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CNP infusion attenuates cardiac dysfunction and inflammation in myocarditis

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Abstract

Myocarditis is an acute inflammatory disease of the myocardium for which there is currently no specific therapy. We investigated the therapeutic potential of C-type natriuretic peptide (CNP) in acute experimental autoimmune myocarditis. One week after injection of porcine myosin into male Lewis rats, CNP (0.05 μg/kg/min) was continuously administered for 2 weeks. CNP infusion significantly increased maximum dP/dt, decreased left ventricular end-diastolic pressure, and improved fractional shortening compared with vehicle administration. In vehicle-treated hearts, severe necrosis and marked infiltration of CD68-positive inflammatory cells were observed. Myocardial and serum levels of monocyte chemoattractant protein-1 were elevated in myocarditis. However, these changes were attenuated by CNP infusion. In addition, treatment with CNP significantly increased myocardial capillary density. Guanylyl cyclase-B, a receptor for CNP, was expressed in myocarditic heart, and cyclic guanosine monophosphate was elevated by CNP infusion. In conclusion, CNP infusion attenuated cardiac function in acute myocarditis through anti-inflammatory and angiogenic effects.

Keywords: Angiogenesis; Inflammation; MCP-1; Myocarditis; Natriuretic peptides

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation. Acute myocarditis is associated with rapidly progressive heart failure, arrhythmias, and sudden death [1]. Immunomodulatory therapies such as immunoglobulin and interferon are regarded as promising for myocarditis [2,3]; however, the efficacy of those treatments still remains controversial [3,4]. Other treatment options are restricted to supportive care for heart failure or arrhythmias. The lack of specific treatment and the potential severity of the illness underlie the importance of novel and effective therapeutic strategies for myocarditis.

There are three main natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), all of which signal through natriuretic receptors and cyclic guanosine monophosphate (cGMP) signaling pathways. ANP and BNP are predominantly secreted from cardiac myocytes. They have anti-hypertrophic effects on cardiac myocytes in an autocrine manner and also have inhibitory effects on collagen synthesis of cardiac fibroblasts in a paracrine manner, and thus have suppressive effects on cardiac remodeling. Cardioprotective effects of ANP and BNP have already been demonstrated, and they are used clinically for the treatment of heart failure. On the other hand, CNP, originally identified in the porcine brain [5], is predominantly

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expressed in vascular endothelial cells and plays a role in the local regulation of vascular tone and remodeling [6]. However, the potential therapeutic effects of CNP in heart disease are not well understood.

Recently, it has been shown that CNP is also synthesized in cardiac fibroblasts and inhibits collagen synthesis of cardiac fibroblasts more potently than ANP and BNP [7]. In addition, CNP proved to have more potent anti-hypertrophic effects than ANP in cultured cardiac myocytes [8]. More recently, infusion of CNP has been shown to improve cardiac function after myocardial infarction through anti-fibrotic and anti-hypertrophic effects [9]. These findings indicate the therapeutic potential of CNP in heart disease. However, it remains unknown whether CNP infusion improves acute myocarditis leading to severe heart failure. In the present study, cardiac myosin purified from pig hearts was injected into rats, and autoimmune myocarditis was induced [10].

Thus, the purposes of this study were (1) to investigate whether infusion of CNP improves cardiac function in a rat model of acute myocarditis, and (2) to investigate the mechanisms responsible for the effect of CNP on the myocarditic heart.

Materials and methods

Model of acute myocarditis. We produced a rat model of acute myocarditis by injecting pig cardiac myosin. In brief, purified myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [11]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and 0.2 ml of the antigen-adjuvant emulsion was injected into the foot pads.

CNP preparation and treatment. CNP was diluted in PBS with 5% glucose and administered via an ALZET mini-osmotic pump (DURECT Corporation, Cupertino, CA, USA) inserted subcutaneously, which discharged CNP at a rate of 0.05 μg/kg/min for the duration of 14 days beginning 1 week after myosin injection.

Experimental groups. Rats with sham operation or those with acute myocarditis were treated with vehicle or CNP. Fifty-four male 10-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were randomly placed into four groups and received the following treatments: (1) sham rats given vehicle ($n = 12$), (2) sham rats given CNP ($n = 12$), (3) myocarditis rats given vehicle ($n = 15$), and (4) myocarditis rats given CNP ($n = 15$).

Echocardiography. Echocardiography was performed at day 21 post-myosin injection. Rats were anesthetized with sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), anterior wall thickness (AWT), and posterior wall thickness (PWT) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as follows;

$$\%FS = (LVDd - LVDs)/LVDd \times 100$$

Hemodynamic study. Hemodynamic measurements were taken at day 21 post-myosin injection. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate was also monitored with electrocardiogram. As indexes of hemodynamics, heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt, and minimum dP/dt were used. Anesthesia was maintained with sodium pentobarbital, and the above mentioned indexes were recorded simultaneously during spontaneous ventilation after an equilibration period of a minimum of 20 min.

Histopathology. The heart was excised above the origin of the great vessels, and the heart and body weights were recorded. A midventricular portion of the heart was fixed with formalin and embedded in paraffin, and 4-μm sections were cut and stained with either hematoxylin and eosin (H&E) or Masson's trichrome stain, or subjected to immunohistochemical staining. H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) for the characterization of myocardial injury and inflammation, without knowledge of the experimental groups, on the following sc ale: (0) no or questionable presence, (1) limited focal distribution, (2 and 3) intermediate severity, and (4) coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue.

Immunohistochemistry. Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Sections were incubated with DakoCytomation protein block, then with anti-von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), CD68 (DakoCytomation), or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, San Jose, CA, USA) antibodies, followed by sequential incubations with HRP-linked rabbit anti-mouse IgG (DakoCytomation). The reaction products were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with hematoxylin. The numbers of vWF-stained capillaries and CD68-stained cells were determined in ten randomly selected fields (vWF; 400×, CD68; 200×).

Enzyme-linked immunosorbent assay (ELISA). Serum MCP-1 level on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA Kit (Biosource International, Camarillo, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of guanylyl cyclase-B (GC-B) mRNA, a receptor for CNP, was examined by RT-PCR. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and myocarditis rats given vehicle ($n = 5$ in each group). Total RNA was extracted from heart with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed (PCR Amplification Kit, Takara, Shiga, Japan). The complementary DNA was amplified by the PCR using specific primers for GC-B or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers for GC-B were as follows [12]: sense primer 5′-AACGGGCG CATTGTGTATATCTGCGGC-3′ and antisense primer 5′-TTATCA CAGGATGGGTCGTCCAAGTCA-3′. For GAPDH, the primers were as follows: sense primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and antisense primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

Radioimmunoassay. To investigate whether subcutaneous administration of CNP has a biological activity in heart, we measured myocardial level of cGMP. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and those given CNP ($n = 10$ in each groups). Myocardial level of cGMP was measured with a radioimmunoassay kit (cGMP assay kit; YAMASA Co., Chiba, Japan).

Statistical analysis. Data were presented as means ± SEM. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P < 0.05$.

Results

Improvement in cardiac function by CNP treatment

Myocarditis rats given vehicle had two deaths 19 and 21 days after myosin injection, respectively, whereas those treated with CNP showed no mortality. At 3 weeks post-myosin injection, Myocarditis rats given vehicle showed decreased maximum dP/dt and minimum dP/dt, and

increased LVEDP compared with the sham rats (Fig. 1A–C), indicating the presence of acute heart failure in this model. Such parameters subsequently returned to baseline with CNP treatment. On echocardiography, rats with myocarditis showed an increase in LVDd and a significant reduction in %FS (Fig. 1D–F). CNP infusion significantly improved %FS in myocarditis rats. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was reduced by CNP treatment (Table 1). MAP was significantly decreased in myocarditis rats, and the decrease was significantly attenuated by CNP treatment. CNP did not significantly influence cardiac function in sham rats.

Attenuation of inflammatory cell infiltration by CNP treatment

Histological examination showed that myocardial necrosis and tissue granulation as well as inflammation and edema were markedly increased in our model of acute myocarditis (Fig. 2A and B). CNP administration significantly attenuated necrotic changes observed in myocarditis rats. CNP-treated hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.) as compared to vehicle-treated hearts. Although, CNP is known to have potent anti-fibrotic activ-

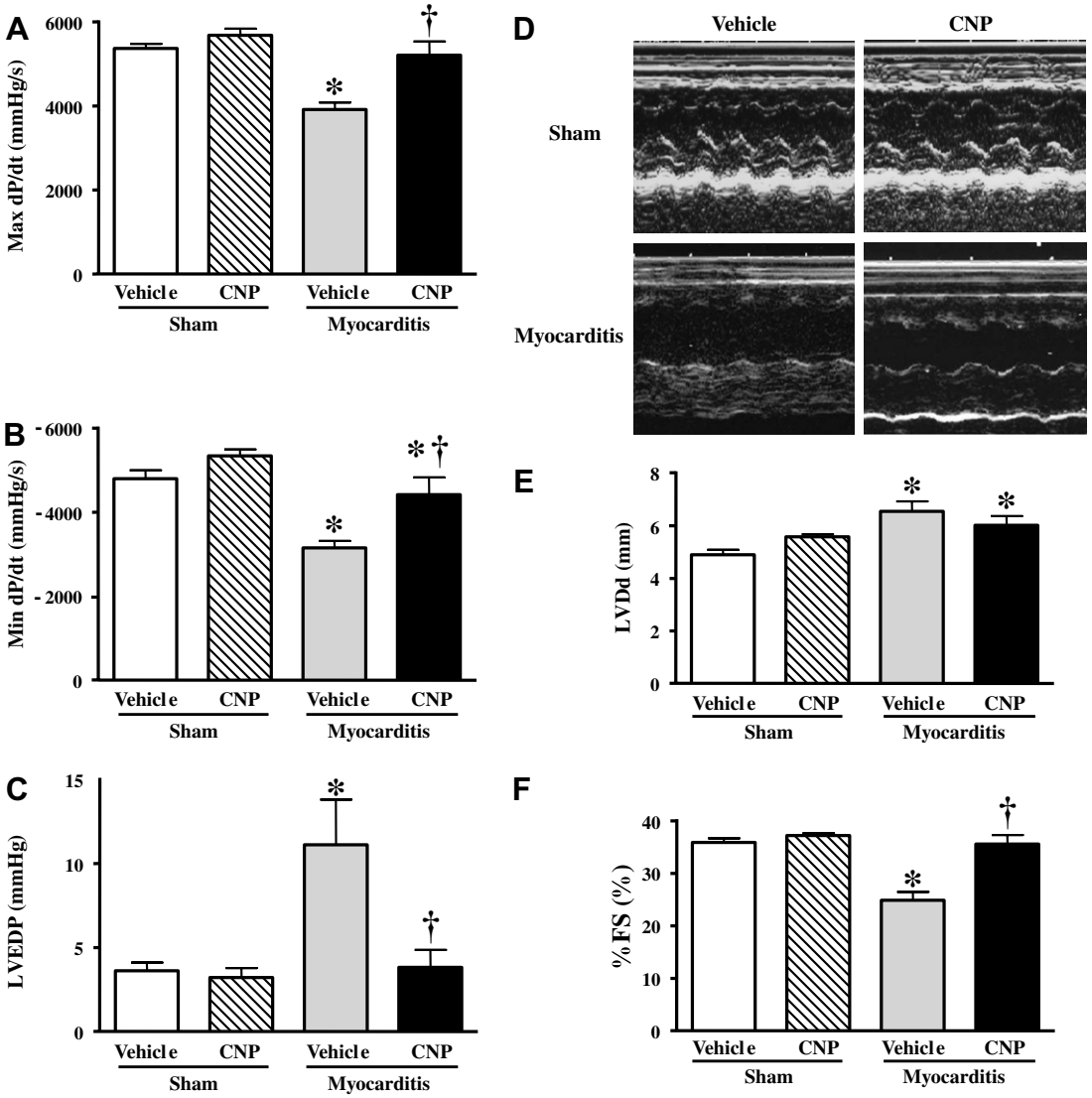

Fig. 1. Effects of CNP administration on hemodynamic parameters in acute myocarditis. (A) Maximum dP/dt (Max dP/dt), (B) minimum dP/dt (Min dP/dt), and (C) left ventricular end-diastolic pressure (LVEDP) were measured in sham rats given vehicle, sham rats given CNP, myocarditis rats given vehicle, and myocarditis rats given CNP. (D) Representative echocardiographic images showing wall thickening and poor myocardial movement in rats with myocarditis and improved cardiac contractility in those treated with CNP. (E,F) CNP administration in myocarditis tended to attenuate the increase in left ventricular diastolic dimension (LVDd) and significantly improved fractional shortening (%FS). Values are means ± SEM. $^{*}P < 0.05$ *vs.* Sham-Vehicle, $^{\dagger}P < 0.05$ *vs.* Myocarditis-Vehicle.

Table 1
Physiological and catheter-based parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 12$)	CNP ($n = 13$)
BW (g)	282 ± 2	282 ± 3	208 ± 4*	224 ± 3*,†
HW/BW (g/kg)	2.86 ± 0.04	2.81 ± 0.03	6.33 ± 0.25*	5.29 ± 0.20*,†
HR (bpm)	428 ± 7	422 ± 5	367 ± 13*	431 ± 13*,†
MAP (mmHg)	111 ± 4	103 ± 4	87 ± 3*	105 ± 5†
LVSP (mm Hg)	124 ± 5	125 ± 4	104 ± 4*	123 ± 6†

BW, body weight; HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

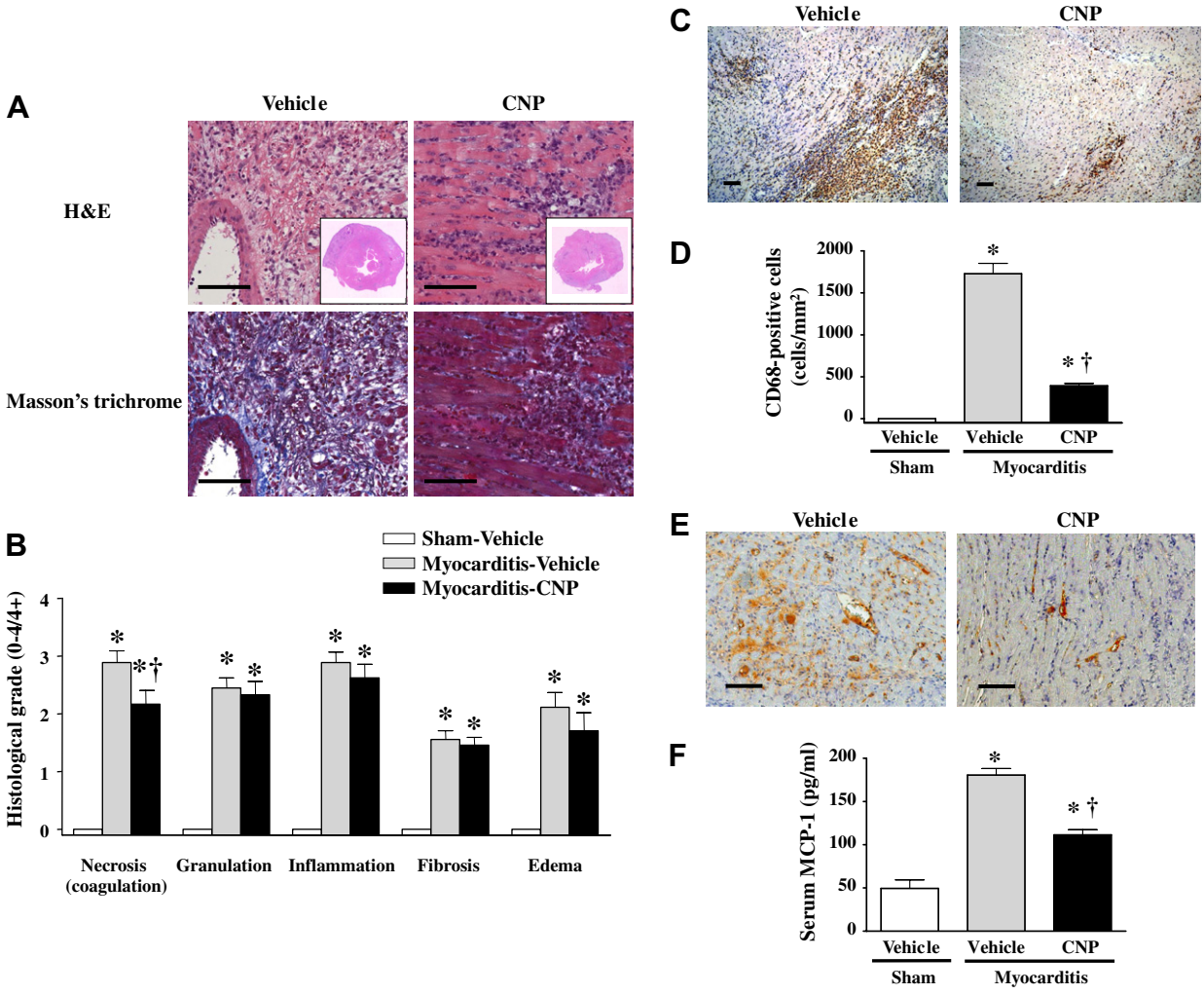

Fig. 2. Histological analysis of the myocardium. (A) Representative myocardial sections showed markedly decreased inflammation and tissue necrosis (H&E) and a comparable degree of early fibrosis (Masson's trichrome) in CNP-treated hearts as compared to myocarditic hearts. Insets are transverse section of the left ventricular section (H&E). (B) Semiquantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were lower in myocarditis rats treated with CNP as compared to untreated rats. Sham rats exhibited no measurable pathological change. Scale bar is 100 μm. (C) Representative myocardial sections immunohistochemically-stained for CD68 demonstrated a marked decrease in CD68-positive cells, including giant cells, in CNP-treated hearts as compared to vehicle-treated hearts. Scale bar is 100 μm. (D) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in CNP-treated hearts. (E) Representative MCP-1-stained myocardial sections from rats with acute myocarditis. Scale bar is 100 μm. (F) Serum level of MCP-1 measured by ELISA. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

ity [9], myocardial fibrosis was not significantly attenuated by CNP infusion (Fig. 2B), probably due to the acute nature of this experiment (Table 2).

Notably, marked histiocytic infiltration was demonstrated by the presence of CD68-positive cells, including multinucleated giant cells, in rats with myocarditis, and this

Table 2
Echocardiographic parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 9$)	CNP ($n = 11$)
LVDd (mm)	5.6 ± 0.1	5.6 ± 0.1	6.5 ± 0.4*	6.0 ± 0.3
LVDs (mm)	3.6 ± 0.1	3.5 ± 0.1	4.9 ± 0.4*	3.9 ± 0.2†
%FS (%)	36 ± 1	37 ± 1	25 ± 2*	36 ± 2†
AWT diastole (mm)	1.9 ± 0.1	1.9 ± 0.1	3.1 ± 0.2*	2.8 ± 0.2*
PWT diastole (mm)	1.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3*	3.6 ± 0.4*

LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

was significantly attenuated by CNP treatment (Fig. 2C and D). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in myocytes surrounding and adjacent to areas of inflammatory infiltration (Fig. 2E). The hearts in myocarditis rats treated with CNP showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in myocarditis rats, whereas it was significantly decreased in those treated with CNP (Fig. 2F).

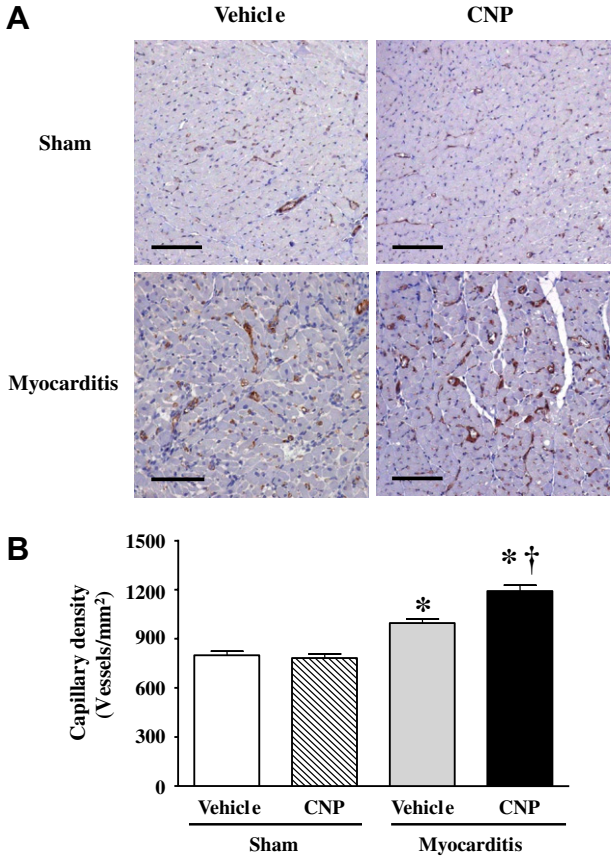

Fig. 3. Angiogenic potential of CNP in acute myocarditis. (A) Representative myocardial sections immunohistochemically-stained for vWF exhibit increased microvasculature in control myocarditic hearts, which was more marked in CNP-treated hearts. (B) Capillary density measured in 10 random representative high powered fields showed a significant increase in rats with acute myocarditis and a further increase in those treated with CNP. Scale bar is 100 μm. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

Effect of CNP on angiogenesis

To determine the angiogenic effect of CNP treatment in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density in the heart was increased in myocarditis, particularly in areas directly adjacent to tissue necrosis (Fig. 3). Notably, capillary density was increased over that in acute myocarditis alone. The clustering of relatively small vessels seen in CNP-treated myocarditic hearts was indicative of recent endothelial regeneration or angiogenesis. On the other hand, CNP did not significantly influence the capillary density in the sham rats.

Expression of GC-B and cGMP in myocardium

RT-PCR demonstrated that GC-B mRNA was expressed in myocarditic heart (Fig. 4A). Myocardial level

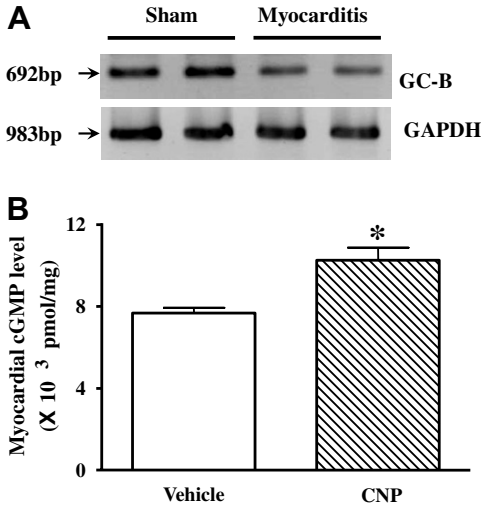

Fig. 4. Expression of GC-B and cGMP in the myocardium. (A) RT-PCR analysis of GC-B mRNA expression in myocarditic heart. (B) Myocardial level of cGMP measured by radioimmunoassay. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle.

of cGMP was significantly elevated by the subcutaneous infusion of CNP (Fig. 4B). These results suggest that subcutaneous infusion of CNP (0.05 μg/kg/min) has biological effects on myocaditic heart.

Discussion

In this study, we focused on the therapeutic potential of CNP in the acute phase of autoimmune myocarditis. We showed that CNP treatment 1 week following myosin injection but prior to the development of myocarditis (1) preserved cardiac function after acute myocarditis, (2) significantly decreased tissue necrosis, inflammatory cell infiltration and MCP-1 expression in the heart and serum, and led to a tendency for reduced overall inflammation, granulation and edema, and (3) stimulated angiogenesis in myocarditic hearts beyond the baseline increase seen in myocarditis.

The rat model of myosin-induced experimental autoimmune myocarditis closely resembles human giant cell myocarditis [11]. This disease model is triphasic, consisting of antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our experiments, CNP was administered 1 week following myosin injection, corresponding to an early time point in the disease process. In the present study, CNP treatment significantly improved cardiac function as determined by increased maximum dP/dt and %FS as well as decreased LVEDP in rats with acute myocarditis. Importantly, earlier studies have shown that the vasodilator effect of CNP is much less potent than that of ANP [5,9,14,15]. ANP and BNP cause vasodilatation and hypotension, thus limiting their use as treatment for patients with severe heart failure. Because the effects of CNP on blood pressure and HR were very small, CNP treatment is considered as a safer alternative for the treatment of those patients [16]. Indeed, administration of CNP did not decrease arterial pressure, but sustained its biological activity.

Our data showed a significant decrease in inflammatory cell infiltration and a consistent tendency for decreased overall inflammation and edema by CNP treatment. In addition, CNP infusion decreased MCP-1 expression in the heart and serum. A previous study has demonstrated that CNP reduces macrophage infiltration by inhibition of MCP-1 expression [17]. These findings suggest that attenuation of inflammatory cell infiltration by CNP may be regulated, at least in part, by suppression of MCP-1 expression.

Recently, it was shown that CNP has anti-fibrotic properties in pulmonary fibrosis and myocardial infarction, through a cGMP-dependent pathway [9,18]. However, since the present experiments were carried out in the acute phase of myocarditis, the anti-fibrotic effect of CNP in the myocarditic heart was not clear. Further studies are necessary to examine the anti-fibrotic effects of CNP in the chronic phase of myocarditis.

We demonstrated that CNP induces endothelial regeneration beyond the increase seen in myocarditis. In rabbit balloon injury, infectious vein graft disease and hindlimb ischemia models, CNP overexpression stimulated reendothelialization via a cGMP-dependent pathway [19]. Endothelial dysfunction including microvascular constriction and microaneurysm formation has previously been reported in myocarditis [20], as well as chronic impairment of endothelial-dependent vasorelaxation of coronary resistance vessels in myocarditis [21]. Thus, the endothelial regenerative effects of CNP are likely to be beneficial in preventing myocardial injury and dysfunction in acute myocarditis. In this study, capillary density in normal heart was not increased by CNP infusion. In inflammatory tissue, it is speculated that CNP does not have an effect on initiation of angiogenesis, but promote angiogenesis at the phase of forming mature blood vessels. However, a further examination is necessary to elucidate the mechanisms of angiogenic effects.

Considering the importance of natriuretic peptides, such as ANP and BNP, in the diagnosis and treatment of cardiovascular diseases, there is currently much interest in the role of CNP. Since, CNP has marked cardioprotective effects including anti-inflammatory and angiogenic effects, and has less vasodilator effects, which enable the use of this peptide in patients with hypotension, this molecule may have great potential for the treatment of patients with acute myocarditis.

In summary, administration of CNP ameliorated cardiac dysfunction in a rat model of acute myocarditis. The beneficial effects may be due, at least in part, to anti-inflammatory and angiogenic effects. This work expands the beneficial effects of CNP to acute myocarditis, and increases our understanding of the role of natriuretic peptides in severe heart failure.

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