hg; CBF: 82 ± 3 to 27 ± 2 ml per 100 g per min) increased cyclic GMP concentrations in the coronary artery from 44 ± 17 to 121 ± 22 fmol per mg protein (Figure 7; $P<0.01$). Moreover, carperitide provided during myocardial ischemia further increased cyclic GMP levels in the involved coronary artery ($P<0.05$); L-NAME attenuated this effect. Furthermore, carperitide increased myocardial cyclic GMP levels (168 ± 30 to 263 ± 30 pmol per mg protein, $n=3$ each); L-NAME attenuated this effect (184 ± 24 pmol per mg protein, $n=3$).

### Effects of carperitide on infarct size following ischemia and reperfusion

Of the 36 dogs, 6 were excluded from the analysis because their subendocardial collateral flow was >15 ml per 100 g per min, and hence 30 dogs completed the protocol satisfactorily. Of these 30 dogs, 6 developed ventricular fibrillation, and hence these animals were also excluded from analysis. The number of dogs that were excluded from the analysis were 2, 0, 2 and 2 in the saline, carperitide, carperitide and L-NAME or L-NAME groups, respectively.

Heart rate and aortic blood pressure were similar among the four groups throughout this protocol. Neither the area at risk nor endocardial collateral blood flow in the LAD region during myocardial ischemia differed among the groups receiving saline, carperitide, carperitide and L-NAME or L-NAME (Table 1). Carperitide, however, decreased the infarct size compared with results from the group treated with saline (18.1 ± 3.6% vs. 39.8 ± 5.1% of the area at risk, respectively; $P<0.05$); this effect was blunted by L-NAME (18.1 ± 3.6% vs. 41.6 ± 2.2% of the area at risk in the groups treated

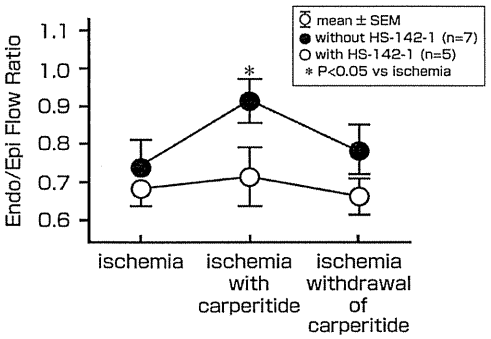


**Figure 3** The ratio of epicardial flow to endocardial flow in the myocardium during ischemia. Carperitide predominantly increased endocardial blood flow relative to epicardial blood flow. This effect was attenuated by HS-142-1. *$P<0.05$ *vs.* ischemia.

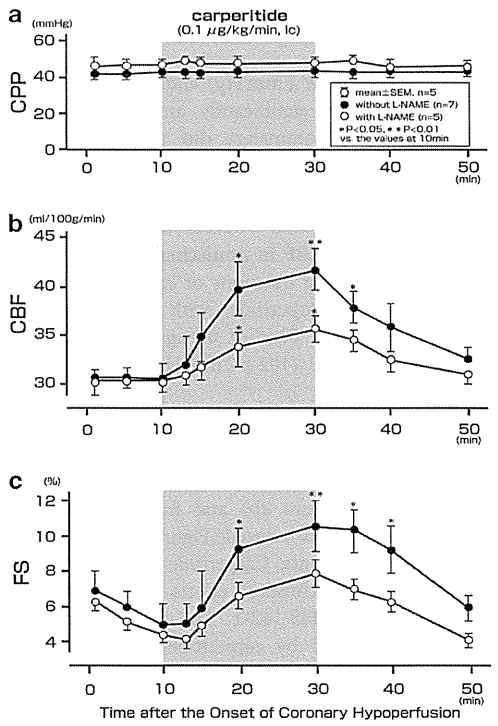


**Figure 5** Effects of carperitide on coronary blood flow (CBF) and fractional shortening (FS) in ischemic hearts with or without $L^{\omega}$-nitroarginine methyl ester (L-NAME). Carperitide increased both CBF (**b**) and FS (**c**) without changes in coronary perfusion pressure (CPP; **a**), effects that were inhibited by an inhibitor of nitric oxide (NO) synthase L-NAME. *$P<0.05$, **$P<0.01$ *vs.* the values at 10 minutes.

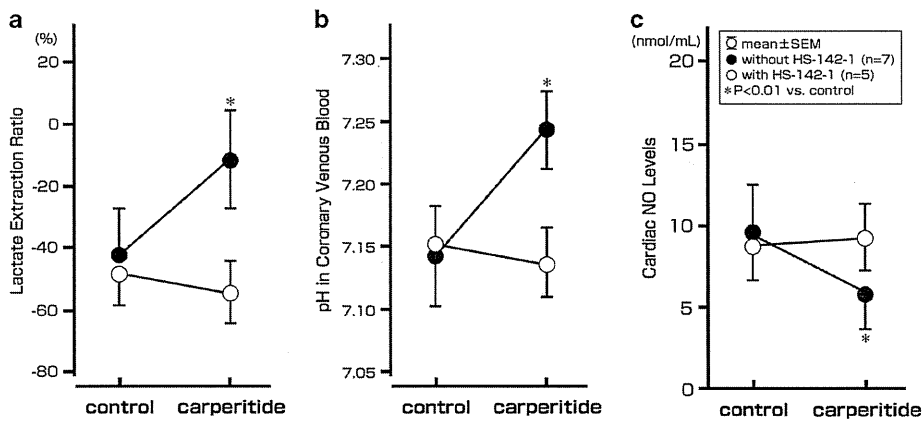


**Figure 4** Effects of carperitide on metabolic function in ischemic hearts with or without HS-142-1. Carperitide increased both (**a**) lactate extraction ratio (LER) and (**b**) pH levels in coronary venous blood from the ischemic area; HS-142-1 inhibited these effects without increasing cardiac nitric oxide (NO) levels (**c**). *$P<0.01$ *vs.* the control.

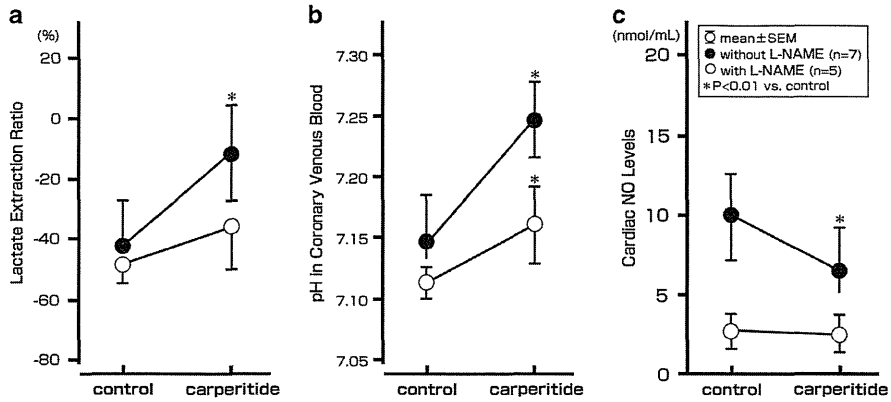


**Figure 6** Effects of carperitide on metabolic function in ischemic hearts with or without L$^{\omega}$-nitroarginine methyl ester (L-NAME). Carperitide increased both (**a**) lactate extraction ratio LER and (**b**) pH levels in coronary venous blood from the ischemic area; L-NAME blunted these effects without increasing cardiac nitric oxide (NO) levels (**c**). *$P<0.01$ *vs.* the control.

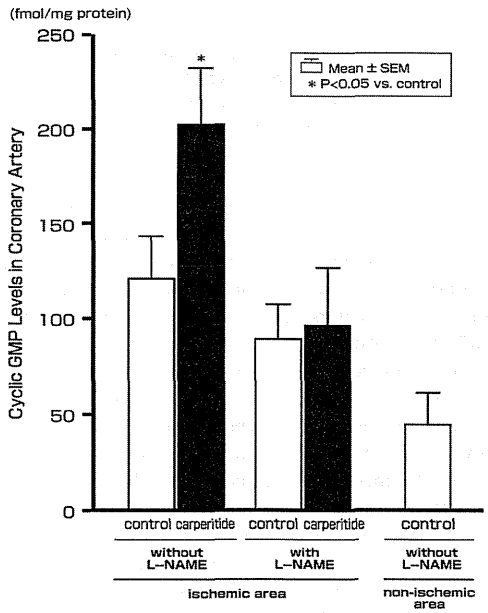


**Figure 7** Cyclic guanosine monophosphate (GMP) levels in the coronary arteries of ischemic hearts. Ischemia *per se* increased cyclic GMP levels that were further elevated by carperitide. The increases in cyclic GMP levels were attenuated by Lω-nitroarginine methyl ester (L-NAME). *$P<0.05$ *vs.* the control.

with carperitide or carperitide and L-NAME, respectively; $P<0.05$; Figure 8a). Figure 8b shows the regression plots of the area at risk and endocardial collateral blood flow during ischemia. Carperitide mediated the substantial cardioprotection irrespective of collateral flow that was again blunted by L-NAME.

## DISCUSSION

The present study showed that carperitide causes coronary vasodilation and promotes myocardial contractility and metabolism in ischemic hearts, the effects that are mediated by accumulation of cyclic GMP in the coronary artery and myocardium. In addition, carperitide potently decreases the infarct size following sustained ischemia and reperfusion. We also showed that inhibiting NO

**Table 1 Risk area and endocardial collateral blood flow during myocardial ischemia in each group**

| Groups | *Risk area* (%) | *Collateral blood flow during myocardial ischemia* (ml per 100g per min) |
|---|---|---|
| 1. Control group | 39.3±2.0 | 8.4±1.6 |
| 2. Carperitide group | 40.6±1.8 | 7.7±1.6 |
| 3. Carperitide + L-NAME group | 42.3±2.5 | 10.4±1.3 |
| 4. L-NAME group | 39.2±1.3 | 7.8±1.9 |

Abbreviation: L-NAME, L$^{\omega}$-nitroarginine methyl ester.
Values are expressed as means±s.e.m. There were no significant differences in the area at risk or coronary blood flow (CBF) among any of the groups.

production attenuates carperitide-induced coronary vasodilation and reductions in the infarct size.

### Role of NO in carperitide-mediated coronary vasodilation in ischemic hearts

The present study showed that carperitide increases CBF in a manner dependent on cyclic GMP. Because cyclic GMP induces vasorelaxation in smooth muscle cells, the carperitide-induced coronary vasodilation was likely a result of increased cyclic GMP levels. Several other effects of carperitide, however, should be considered. First of all, because carperitide attenuates catecholamine-induced cellular responses,[23] the reduction of α-adrenoceptor activation by carperitide may cause coronary vasodilation. Indeed, we could not exclude the possibility that the carperitide-induced coronary vasodilation is mediated by coronary $\alpha_2$-adrenoceptor blockade. On the other hand, myocardial effects of carperitide may also be involved in the present observation. If this myocardial effect of carperitide was the case, carperitide should have also reduced norepinephrine-induced myocardial hypercontraction, leading to lower myocardial contractility and oxygen consumption. However, the present study revealed that carperitide did not alter myocardial oxygen consumption (Figure 1). It is well known that an increase in coronary perfusion increases myocardial oxygen consumption, termed the Gregg phenomenon. In turn, carperitide has a potency to decrease myocardial oxygen consumption via increased myocardial cyclic GMP levels that may blunt the Gregg phenomenon. We may have observed the mixed effects of carperitide

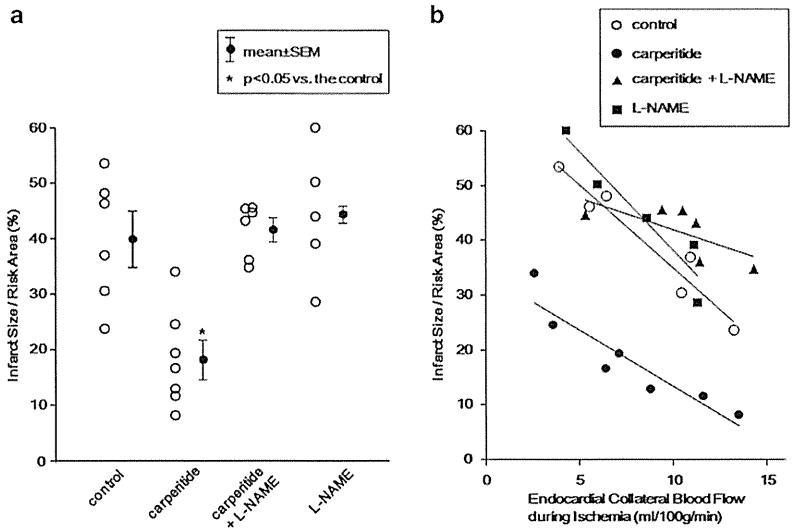


**Figure 8** Effects of carperitide on myocardial ischemia and reperfusion. (**a**) Infarcts were smaller in the carperitide group compared with the control group, a difference that was abolished by $L^{\omega}$-nitroarginine methyl ester (L-NAME). Infarct size expressed as the plot of infarct size because of 90 min of ischemia and regional collateral flow during ischemia (**b**). There are inverse relations between normalized infarct area and collateral flow, and a significant difference ($P<0.05$) is seen in the carperitide group compared with the control group. ANP, atrial natriuretic peptide. *$P<0.05$ *vs.* the control group.

that may culminate in no changes in myocardial oxygen consumption. Second, because (1) NO increases CBF[24] and (2) L-NAME attenuates carperitide-induced coronary vasodilation, carperitide may enhance NO production. Because the levels of the end-product of cardiac NO (Figures 4c and 6c) did not increase in response to carperitide, this possibility appears unlikely. Third, carperitide may inhibit leukocyte activation and adhesion that occur in ischemic hearts.[25] Because leukocyte adhesion decreases CBF by plugging small coronary arteries, reduced leukocyte adhesion may restore CBF. However, this would not be likely. A significant amount of binding between leukocytes and coronary endothelial cells would be required to reduce CBF, and this degree of adhesion would not likely be attenuated quickly following carperitide infusion. The effects of carperitide, however, were reversible as shown in Figures 2 and 3. Fourth, carperitide may open collateral flow that may increase CBF. In the present study, carperitide increased CBF in the perfused region that was measured with an electromagnetic flow probe attached to the bypass tube in the coronary hypoperfusion model (Figure 2b), indicating that carperitide seemed to increase coronary forward flow independent of the collateral flow. Furthermore, the myocardial End/Epi flow ratio was augmented by carperitide (Figure 3), suggesting that carperitide-induced increases in coronary flow is not luxury flow but effective flow to the ischemic myocardium. On the other hand, the present study also showed that carperitide did not increase the myocardial collateral blood flow during myocardial ischemia in the total coronary occlusion model (Table 1), suggesting that carperitide may not increase collateral flow. Further study is needed to study the effects of carperitide on collateral flow in the animal model with less collateral flow in the future. Fifth, carperitide attenuates oxidative stress[26,27] that may contribute to coronary vasodilation in ischemic hearts because oxygen-derived free radicals reduce coronary vasodilation by reducing NO bioavailability.[28,29] However, cardiac NO levels decreased in response to carperitide, arguing against this possibility.

How does carperitide affect NO in ischemic hearts? Our results demonstrate that carperitide does not increase NO production in ischemic hearts. Alternatively, the inhibition of NOS may deactivate downstream ANP receptors because L-NAME attenuates increases in cyclic GMP levels following ANP receptor activation. Several lines of evidence support the idea that NO modulates carperitide activity:[11–14] both factors signal via cyclic GMP and the soluble and particulate guanylate cyclase pathways. It is reported that ANP increases cardiac NOS activity and thus cardiac NO synthesis;[30] the same authors reported that ANP increased NOS activity, but the activation was lower in spontaneously hypertensive rats than Wistar-Kyoto rats.[31] Intriguingly, carperitide significantly decreased NO production in ischemic hearts (Figures 4c and 6c) in the present study. These data seem to be contradictory, but this is not the case. One possible explanation is that cardiac NOS activity may be already saturated in the coronary hypoperfusion model of the present study, and carperitide-induced NO-independent coronary vasodilation may become a major determinant of coronary vascular tone. Another possibility is that these differences may be attributable to the variations in experimental models (normotensive vs. hypertensive hearts and nonischemic vs. ischemic hearts). Whatever the mechanisms are, this study is the first to reveal a relationship between carperitide and endogenous NO in ischemic hearts. At present, however, no available evidence details how the sensitivity of ANP receptors or signal transduction following ANP receptor activation is reduced by inhibiting NOS.

Furthermore, it is reported that ANP may ameliorate endothelial dysfunction by upregulating endothelial NOS (coded by NOS3 gene) and downregulating inducible NOS (coded by NOS2 gene).[32] However, as we used L-NAME, a nonselective NOS inhibitor, to examine the involvement of NOS in the carperitide-induced cardioprotection, we could not clarify what type of NOS is activated in hypoperfused and/or ischemic canine hearts by treatment with carperitide. This would be the next target to elucidate the relationship between carperitide and NO.

### Role of NO in carperitide-mediated cardiac function in ischemic hearts

It is reported that NO prevents cellular damages by induction of the rapid recovery to normal pH after ischemia/reperfusion via a guanylyl cyclase/cyclic GMP/protein kinase G (PKG) signaling cascade, and thus inhibits mitochondrial permeability transition (MPT).[33] Yang *et al.*[34] reported that ANP infusion decreased infarct size of the risk area, and this effect was mimicked by a cyclic GMP analog that directly activates PKG and likely by opening of mitochondrial KATP channel and stimulation of downstream kinases. Furthermore, Cohen *et al.*[35] reported that postconditioning prevents mitochondrial permeability transition pore (MPTP) formation by maintaining acidosis during the first minutes of reperfusion. Recently, soluble and particulate guanylate cyclase activator exerts cardioprotective effects via cyclic GMP/PKG signaling cascade and activates phospholamban phosphorylation.[36] Therefore, these myocardioprotective mechanistics of carperitide in addition to the coronary vasodilation may be involved in the cardioprotective effects of carperitide in ischemic heart.

### Carperitide limits the infarct size

We showed that carperitide reduces infarct size, and this was also revealed in a previous study.[37] Myocardial infarction is caused by many factors, including free radial generation, platelet aggregation, myocardial calcium ion overload, leukocyte activation and excess catecholamines, each of which is reportedly attenuated by carperitide. Carperitide exerts protective effect of cyclic GMP/PKG signaling pathway during reperfusion in isolated myocytes or isolated hearts on top of the vasodilatory effects of carperitide to reduce cardiac preload and afterload, all of which may mediate cardioprotection. However, even if collateral flow is increased by carperitide, the collateral flow-independent infarct size limitation caused by carperitide largely exists because the infarct size-limiting effect of carperitide is observed even after normalization by the collateral flow (Figure 8b). More importantly, clinical observations have shown that carperitide is cardioprotective against ischemia and reperfusion injury.[10,38]

### Clinical implications

Carperitide improves the pathophysiology of acute decompensated HF,[8,9] whereas a recent large clinical trial (ASCEND study) suggested that nesiritide, the recombinant human brain natriuretic peptide (BNP), does not reduce mortality and morbidity in patients with acute decompensated HF.[39] Our group and others have reported that carperitide limits infarct size in humans,[10,38] and the present study showed this cardioprotection may require NO. It is to be noted that nitrate is usually administered to patients with AMI that may enhance the effects of carperitide. If this is the case, it would be important to maintain NO at levels that are sufficient to strengthen the effects of carperitide.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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# Noninvasive and quantitative live imaging reveals a potential stress-responsive enhancer in the failing heart

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ABSTRACT **Recent advances in genome analysis have enabled the identification of numerous distal enhancers that regulate gene expression in various conditions. However, the enhancers involved in pathological conditions are largely unknown because of the lack of *in vivo* quantitative assessment of enhancer activity in live animals. Here, we established a noninvasive and quantitative live imaging system for monitoring transcriptional activity and identified a novel stress-responsive enhancer of *Nppa* and *Nppb*, the most common markers of heart failure. The enhancer is a 650-bp fragment within 50 kb of the *Nppa* and *Nppb* loci. A chromosome conformation capture (3C) assay revealed that this distal enhancer directly interacts with the 5′-flanking regions of *Nppa* and *Nppb*. To monitor the enhancer activity in a live heart, we established an imaging system using the firefly luciferase reporter. Using this imaging system, we observed that the novel enhancer activated the reporter gene in pressure overload-induced failing hearts (failing hearts: 5.7±1.3-fold; sham-surgery hearts: 1.0±0.2-fold; $P<0.001$, repeated-measures ANOVA). This method will be particularly useful for identifying enhancers that function only during pathological conditions.—Matsuoka, K., Asano, Y., Higo, S., Tsukamoto, O., Yan, Y., Yamazaki, S., Matsuzaki, T., Kioka, H., Kato, H., Uno, Y., Asakura, M., Asanuma, H., Minamino, T., Aburatani, H., Kitakaze, M., Komuro, I., and Takashima, S. Noninvasive and quantitative live imaging reveals a potential stress-responsive enhancer in the failing heart. *FASEB J.* 28, 1870–1879 (2014). www.fasebj.org**

*Key Words: natriuretic peptide · transcriptional regulation · in vivo assessment*

GENE EXPRESSION IS REGULATED through the integrated action of many *cis*-regulatory elements, including core promoters, proximal promoters, distant enhancers, and insulators (1). Several methods have been used to explore the function of *cis*-regulatory elements during a variety of developmental stages (2, 3). However, the identification of gene regulatory elements with pathophysiological roles has been technically difficult because there are few appropriate models for monitoring transcriptional activity in live animals under pathological conditions.

Here, we focused on the regulatory elements that are responsive to heart failure. The natriuretic peptides, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), encoded by the neighboring genes *Nppa* and *Nppb* are activated in the embryonic heart, down-regulated after birth, and then reactivated during heart failure. Both peptides are well-known biomarkers that are strongly induced during heart failure and represent its severity. Cardiologists frequently use these peptides as natriuretic and vasorelaxant agents to treat various clinical conditions (4–8). Many studies have tried to elucidate the mechanisms of their transcriptional regulation because factors that regulate these

Abbreviations: 3C, chromosome conformation capture; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; ChIP-seq, chromatin immunoprecipitation sequencing; CMV, cytomegalovirus; CR, conserved region; CTCF, CCCTC-binding factor; H3K4me1, histone H3 monomethylated at lysine 4; H3K4me3, histone H3 trimethylated at lysine 4; PE, phenylephrine; TAC, transverse aortic constriction

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natriuretic peptides are potential therapeutic targets for heart disease (9–14).

Mice transgenic for various loci, including the 5′-flanking regions of the natriuretic peptide genes, have been used to identify the regulatory elements required for transcriptional activation either during heart development or in the diseased heart. These studies reported that the 5′-flanking regions of the natriuretic peptide genes regulated their expression during heart development (9, 10, 13); however, the 5′-flanking regions were not responsible for their specific reactivation in the diseased heart (11, 12). A recent study identified the distal enhancer elements regulating the natriuretic peptide genes in the developing heart by examining cardiac-specific transcription factor binding sites; however, these enhancer elements did not respond to heart failure (14). Therefore, the stress-responsive regulatory elements that function during heart failure have not yet been identified and are potentially located outside the 5′-flanking regions.

In this study, we aimed to identify the novel stress-responsive enhancer elements of the *Nppa* and *Nppb* genes in the failing heart. Furthermore, we established a noninvasive and quantitative live imaging assay to monitor the transcriptional activity of candidate enhancers in the failing heart. *In vivo* live imaging of the firefly luciferase reporter in a single mouse enabled us to analyze the sequential changes in enhancer activity during the progression of heart failure. Combined with a fine mapping technique using epigenetic markers, we identified a 650-bp stress-responsive enhancer that was strongly activated by cardiac hypertrophy and heart failure.

## MATERIALS AND METHODS

### Animals

All procedures were performed according to the U.S. National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) and were approved by the Animal Experiments Committee, Osaka University (approval no. 21-78-10).

### Reagents and antibodies

Phenylephrine (PE) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The anti-RNA polymerase II and anti-histone H3 trimethylated at lysine 4 (H3K4me3) antibodies used for chromatin immunoprecipitation sequencing (ChIP-seq) were kind gifts from Dr. H. Kimura (Graduate School of Frontier Biosciences, Osaka University).

### Primary culture of neonatal rat cardiomyocytes

Ventricular myocytes obtained from 1- or 2-d-old Wistar rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% FBS, as described previously (15).

### Comparative genomics

Genome-wide multiple alignments of the genomic sequences containing the *Nppa* and *Nppb* genes were performed using the University of California Santa Cruz (UCSC) Genome Browser (16); 8 vertebrate species were compared, including mouse (mm9, July 2007), rat (m4, Nov. 2004), human (hg18, Mar. 2006), orangutan (ponAbe2, July 2007), dog (canFam2, May 2005), horse (equCab1, Jan. 2007), opossum (monDom4, Jan. 2006), and chicken (galGal3, May 2006). We used vertebrate Multiz alignment of DNA sequences (17) to analyze the homology of DNA sequences among mouse and other species. We used the Placental Mammal Basewise Conservation assessed by PhyloP (18) to assess the degree of mammalian conservation. Next, we identified discrete conserved fragments. The transcribed sequences within the conserved set were filtered out using known genes, spliced ESTs, and mRNA annotations obtained from the UCSC genome browser. Finally, we manually curated the data set to remove any additional false positives by visual examination of the UCSC genomic data. We defined the noncoding conserved regions (CRs) that were homologous at least in the human and mouse genomes and at least 1 kb away from the transcription start sites as the enhancer candidates.

### ChIP sequencing on mouse heart tissues

Whole hearts were isolated from 8-wk-old C57BL6 mice, perfused rapidly with cold PBS, flash-frozen in liquid nitrogen, homogenized using a sterile tissue grinder, and cross-linked with 0.3% paraformaldehyde. Subsequently, chromatin isolation, sonication, and immunoprecipitation using an anti-RNA polymerase II antibody and an anti-H3K4me3 antibody were performed. The ChIP DNA and input samples were sheared by sonication, end-repaired, ligated to the sequencing adapters, and amplified. The purified ChIP DNA library samples were sequenced using the Illumina Genome Analyzer II (Illumina, Inc., San Diego, CA, USA). Unfiltered sequence reads were aligned to the mouse reference genome [U.S. National Center for Biotechnology Information (NCBI) build 37, mm9] using Bowtie. RNA polymerase II- and H3K4me3-enriched regions were identified using MACS (19) with the default parameters.

### Lentiviral enhancer assay

Eleven CRs were PCR amplified from the mouse BAC clone containing the *Nppa* and *Nppb* loci (clone RP23-128E8; BACPAC Resources Center, Children's Hospital Oakland, Oakland, CA, USA; primers and probes are listed in Supplemental Table S1). The PCR fragments were subcloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA) and recombined into a lentiviral vector encoding the firefly luciferase reporter (pGreenFire Transcriptional Reporter Lentivector; System Biosciences, Mountain View, CA, USA). The lentiviral particles were produced by transfection of 293T cells with the 3 lentiviral packaging plasmids (*i.e.*, pMDLg/pRRE, pRSV-Rev, and pMD2.VSV.G) using Lipofectamine 2000 (Invitrogen). The supernatant from 293T cells containing the lentiviral particles was collected 48 h after transfection, sterilized using a 0.45-μm cellulose acetate filter, and concentrated by centrifugation (Peg-it Virus Precipitation Solution, System Biosciences).

Rat neonatal cardiomyocytes isolated as described above were plated in 96-well plates. The next day, the medium was replaced with a serum-free medium containing the lentiviral vector, and the cells were incubated for 12 h. Subsequently, the cardiomyocytes were exposed to 100 μM PE for 48 h prior to the luciferase assay.

### RNA extraction and quantitative RT-PCR

The total RNA was prepared from rat cardiomyocytes, rat cardiac fibroblasts, murine hearts, and murine brains using the RNA-Bee RNA isolation reagent (Tel-Test, Friendswood, TX, USA) and then converted to cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The quantitative RT-PCR was performed using the TaqMan technology and the StepOnePlus real-time PCR System (Applied Biosystems). All samples were processed in duplicate. The level of each transcript was quantified according to the threshold cycle ($C_t$) method using GAPDH as an internal control. Inventoried TaqMan gene expression assays were used: *Nppa*, Rn0056661, Mm01255748; *Nppb*, Rn00580641, Mm01255770; *Gapdh*, rodent GAPDH control reagent.

### 3C analysis

The whole hearts of the mice were isolated, perfused rapidly with cold PBS, flash-frozen in liquid nitrogen, homogenized using a sterile tissue grinder, and fixed with 1% paraformaldehyde. The cross-linked tissues utilized for 3C experiments were subjected to digestion with *Bam*HI following standard protocols (20, 21). The mouse BAC DNA containing *Nppa* and *Nppb* (clone RP23-128E8) was used as a control. The TaqMan real-time PCR was performed using probes near the restriction sites; the primers and probes are listed in Supplemental Table S2.

### Transgenic mouse enhancer assay

The candidate enhancer regions were cloned into a vector encoding the minimal CMV promoter driving the luciferase gene as described above. Transgenic mouse embryos were generated by pronuclear injection into the zygotes of BDF1 mice using standard methods. Because black fur attenuates light transmission, albino mice were generated by crossing the transgenic founders to ICR albino mice.

### *In vivo* bioluminescence imaging

Prior to *in vivo* imaging, the mice were anesthetized using isoflurane, and the black mice were shaved from the neck to the lower torso to allow the optimal visualization of fluorescence without interference from the black fur. A D-luciferin solution was injected intraperitoneally (150 mg/kg i.p.) or intravenously (75 mg/kg i.v.). The mice were imaged using an *in vivo* live imaging system (IVIS Lumina II; Caliper Life Sciences, Waltham, MA, USA). For quantification, the bioluminescence light intensity was measured at the region of interest and expressed in relative light units (RLU/min) using Living Image 4.0 (Caliper Life Sciences). To calculate the enhancer activity in the heart, we defined the ratio of heart to brain luciferase intensities as the cardiac-specific enhancer activity.

### Transverse aortic constriction (TAC)

Transgenic mice aged 8 wk and weighing 20–25 g were subjected to pressure overload, as described previously (22). Briefly, the chest was entered *via* the second intercostal space at the upper left sternal border. After the arch of the aorta was isolated, a TAC was created using a 7-0 suture tied twice around a 27-gauge needle and the aortic arch, between the innominate and left common carotid arteries. After the suture was tied, the needle was gently removed, yielding 60–80% constriction of the aorta.

### PE-induced hypertrophy

Transgenic mice aged 8 wk and weighing 20–25 g were treated with PE (75 mg/kg/d) using an osmotic minipump (Alzet, Cupertino, CA, USA) to induce cardiac hypertrophy, as previously reported (23, 24).

### Statistical analysis

Data are expressed as means ± SE. The 2-tailed Student's *t* test and repeated ANOVA were used to analyze differences between the groups. Values of $P < 0.05$ were considered to represent a significant difference.

## RESULTS

### Identification of candidate enhancers near the *Nppa-Nppb* locus using comparative genomics and ChIP-seq

To identify potential enhancers, we performed a comparative analysis of the genomic sequences of mouse and divergent species and identified CRs that may function as common regulatory sequences (25–27). We defined CRs that were homologous at least in the human and mouse genomes and at least 1 kb away from the transcription start sites of *Nppa* and *Nppb* as the candidate enhancers. First, we analyzed the 50-kb *Nppa-Nppb* locus bounded by the binding sites of 2 CCCTC-binding factors (CTCFs), which can function as insulators (28, 29). Using a genome database (30), we identified 11 CRs, including the *Nppa* and *Nppb* introns in the 50-kb region (**Fig. 1**).

Next, we performed a ChIP-seq analysis on RNA polymerase II and H3K4me3 in the adult mouse heart. We analyzed the epigenetic modifications near the *Nppa* and *Nppb* genes combined with the ChIP-seq analysis using a public database of the adult mouse heart (30). We hypothesized that the normal heart would have activated epigenetic marks because *Nppa* and *Nppb* are expressed, albeit at low levels, in normal conditions. Recent genome-wide studies have determined that enhancers can be defined as DNA sequences bound by the RNA polymerase II and transcriptional coactivator protein p300, and where histone H3 monomethylated at lysine 4 (H3K4me1) accumulates instead of H3K4me3 (31–34). Among the 11 CRs identified, only CR9 coincided with the binding sites of RNA polymerase II and p300, and overlapped with the gene areas modified by H3K4me1, and filled all criteria for the enhancer (Fig. 1). In addition, H3K4me1 modifications in CR9 were only observed in the heart but not in the other organs (Fig. 1 and Supplemental Fig. S1). Therefore, we analyzed the 11 CRs, including CR9, as the most likely distal candidate enhancers for the stress-responsive regulatory regions of the natriuretic peptide genes.

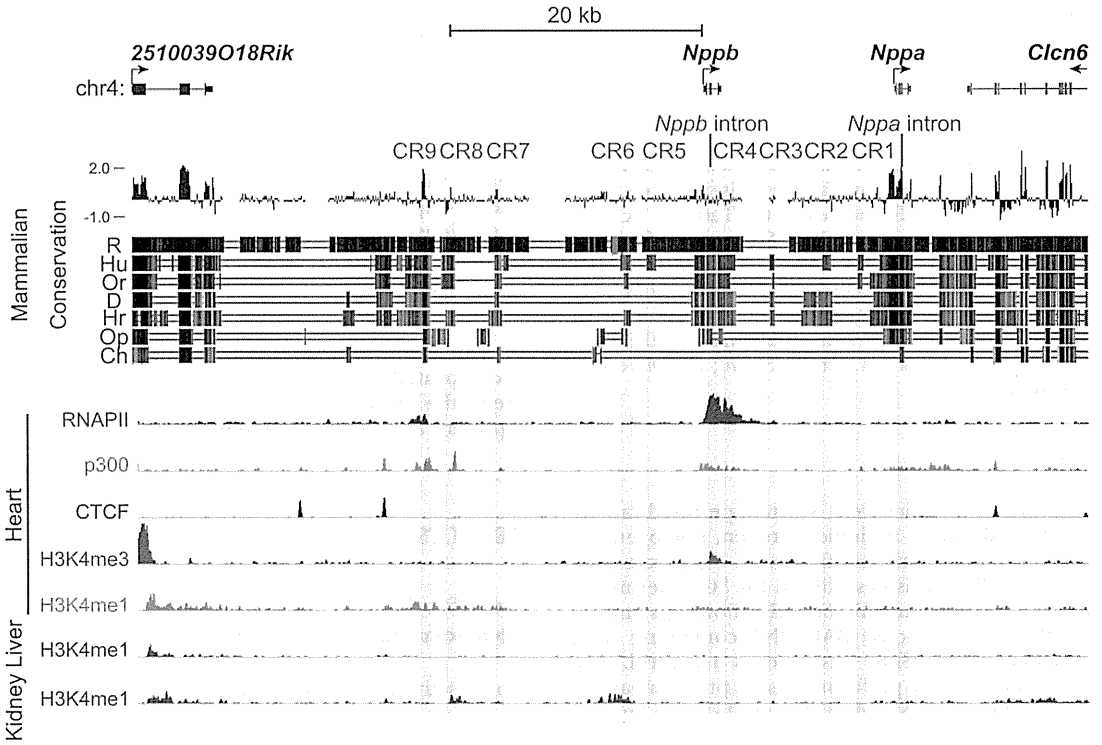


**Figure 1.** Mammalian evolutionarily conserved regions and ChIP-seq data surrounding the murine *Nppa* and *Nppb* loci. We used an open database on the University of California Santa Cruz (UCSC) Genome Browser to assess the degree of DNA sequence conservation around *Nppa* and *Nppb* gene loci. Blue and red vertical lines, the Placental Mammal Basewise Conservation assessed by PhyloP; black vertical lines, the vertebrate Multiz alignment of DNA sequences among mice and 7 other species (rats, humans, orangutans, dogs, horses, opossums, and chickens). We defined noncoding conserved regions (CRs) that were homologous at least in the human and mouse genomes and at least 1 kb away from the transcription start sites of *Nppa* and *Nppb* as the candidate enhancers. CRs are highlighted as light red vertical bars (CR1-9, *Nppa* intron, and *Nppb* intron). ChIP-seq data for H3K4me1, p300, and CTCF were obtained from an open database of the adult mouse heart. Some CRs coincided with the peaks for H3K4me1, RNA polymerase II, and the transcriptional coactivator protein p300. R, rat; Hu, human; Or, orangutan; D, dog; Hr, horse; Op, opossum; Ch, chicken.

### Identification of a distal enhancer element responsive to an $\alpha_1$-adrenergic receptor agonist

We screened the candidate enhancers for potential stress-responsive regulatory regions. We analyzed the enhancer activity of these 11 CRs after treatment with PE, an $\alpha_1$-adrenergic receptor agonist, which mimics cardiac overload and induces *Nppa* and *Nppb* expression in cardiomyocytes (35). We confirmed that PE induced the expression of endogenous *Nppa* and *Nppb* specifically in cardiomyocytes but not in cardiac fibroblasts (**Fig. 2*A***). Then, we introduced the 11 CRs with a minimum human cytomegalovirus (CMV) promoter and the luciferase gene into rat cardiomyocytes using a lentiviral vector system.

Among the 11 CRs tested, only CR9, which is located 22 kb upstream from the *Nppb* transcription start site and shows high mammalian conservation score in the Placental Mammal Basewise Conservation by PhyloP (Fig. 2*B*), reproducibly increased the PE-induced luciferase activity by ~5-fold compared to the minimal CMV promoter alone (Fig. 2*C*). However, CR9 did not respond to PE in cardiac fibroblasts (Fig. 2*C*). These results suggest that CR9 is the regulatory element that is responsive to PE specifically in cardiomyocytes.

### Long-range physical interaction between the distal enhancer element and the proximal promoters of the *Nppa* and *Nppb* genes

Confirming the looping interactions between distal elements and promoters is one way to demonstrate the transcriptional regulatory activity of distal elements. We performed a 3C assay (20) to comprehensively investigate whether the genomic region containing CR9 moved closer to the *Nppa* or *Nppb* promoter in an adult murine heart treated with a continuous infusion of PE *in vivo*.

The ligation frequencies were quantified by TaqMan real-time PCR using specific primers and probes and were compared to the ligation frequency of noncross-linked *Bam*HI-digested BAC DNA containing the *Nppa-Nppb* locus. We observed that CR9 interacts with both the *Nppa* and *Nppb* promoter regions at a higher frequency relative to other gene areas (Fig. 2*D*); furthermore, PE treatment strengthened these interac-

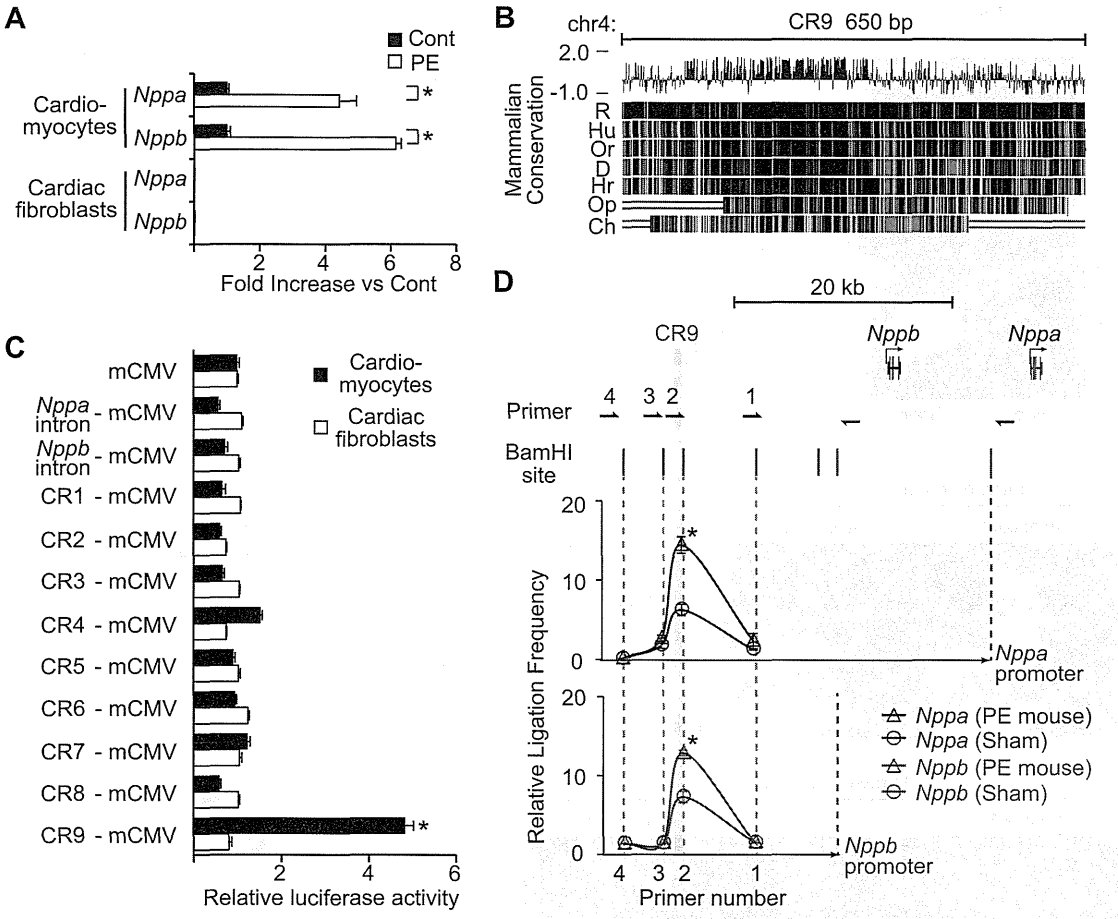


**Figure 2.** Identification of a distal enhancer element that is responsive to an $\alpha_1$-adrenergic receptor agonist. *A*) Relative transcript levels of *Nppa* and *Nppb* in rat neonatal cardiomyocytes and cardiac fibroblasts 48 h after treatment with PE (100 μM). Values are means ± SE ($n$=3 cultures). *$P < 0.01$ *vs.* control; *t* test. *B*) CR9 is a highly conserved genomic region in vertebrates. *C*) Relative luciferase reporter activities of CRs in rat neonatal cardiomyocytes and cardiac fibroblasts 48 h after treatment with PE (100 μM). PE-induced luciferase activity driven by the mCMV promoter was defined as 1. Values are means ± SE ($n$=5 cultures). *$P < 0.001$ *vs.* mCMV alone; *t* test. *D*) *In vivo* 3C analysis of the murine *Nppa* and *Nppb* loci, showing relative ligation frequencies of each primer to the *Nppa* promoter (blue triangle, mouse with PE treatment; blue circle, mouse without PE) and the *Nppb* promoter (red triangle, mouse with PE treatment; red circle, mouse without PE). Vertical bars and arrows show the positions of *Bam*HI sites and primers. Data were normalized to the amplification value of a *Bam*HI-digested and religated BAC clone, which included the *Nppa* and *Nppb* loci (means ± SE; $n$=2 hearts). R, rat; Hu, human; Or, orangutan; D, dog; Hr, horse; Op, opossum; Ch, chicken. *$P < 0.05$ *vs.* control; *t* test.

tions (Fig. 2*D*). These results suggest that there is a close proximity between the distal genomic region containing CR9 and the proximal promoters of the *Nppa* and *Nppb* genes in the PE-induced hypertrophic heart.

### Establishment of an *in vivo* live imaging system for gene expression in a murine model of heart disease

We confirmed the activity of the newly identified enhancer CR9 in the heart *in vivo*. The conventional histological evaluation of LacZ reporter expression in the heart only provides data at a single time point; therefore, this method cannot be employed for kinetic assessments or time course analyses of reporter expression in a live heart.

To overcome this difficulty, we established a noninvasive and quantitative live imaging system that allowed real-time monitoring of the firefly luciferase reporter. We generated 3 transgenic mouse lines (Tg-line1, Tg-line2, and Tg-line3) in which the CR9 enhancer element and a minimal CMV promoter driving the luciferase reporter gene were introduced into the germline. The live-imaging system detected luciferase expression in the heart, brain, and intestine of the Tg-line1 (**Fig. 3*A***), in the heart, salivary glands, and skin of the Tg-line2 (Supplemental Fig. S2*A*), and in the heart of the Tg-line3 (Supplemental Fig. S2*E*).

To identify the organs in which CR9 functioned as a stress-responsive enhancer, we examined the luciferase reporter expression in each organ by quantitative PCR. Continuous infusion of PE increased the blood pressure and resulted in cardiac hypertrophy (24, 36). The

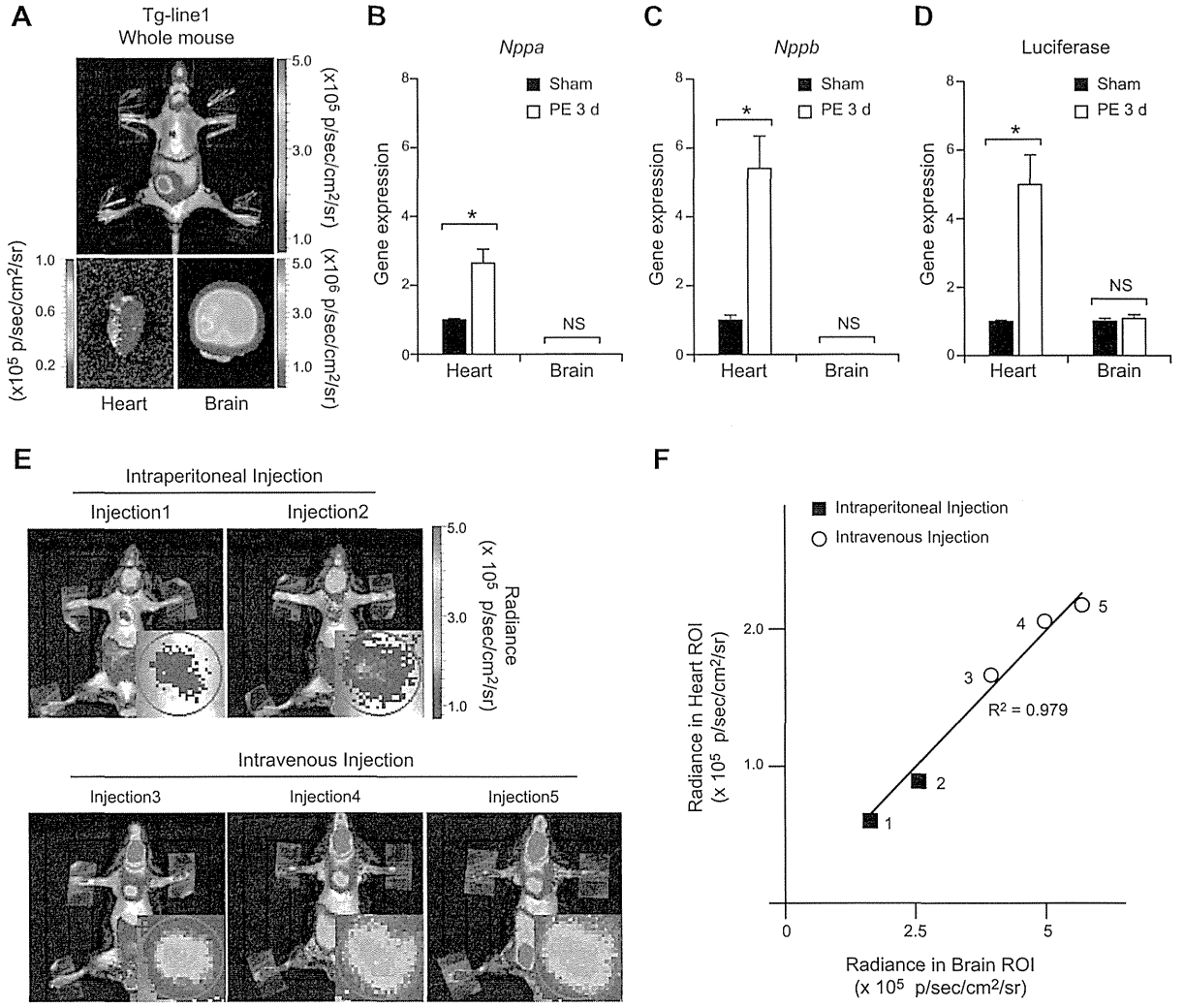


**Figure 3.** Establishment of an *in vivo* live imaging system for enhancer activity. *A*) Chemiluminescence imaging of CR9 in a mouse of Tg-line1. Top panel: result from whole-animal *in vivo* live imaging. Bottom panels: chemiluminescence images of the heart and brain in the same mouse. *B, C*) Relative transcript levels of *Nppa* and *Nppb* in the ventricular myocardium and brain of CR9 Tg-line1 mice treated with continuous infusion of PE for 3 d. Average transcript level in the ventricular myocardium of preinfused mice was defined as 1 (means ± SE; $n$=5 hearts). *$P < 0.01$ *vs.* sham-infused mice; *t* test. *D*) Relative transcript levels of luciferase reporter in the ventricular myocardium and brain of the CR9 Tg-line1 mice continuously infused with PE for 3 d. Average transcript level in the ventricular myocardium and brain of preinfused mice was defined as 1. (means ± SE; $n$=5 hearts). *$P < 0.01$ *vs.* sham-infused mice; *t* test. *E*) Comparison of the chemiluminescence intensities obtained using different luciferin injection methods in a Tg-line1 mouse; injections 1 and 2, intraperitoneal injections (top panels), injections 3, 4, and 5, intravenous injections (bottom panels). Injections were performed ≥4 h apart to eliminate the effect of the previous injection. Inset in each panel shows a magnified image of the heart. *F*) Scatterplots of the chemiluminescence intensities in the heart and brain. Plots indicate the independent experiments shown in each panel in *E*. There is a linear relationship between the expression in the heart and the brain, $R^2 = 0.979$.

expression of endogenous *Nppa* and *Nppb* mRNA increased 3 d after the PE infusion began (Fig. 3*B, C* and Supplemental Fig. S2*B, C, F, G*). Concomitantly, the quantitative PCR analysis of the CR9 luciferase mRNA expression showed enhanced expression in the ventricular myocardium 3 d after the PE infusion (Fig. 3*D* and Supplemental Fig. S2*D, H*). On the other hand, in the brain and the salivary glands where neither *Nppa* nor *Nppb* is highly expressed, the CR9-driven luciferase mRNA expression did not respond to PE (Fig. 3*B–D* and Supplemental Fig. S2*B–D, F–H*). Therefore, the patterns of PE-induced luciferase expression suggest that CR9 is almost exclusively active in the heart. Because the integration sites were random in these three lines, the patterns of luciferase expression depend on CR9 or other enhancers near the integrated sites. The expression of luciferase in the brain of Tg-line1 and salivary glands of Tg-line2, both of which express neither *Nppa* nor *Nppb,* might be driven by other enhancers near the integrated sites.

To evaluate the accuracy and reproducibility of this method, we measured the luminescence in the heart of

a mouse from Tg-line1. In this transgenic line, the brain, intestine, and testis expressed the reporter protein due to positional effects of the insertion site and most likely not due to CR9 activity. Because the luciferase mRNA expression in the brain remained unchanged after PE treatment (Fig. 3*D*), we used the reporter activity in the brain as a control. The absolute luminescence values of the heart were affected by the injection method and the amount of luciferase substrate injected (Fig. 3*E*). However, using brain luminescence as a control, we successfully eliminated the signal variations caused by these differences. The ratio of the luminescence in the heart and brain remained constant within each mouse, independent of the injection method (Fig. 3*F*). Therefore, we defined the ratio of heart to brain luciferase intensities as the cardiac-specific enhancer activity.

### Distal enhancer element was activated in the murine model of heart failure

To examine whether the CR9 enhancer was also responsible for gene expression in other pathological conditions, we subjected Tg-line1 mice to heart failure induced by TAC and compared them with sham-surgery mice. This model mimics the heart condition of patients with hypertension who suffer a continuous pressure overload on the heart. The pressure overload by TAC caused potent cardiac hypertrophy at 2 wk postsurgery and reduced cardiac contractility at 3 wk postsurgery (**Fig. 4*A*, *B***), as previously reported (22). The endogenous *Nppa* and *Nppb* expression increased severalfold in the ventricular myocardium 3 wk after the TAC surgery (Fig. 4*C*). The heart to brain luciferase intensity ratio also increased severalfold 3 wk following the TAC surgery (Fig. 4*D*, *E* and Supplemental Fig. S3). However, the heart to brain luciferase intensity ratio of sham-surgery mice did not change after the surgery (Fig. 4*D*, *E* and Supplemental Fig. S3; 3 wk after TAC surgery: 5.7±1.3 fold; 3 wk after sham surgery: 1.0±0.2 fold; $P < 0.001$, repeated ANOVA). These results suggest that CR9 increases transcriptional activity during mechanical pressure overload-induced hypertrophy and subsequent heart failure.

## DISCUSSION

Here, we focused on the stress-responsive regulatory elements of *Nppa* and *Nppb* in heart failure. By screening the evolutionarily conserved and epigenetically modified regions around the *Nppa* and *Nppb* gene loci, we identified a 650-bp transcriptional enhancer that was responsive to an $\alpha_1$-adrenergic receptor agonist *in vitro*. Furthermore, *in vivo* 3C analysis revealed that this distal enhancer directly interacted with the 5′-flanking regions of both *Nppa* and *Nppb*. Using *in vivo* live imaging of luciferase reporter gene expression, we observed that this 650-bp enhancer caused cardiac-specific activation of reporter gene expression during the progression of pressure overload-induced heart failure. Notably, this is the first study to provide a time series analysis for monitoring enhancer activity under pathological conditions in an individual live mouse.

Although numerous approaches have been used to explore the stress-responsive regulatory elements driving gene transcription during heart failure (11, 12, 14), these elements have not yet been identified due to the technical difficulty involved. To detect the elements that are responsive to pathological conditions such as heart failure, it is essential to confirm the activity of the responsive element using a beating heart that remains connected to the systemic cardiovascular system. Therefore, it would be beneficial to establish transgenic mouse lines carrying a reporter plasmid to assess the responsive elements driving the expression of specific genes. However, the creation of multiple stable adult mouse lines to identify these elements is time-consuming.

In this study, we utilized two improved methods for reporter analysis and successfully identified a novel potent enhancer.

First, by performing an enhancer analysis using a lentiviral vector, we accurately identified candidate enhancers in cardiomyocytes and subsequently generated transgenic reporter mice. Previous promoter analyses used electroporation or lipofection to transfect cultured cardiomyocytes with plasmids (37, 38), but the transfection efficiency of these methods in primary cardiomyocytes is too low to accurately measure reporter activity during the stress response. In this study, greater than 90% transduction efficiency of cardiomyocytes was achieved using a lentiviral vector, which enabled us to accurately identify a specific enhancer fragment. Using this method, we efficiently minimized the number of reporter plasmids to be subsequently integrated into the mouse genome to screen for potential enhancers.

Second, by sequentially measuring the enhancer activity in a single live mouse, we collected robust data to assess enhancer activity in the heart *in vivo*. LacZ is not a suitable reporter for this purpose because LacZ activity can only be assessed after animal euthanization. Therefore, we overcame this limitation using the luciferase reporter plasmid. Recent advances in high-sensitivity luminescence imaging have made it possible to evaluate enhancer-driven luciferase activity without operating on the mice. Therefore, we sequentially assessed reporter activity and hemodynamic changes in the same mouse throughout the time course of the development of heart failure. These data were highly reproducible and enabled us to identify an enhancer element that was activated by cardiac overload. Because this method can be applied to any organ, the *in vivo* luciferase reporter assay may be used for assessing the *in vivo* enhancer or promoter activities responsible for clinically important diseases. The noninvasive nature of this method also enabled us to simultaneously assess the hemodynamic and metabolic parameters *in vivo* along with reporter activity. Specifically, the Tg-line1

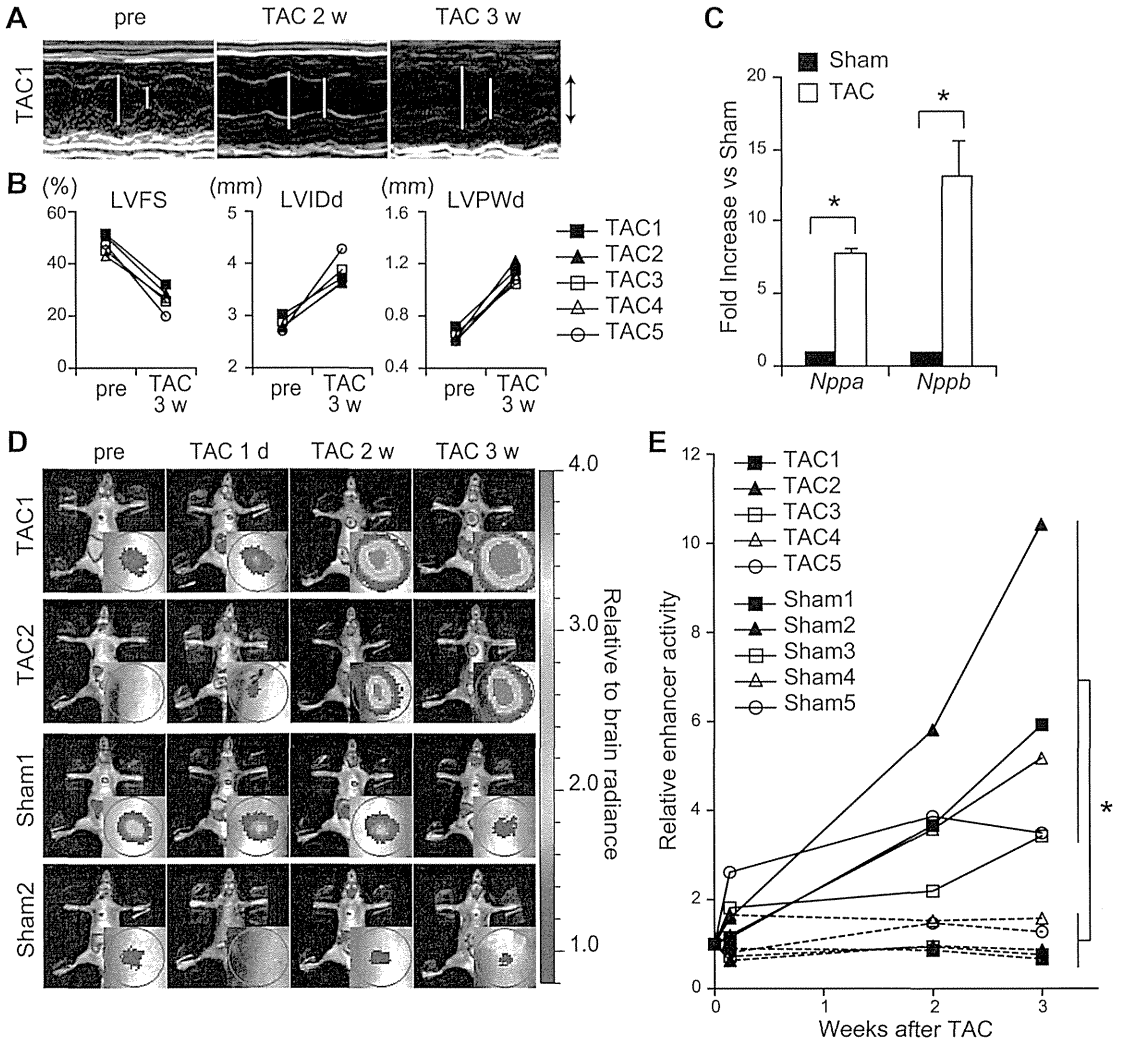


**Figure 4.** Distal enhancer element is reactivated in the murine model of heart failure. *A*) Representative M-mode echocardiograms in a mouse of Tg-line1 (TAC1) before and after TAC. Open bars indicate maximal left ventricular internal dimension in diastole (LVIDd) and maximal left ventricular internal dimension in systole (LVIDs). Up and down arrows represent 3 mm. *B)* Echocardiographic changes in left ventricular fractional shortening (LVFS), LVIDd, and left ventricular posterior wall thickness in diastole (LVPWd) in 5 mice of Tg-line1 (TAC1-5) before and after TAC. *C*) Relative *Nppa* and *Nppb* transcript levels in the ventricular myocardium 3 wk after the TAC procedure (means ± SE; $n$=3 hearts). *$P < 0.05$ *vs.* sham-surgery mice; *t* test. *D*) Sequential *in vivo* live imaging of 4 representative Tg-line1 mice before and after TAC or sham surgery at each time point. Top 2 and bottom 2 panels represent sequential imaging data of TAC and sham-surgery mice, respectively. Sequential imaging of the 6 other surgically treated mice is shown in Supplemental Fig. S3. Insets in images show magnified images of the heart. Color scale depends on the ratio relative to brain intensity. *E*) Cardiac-specific enhancer activity plots of 10 Tg-line1 mice (TAC1, TAC2, and Sham1, Sham2, shown in *D*) and TAC3–5 and Sham3–5 shown in Supplemental Fig. S3). Heart to brain luciferase intensity ratio represents the cardiac-specific enhancer activity; enhancer activity in presurgery mice was defined as 1. 3 wk after TAC surgery: 5.7 ± 1.3 fold; 3 wk after sham surgery: 1.0 ± 0.2 fold; means ± SE; $n$ = 5. *$P < 0.001$, repeated ANOVA.

mice enabled us to accurately quantify the expression level of the natriuretic peptides. These mice are useful tools for repeatedly assessing the degree of heart failure to screen various cardiovascular drugs.

The integration of activities from multiple enhancers could confer specificity and robustness to transcriptional regulation (1). Warren *et al.* (14) identified the *Nppa* enhancer in the embryonic heart by examining Nkx2-5 binding regions around the *Nppa* locus, but the enhancer did not respond to heart failure. This enhancer does not overlap with CR9 and might regulate *Nppa* expression only during the embryonic stage (14). On the other hand, Horsthuis *et al.* (11) showed that the regulatory region from −27 to +58 kb relative to the transcription start site of *Nppa* was sufficient for *Nppa* gene expression in the failing heart, similar to CR9. However, because this 85-kb regulatory region does not include CR9, *Nppa* may have multiple enhanc-

ers that regulate its expression during heart failure. Furthermore, the length of the 85-kb region poses a challenge for understanding its specific biological role.

This is the first study to provide a time course imaging analysis of enhancer activity using an individual live diseased mouse model. Using this new method, we identified a novel heart enhancer. This method can be widely used for identifying enhancers that regulate transcriptional activity only under pathological conditions. FJ

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