Cardioprotective Effect of Apelin-13 on Cardiac Performance and Remodeling in End-Stage Heart Failure

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Background: Apelin and its cognate G protein-coupled receptor, APJ, constitute a signaling pathway with a positive inotropic effect on cardiac function. Recently, we and other investigators demonstrated that a reduction in myocardial apelin/APJ expression might play a critical role in experimental models of end-stage heart failure (HF). Therefore, we evaluated whether exogenous apelin infusion restores apelin/APJ expression and improves cardiac function in the failing heart of Dahl salt-sensitive hypertensive (DS) rats.

Methods and Results: High salt-loaded DS rats were treated with vehicle and pyroglutamylated apelin-13 (Pyr-AP13; 200 µg·kg⁻¹·day⁻¹, IP) from the age of 11 to 18 weeks. Decreased end-systolic elastance and percent fractional shortening in failing rats was significantly ameliorated by Pyr-AP13. Pyr-AP13 effectively inhibited vascular lesion formation and suppressed expression of inflammation factors such as tumor necrosis factor-α and interleukin-1β protein. Downregulation of apelin and APJ expression, and phosphorylation of endothelial nitric oxide synthase at Ser^473 and Akt at Ser^473 in failing rats was significantly increased by Pyr-AP13. Upregulation of NAD(P)H oxidase p22^phox, p47^phox, and gp91^phox in DS rats was significantly suppressed by Pyr-AP13.

Conclusions: Exogenous apelin-13 may ameliorate cardiac dysfunction and remodeling and restore apelin/APJ expression in DS rats with end-stage HF. Thus, apelin-13 may have significant therapeutic potential for end-stage HF. (Circ J 2012; 76: 137–144)

Key Words: Apelin; Cardiac function; Heart failure; Remodeling
angiotensin II (Ang II) type 1 receptor (AT1R) blocker in end-stage HF with severe left ventricular (LV) dysfunction. However, the effect of exogenous apelin on the apelin/APJ pathway in end-stage HF remain unknown. Accordingly, we investigated whether exogenous apelin-13 restores expression of apelin and APJ and improves cardiac function and cardiovascular remodeling in DS rats with end-stage severe HF.

**Methods**

All experimental procedures and protocols in this study were in accordance with the Dokkyo Medical University School of Medicine institutional guidelines for animal research and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Animal Models and Experimental Designs**

Male, inbred DS rats and Dahl salt-resistant (DR) rats (Eisai, Tokyo, Japan) were weaned and fed a diet containing 0.3% NaCl until the age of 6 weeks. Thereafter, they were fed a diet containing 8% NaCl until the age of 18 weeks. Systolic blood pressure (SBP) was measured by the tail-cuff method at the start of the study and at 1-week intervals thereafter. Transthoracic echocardiography evaluating the LV end-diastolic diameter (LVEDD), and percent fractional shortening (%FS) were performed at 18 weeks, as described previously. At the age 11 weeks, when LV hypertrophy developed, DS rats were randomly divided into two groups: treated with vehicle (DSHF-V), and automatic infusion of pyroglutamylated apelin (Pyr-AP13) (Pyr-AP13, 200 μg kg⁻¹ day⁻¹; DSHF-AP) via an Alzet osmotic minipump implanted intraperitoneally in the abdominal cavity followed by closure of the abdominal wall in layers. This common posttranslational modification preserves biological activity by rendering the peptide more resistant to enzymatic cleavage. Therefore, we used Pyr-AP13 for this chronic study. Age-matched male DR rats served as a control group (DR-C).

**LV Pressure-Volume Relation**

The chest was opened via a midline sternotomy, and the pericardium was dissected to expose the heart. The LV end-systolic pressure—volume relationship (contractility: end-systolic elastance (Ees)) was modified for the conductance catheter technique as described previously. Briefly, the conductance catheter was inserted into the LV through the apex and pushed until the distal tip was placed in the ascending aorta along the longitudinal axis of the LV. A 3F catheter-tip micromanometer (SPR-524, Millar instruments) was also inserted into the LV from the apex. To change the preload, a snare was placed around the inferior vena cava. We recorded conductance volume and LV pressure simultaneously during gradual inferior vena cava occlusion. Electrical signals were digitized through an analog-to-digital converter (AD12-8, Contec) at a sampling frequency of 1,000 Hz with 12-bit resolution and stored on a personal computer (Dynabook SS 330, Toshiba). The points of the end-systolic pressure—volume relation were determined by an iterative technique reported previously. In several consecutive pressure—volume loops, the points of each cardiac cycle with maximum pressure-volume ratio were first determined. Linear regression of these points with expression:

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\text{Pes=Ees(Ves–Vo)}
\]

yielded estimates for the slope, or Ees, and the volume-axis intercept (Vo), where Pes and Ves are end-systolic pressure and volume, respectively.

**Western Blot Analysis**

Apelin, APJ, Akt, NAD(P)H oxidase p22phox, p47phox, gp91phox, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), monocyte chemotaxin protein-1 (MCP-1), angiotensin-converting enzyme (ACE), AT1R, and nuclear factor-κ B (NF-κB) protein expressions were measured as described previously. LV was homogenized (25% W/vol) in 10mmol/L HEPES buffer, pH 7.4, containing 320mmol/L sucrose, 1mmol/L EDTA, 1mmol/L DTT, 10μg/ml leupeptin, and 2μg/ml aprotonin at 0°C to 4°C with a polytron homogenizer. Protein concentrations were determined with bovine serum albumin as a standard protein. Equal amounts of protein were loaded in each lane of SDS-PAGE using 13% gels. The proteins in the gels were transferred electrophoretically to PVDF sheets for 1h at 2mA/cm². The sheets were immunoblotted with an anti-apelin, anti-APJ, anti-Akt, anti-NAD(P)/H oxidase subunits, anti-TNF-α, anti-IL-1β, and anti-MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a buffer containing 10mmol/L Tris-HCl, pH 7.5, 100mmol/L NaCl, 0.1% Tween 20, and 5% skim milk. The proteins transferred to the sheets were detected using the ECL immunoblotting detection system (Amersham Life Science Inc).

**Phosphorylation of eNOS at Ser1177, Akt at Ser473, and NF-κB at Ser536**

Phosphorylation of eNOS at Ser1177, Akt at Ser473, and NF-κB at Ser536 was measured in detail as described previously.

**Detection of Superoxide Anion in the LV**

Histological detection of superoxide anion in the LV was performed using dihydroethidium (DHE) as described previously.

**Nitrite Production**

Nitrite production was measured as described in detail previously. The LV was used for the assay of nitrite production within 24h. Three 50-μm sections of myocardium from each rat were cut on a vibratome and incubated in a buffer (pH 7.2) containing 25mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) (Sigma, St Louis, MO, USA); 140mmol/L NaCl, 5.4mmol/L KCl, 1.8mmol/L CaCl2, 1mmol/L MgCl2, and 5mmol/L glucose for 48h at 37°C. The supernatant was used for the assay of NO2⁻ production, and the amount of NO2⁻ was corrected via the protein amount measured using the Bradford method (Bio-Rad, Richmond, CA, USA). Nitrite was measured with an autoanalyzer (TCA-NOX 1000m; Tokyo Kasei Kogyo, Tokyo, Japan) using the Griess method.

**Histologic Examination and Evaluation of Cardiovascular Remodeling**

Histologic examination was performed as described in detail previously. Histologic examination was performed using dihydroethidium (DHE) as described previously. The wall-to-lumen ratio (the area of the vessel wall divided by the area of the total blood vessel lumen) was determined. The area of fibrosis immediately surrounding the blood vessels was calculated, and perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total area of the vessel. To assess the area of myocardial interstitial fibrosis, the area of pathologic collagen deposition was measured in the microscopic field of each Masson’s trichrome-stained section. The ratio of the total area of fibrosis within the LV myocardial sample to the total area of the LV myocardium in each heart was calculated and used for analysis.
Statistical Analysis
All values are expressed as mean±SEM. Mean values were compared among the 3 groups by ANOVA and the Bonferroni post hoc test for multiple comparisons. A value of P<0.05 was considered statistically significant.

Results
Physiological Profiles After 7 Weeks’ Treatment With Pyr-AP13 in DS Rats
Body weight (BW), SBP, LV weight (LVW) to BW ratio, and heart rate (HR) in the 3 groups are presented in Table. BW was significantly lower in DSHF-V than in DR-C rats. Long-term Pyr-AP13 therapy in DS rats significantly increased BW. In contrast, DSHF-V rats had a higher LVW/BW than did DR-C rats. Long-term Pyr-AP13 therapy in DS rats significantly decreased LVW/BW.

Time-related changes in SBP among the 3 groups are shown in Figure 1. Before feeding with 8% NaCl diet, SBP was 114±3 mmHg in DR-C, 117±4 mmHg in DSHF-V and 118±4 mmHg in DSHF-AP. As shown in Figure 1 and Table, SBP in DSHF-V and DSHF-AP rats was similar and significantly higher than that in DR-C rats. HR was significantly higher in DSHF-V than in DR-C rats. Long-term Pyr-AP13 therapy in DS rats significantly decreased HR (Table, Figure 1).

LV Ees in End-Systolic Pressure-Volume Relation and Cardiac Function for LVEDD and %FS
LVEDD was significantly higher in DSHF-V than in DR-C rats. Long-term Pyr-AP13 therapy in DS rats significantly decreased LVEDD. In contrast, DSHF-V rats had lower Ees and %FS than did DR-C rats. Long-term Pyr-AP13 therapy in DS rats significantly improved Ees and %FS (Table).

Cardiovascular Remodeling
The wall-to-lumen ratio, perivascular fibrosis, and area of interstitial fibrosis of the heart in the 3 groups are shown in Figure 2F and Table (n=10 tissue sections per group). The wall-to-lumen ratio was significantly increased in DSHF-V rats compared with DR-C. Long-term Pyr-AP13 treatment caused significant amelioration of this ratio. Perivascular and interstitial fibrosis was significantly greater in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 treatment also caused significant improvement in perivascular and interstitial fibrosis.

Apelin and APJ Expression
Apelin and APJ protein expressions in the LV of DS rats were significantly lower in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 significantly increased these expressions in DS rats (Figure 3).

Phosphorylation of eNOS at Ser1177 and Akt at Ser473 and Nitrite Production
Phosphorylation of eNOS at Ser1177 and Akt at Ser473 and
nitrite production in the LV was significantly lower in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 significantly increased eNOS and Akt phosphorylation and nitrite production in DS rats (Figure 4).

NAD(P)H Oxidase Subunits Expression and Superoxide Anion Production
NAD(P)H oxidase p22phox, p47phox, and gp91phox protein levels, and superoxide anion production by DHE in the LV were significantly higher in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 therapy significantly decreased these expressions in DS rats (Figure 5).

TNF-α, IL-1β, and MCP-1 Expression
TNF-α, IL-1β, and MCP-1 protein levels in the LV were significantly higher in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 therapy significantly decreased these expressions in DS rats (Figure 6).

ACE and AT1R Expression and Phosphorylation of NF-κB at Ser536
ACE and AT1R protein levels, and phosphorylation of NF-κB at Ser536 in the LV were significantly higher in DSHF-V rats than in DR-C rats. Long-term Pyr-AP13 therapy significantly decreased ACE and AT1R expression and NF-κB phosphorylation.
Effect of Apelin-13 in CHF

Discussion

We administered Pyr-AP13 to DS rats with end-stage HF and observed an inhibition of cardiac dysfunction and cardiovascular remodeling, and expression of inflammation factors such as TNF-α, IL-1β, and MCP-1. In addition, we demonstrated that the cardiac apelin/APJ, Akt/eNOS, oxidative stress pathway had deteriorated in end-stage HF with severe LV dysfunction, but was restored by Pyr-AP13. These findings suggest that Pyr-AP13 may improve cardiac dysfunction and remodeling and the apelin/APJ expression associated with Akt/eNOS and oxidative stress pathways in the failing heart of DS rats.

Figure 4. Effects of chronic apelin-13 infusion on phosphorylation of eNOS at Ser1177 and Akt at Ser473 and nitrite production. Values are mean±SEM, n=5–6 per group. *P<0.05 vs. DR-C; †P<0.05 vs. DSHF-V. DSHF-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DR-C, Dahl salt-resistant control rats; eNOS, endothelial nitric oxide synthase.

Figure 5. Effects of chronic apelin-13 infusion on NAD(P)H oxidase p22phox, p47phox, and gp91phox protein expression (A) and superoxide anion production (B–D). Values are mean±SEM, n=5–6 per group. *P<0.05 vs. DR-C; †P<0.05 vs. DSHF-V. (B) DR-C; (C) DSHF-V; (D) DSHF-AP. Bar=500 μm. DSHF-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DSHF-AP, Dahl salt-sensitive hypertensive rats treated with apelin-13; DR-C, Dahl salt-resistant control rats.
Thus, the apelin/APJ pathway may play a pivotal role in end-stage HF with severe LV dysfunction, and apelin-13 may be a potential therapeutic target in end-stage severe HF.

Enormous effort has been directed toward identifying new therapeutic strategies with long-term efficacy in HF. Recent studies have confirmed that the endogenous apelin/APJ signaling pathway contributes to cardiac function in the failing heart. Although apelin-deficient mice display normal or only slightly impaired basal cardiac function, they have marked reductions in exercise capacity and maximum oxygen consumption. They manifest progressive cardiac dysfunction from 6 months of age and develop severe HF when subjected to chronic pressure overload via surgical aortic banding. Iwanaga et al demonstrated that although apelin mRNA was unchanged in the compensatory LH hypertrophy phase, it decreased together with the progression of LV dysfunction, and the expression levels APJ also decreased. These findings indicated that myocardial apelin expression might be downregulated, accompanied by a decrease in APJ expression, in end-stage HF. Recently, we also reported that a similar reduction in cardiac apelin/APJ protein expression was seen in end-stage HF with severe LV dysfunction. Moreover, exogenous apelin potently enhances myocardial contractility. In isoproterenol-induced myocardial injury in rats, apelin and APJ genes were downregulated and apelin infusion ameliorated the effects of cardiac injury. In vivo, acute apelin infusion restores ejection fraction, increases cardiac output and reduces LV end-diastolic pressure in rats with chronic HF. Kuba et al showed that apelin KO mice exhibited normal cardiac development in adulthood, but which deteriorated with aging or with chronic pressure overload, and a con-

![Figure 6. Effects of chronic apelin-13 infusion on TNF-α, IL-1β, and MCP-1 protein expressions. Values are mean±SEM. n=5–6 per group. *P<0.05 vs. DR-C; †P<0.05 vs. DSF-V. DSF-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DR-C, Dahl salt-resistant control rats; IL, interleukin; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.](image6)

![Figure 7. Effects of chronic apelin-13 infusion on ACE and AT1R protein expression and phosphorylation of NF-κB at Ser536. Values are mean±SEM. n=5–6 per group. *P<0.05 vs. DR-C; †P<0.05 vs. DSF-V. ACE, angiotensin-converting enzyme; AT1R, angiotensin II type 1 receptor; DSF-V, Dahl salt-sensitive hypertensive rats treated with vehicle; DR-C, Dahl salt-resistant control rats; NF, nuclear factor.](image7)
The vasodilatory response to apelin is endothelium-dependent because vasoconstriction occurs in endothelium-denuded vessels. This endothelium-dependent vasodilatation appears to be mediated predominantly through eNOS-dependent pathways. The current study found diminished phosphorylation of Akt and eNOS in aortic tissues form diabetic db/db mice. In addition, an abnormal Akt/eNOS phosphorylation pathway might serve to dampen endothelial NO signaling in aortic rings, thereby leading to abnormal vasoreactive responses to Ang II and acetylcholine. In aortic rings from db/db mice, apelin treatment significantly enhanced phosphorylation of Akt and eNOS, which is consistent with previously published findings. Apelin can facilitate the activity of eNOS in endothelial cells and APJ is crucially involved in apelin-induced phosphorylation of eNOS, which promotes the release of NO.

In addition, the apelin/APJ pathway mediated amelioration of atherosclerosis might be related to increased NO production, and the NOS inhibitor, L-NAME, blocks apelin-mediated amelioration of Ang II-exacerbated vascular disease in the ApoE-KO model. These results suggest that chronic apelin infusion potentiates the activity of the apelin/APJ signaling pathway, which may, at least in part, contribute to increased Akt/eNOS phosphorylation. On the other hand, ROS act as intercellular and intracellular messengers that play an important pathophysiologic role in vascular biology. Increased oxidative stress resulting from enhanced production of ROS and/or inadequate mechanisms of antioxidant defense has been recognized as a critical mechanism in the initiation of cardiac dysfunction and the transition from hypertrophy to HF. Moreover, numerous studies have reported that the failing heart is subjected to increased oxidative stress, and antioxidant strategies have been proposed to prevent the progression and development of chronic HF. Recently, Fousali et al. showed that chronic treatment of mice with apelin attenuated pressure-overload-induced LV hypertrophy, and that the prevention of hypertrophy by apelin was associated with increased myocardial catalase activity and decreased plasma lipid hydroperoxide, as an index of oxidative stress. Apelin preserved cardiac function by inhibiting oxidative stress and stimulating catalase activity, supporting the premise that apelin is a potent regulator of cardiomyocyte antioxidant reserve against oxidative stress during hypertrophic remodeling of the heart. In the present study, we demonstrated that apelin significantly prevented the progression of cardiac dysfunction and cardiovascular remodeling in HF, and inhibited NAD(P)H oxidase subunit expression and ROS production. These findings suggest that chronic apelin infusion may play a pivotal role in activation of the antioxidant system in DS rats with end-stage severe HF.

In the present study, we showed that long-term apelin-13 infusion ameliorated LV weight, cardiovascular remodeling, and cardiac function, and inhibited inflammation and oxidative stress, and restored apelin/APJ expression. However, the underlying cardioprotective mechanism of apelin-13 is unclear. The apelin/APJ pathway has opposing actions to the renin–angiotensin system (RAS) in a number of physiologic and pathophysiologic settings. There is some evidence for direct counterregulation of the RAS by the apelin/APJ pathway. Ishida et al. have demonstrated differences in blood pressure response in mice lacking both AT1R and APJ compared with mice lacking only AT1R. In addition, Ang II stimulation produces superoxide via AT1R and activation of NAD(P)H oxidases in endothelial and vascular smooth muscle cells. Moreover, chronic activation of NF-κB may induce expression of inflammatory cytokines and produce detrimental consequences. Therefore, to evaluate the mechanism, we performed experiments to determine whether long-term apelin-13 infusion inhibited ACE and AT1R expression and NF-κB phosphorylation. We found that ACE and AT1R protein expressions and NF-κB phosphorylation were upregulated in DS rats, and apelin-13 infusion suppressed these changes. These findings suggest that the cardioprotective mechanism of apelin-13 may in part be explained by inhibition of the RAS and NF-κB signaling pathway.

Conclusions

We administered long-term apelin infusion DS rats with severe end-stage HF and observed an inhibition of both cardiovascular remodeling and cardiac dysfunction. Moreover, we showed that the cardiac apelin/APJ pathway is suppressed in end-stage HF with severe LV dysfunction, and that this signaling pathway is restored by apelin infusion. In addition, apelin infusion stimulated Akt/eNOS phosphorylation, and inhibited expression of NAD(P)H oxidase subunits and ROS production. These findings suggest that the apelin/APJ pathway may play a critical role in the pathophysiology of HF, and that apelin infusion may be a therapeutic strategy for severe HF.

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Disclosure

All the authors declared no conflicts of interest.

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