Endothelin-Converting Enzyme Inhibition in the Rat Model of Acute Heart Failure: Heart Function and Neurohormonal Activation

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Endothelin-1 (ET-1) has been implicated in many cardiovascular diseases, including acute heart failure (AHF) due to myocardial ischemia. Previously we described the oral endothelin-converting enzyme (ECE) inhibitor, PP36, and in this study, we investigated its cardioprotective effect in more detail, and examined the role of PP36 in the neurohormonal activation in rats that had been subjected to acute myocardial ischemia due to the microsphere embolization of coronary microcirculation. PP36 treatment (3.5×10^{-5} M/kg/day) led to a significant four-fold decrease in hypertensive response when big-ET-1 was administered to healthy, conscious rats. ECE inhibition did not affect mortality during the first 48 hours after ischemia initiation. Systemic hemodynamic, heart function, and neurohormonal activation were analyzed in the healthy control group, the AHF group, and the AHF+PP36 group two days after AHF induction. In conscious rats in the AHF+PP36 group, mean arterial pressure (MAP) was restored and became similar to that of the MAP of the control group. In anesthetized rats, in the AHF+PP36 group, MAP was not restored and was 22% lower than the MAP of the control group. Myocardial contractility was partially restored and cardiac relaxation significantly improved after PP36 application. Further analysis of cardiac output and peripheral resistance in anesthetized rats revealed no differences between the AHF group and the AHF+PP36 group. There were no differences in plasma ET-1 concentration, serum angiotensin converting enzyme activity, and in the adrenal glands' catecholamine content between the AHF group and the AHF+PP36 group. However, rats in the AHF+PP36 group demonstrated a 60% decrease in cardiac endothelial nitric oxide synthase (eNOS) protein expression, and a 56% reduction of myocardial norepinephrine release, when compared with the AHF group's animals. These results suggest that PP36 can preserve heart function during the recovery from acute ischemic injury, and may modulate the cardiac norepinephrine release and eNOS protein level. Exp Biol Med 234:1201–1211, 2009

Key words: endothelin converting enzyme inhibitor; PP36; coronary microembolization; myocardial eNOS; acute heart failure; myocardial norepinephrine release

Introduction

Acute heart failure (AHF) syndrome causes almost one million hospitalizations annually in the United States with similar numbers occurring in the European population aged 65 and older. This places enormous social and financial pressures on society. Several therapies commonly used for the treatment of AHF syndrome present some well-known limitations and have been associated with an increased risk of death at an earlier age (1). New therapeutic targets for AHF alleviation may include blood pressure control, preservation of renal function, neurohormonal modulation, and myocardial protection. There is, therefore, an unmet need for new pharmacologic agents for the early management of AHF that may improve both short- and long-term outcomes (1). Cardiac dysfunction induced by myocardial injury starts the activation of complex neuroendocrine mechanisms that work to compensate for the injury. The endothelin, nitric oxide (NO), catecholamine, and renin-angiotensin systems are involved and exhibit complex interactions between each other *in vivo*. That is why modulating a single component of neurohormonal activity

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may be an important therapeutic goal. It could potentially affect different systems in response.

Endothelin-1 (ET-1) is derived from the conversion of the inactive precursor "big-ET-1" by an enzyme called endothelin-converting enzyme (ECE). The most evident effect of ET-1 is potent arterial and venous vasoconstriction (2). In plasma, ET-1 levels increase in heart failure, pulmonary hypertension, systemic hypertension, and all forms of acute coronary syndromes (3, 4). Endothelin could activate deleterious long-term vascular remodeling via the promotion of growth and proliferation of smooth muscle and myocardial hypertrophy (3, 4). ET-1 signals via two main types of receptors, ETA and ETB. The ETA receptor can be considered the primary vasoconstrictor and growth-promoting receptor, while the ETB receptor inhibits cell growth and vasoconstriction in the vascular system and also functions as a "clearance receptor" (5, 6). About 80% of ET-1 circulating in the main blood stream is eliminated after going through the lung vessels (5, 6). A number of experimental studies have demonstrated the beneficial effects of ETA and mixed ETA-ETB receptor antagonists on survival and left ventricular remodeling in animals after myocardial infarction (7, 8). Although short-term clinical studies look promising, long-term use of ET-1 receptor antagonists in human heart failure is negative (7, 9). Comparing acute effects of the ETA receptor blocker with the ECE inhibitor on a model of rapid right ventricular pacing in dogs demonstrated that the ETA receptor antagonist appeared to induce greater vasodilative effects on systemic and renal vasculature in heart failure than the ECE inhibitor (10). However, the ECE inhibitor reduced the secretion of neurohumoral factors activated in proportion to the severity of the heart failure (10). Another advantage of an ECE inhibitor compared to a mixed ET receptor blocker is that it appears to preserve the ETB receptor mediated ET-1 clearance. Since ECE is a major, but not unique way to produce active ET-1 (11, 12), even with blocked ECE activity, ET-1 clearance might be important for some pathologies with high tissue and/or plasma ET-1 content. Thus, these data may provide the rationale for long-term ECE inhibitor treatment in heart failure.

Levels of big-ET-1 in plasma have been demonstrated to predict a one-year mortality and have been shown to be a better predictor of a one-year outcome than plasma atrial natriuretic peptide and norepinephrine, NYHA class, age, and echocardiographic left ventricular parameters (4). This highlights the key role ECE inhibition plays as an alternative approach to ET-1 in managing AHF. To date, ECE inhibition was beneficial in experimental models of neuronal disorders, hypertension, diabetes, and chronic heart failure where i) only ECE was blocked; ii) ECE and neutral endopeptidase were blocked; or iii) ECE, neutral endopeptidase, and angiotensin converting enzyme were blocked (13–17). However, little is known about the significance of ECE inhibition during AHF. Additionally, new ECE inhibitors have continued to emerge during the last decade including peptide-, indole-, and sulfonylureid-pyrazole-based compounds (18, 19). These inhibitors provide valuable new tools for elucidating the pathophysiological role of ECE.

A recent finding suggests that ET-1 can promote post-ischemic norepinephrine overflow, contributing, at least in part, to subsequent cardiac dysfunction. Indeed, a selective endothelin ETA receptor antagonist or the combination of endothelin ETA and endothelin ETB receptor antagonists suppresses excessive norepinephrine release from sympathetic nerve endings in the hearts of post-ischemic rats and improves cardiac dysfunction after ischemia/reperfusion (20). Moreover, a recent study demonstrated that both endothelin ETA and endothelin ETB receptors exist in the sympathetic nerve varicosities of guinea pig hearts, and both modulate norepinephrine release in association with reperfusion arrhythmias (21) In the isolated heart model, using ECE inhibitors, it was demonstrated that endogenous ET-1 production is enhanced in ischemia/reperfusion conditions (22). This increased ET-1 is responsible for the post-ischemic cardiac dysfunction following norepinephrine overflow (22). However, little is known about the ability of ECE inhibitors to modulate post-ischemic norepinephrine overflow in *in vivo* settings.

The production of nitric oxide by nitric oxide synthase (NOS) plays important roles in physiological and pathological events in the cardiovascular system. It has been established that endothelial NOS (eNOS) can produce NO, resulting in vasodilatation and cardiac muscle relaxation. Currently, it is believed that eNOS activity is important to support nitric oxide amounts in the range of physiological concentrations. Heart function can be adversely affected if eNOS activity changes, either producing too little or too much NO during heart failure development. Indeed, decreased eNOS activity and/or expression were linked to the depression of cardiac contractility in the models of heart failure due to myocardial infarction or ischemia-reperfusion (24). Conversely, some studies did not find any beneficial role of increased eNOS expression in heart function after ischemia-reperfusion (25). The role of heart eNOS is even more complicated when heart failure is accompanied by diabetes, hypertension, or vascular disorder (26, 27), suggesting cardiomyocyte and vascular eNOS interaction. Additionally, heart failure can lead to abnormal vascular regulation as early as three hours following myocardial infarction (28, 29). Several studies have shown that cardiovascular risk factors are associated with an increase in eNOS expression rather than a decrease (26). The increased expression of eNOS in vascular disease is likely to be a consequence of an excess production of H_2O_2 (30). H_2O_2, the dismutation product of O_2, can increase eNOS expression through transcriptional and posttranscriptional mechanisms. This leads to the excessive production of nitric oxide. Elevated concentrations of NO can contribute to myocardial ischemia-reperfusion injury by generating highly reactive NO derivatives. It has been mentioned that

ET-1 may possibly regulate nitric oxide cascade under physiological conditions (31), and in ischemic injury (32). Yet the relationship between ET-1 and the cardiac eNOS expression level during myocardial injury is controversial, probably because of the multiple cytokine contributions to expression regulation during heart ischemia and/or the different experimental models used.

The renin-angiotensin system (RAS) appears to play a key role in the pathophysiology of heart failure, contributing to cardiac hypertrophy and remodeling. The existence of multilevel interactions between the RAS and endothelin (33) suggests their complex involvement in acute heart failure. A growing body of evidence indicates that angiotensin-II and ET-1 may potentially induce an adaptive cardiac response during diastolic dysfunction development (34). Angiotensin-converting enzyme (ACE) inhibitors exert beneficial effects limiting disease progression in all stages of heart failure (1). Using the orally active ETA receptor antagonist LU135252, it was demonstrated that ET-1 is involved in the regulation of tissue ACE activity *in vivo* independent of ACE mRNA expression (35). However, limited information is available regarding the potential of the inhibition of ECE towards ACE activity in *in vivo* acute heart failure settings.

A new ECE inhibitor, PP35, was developed by Professor V. Pozdnev of V.N. Orechovich Institute of Biomedical Chemistry, Moscow, Russia (36). The structure of PP35 includes the N^{α}-benzylsuccinyl group and Leu-Trp-OH dipeptide. Like the non-selective ECE inhibitor phosphoramidon, PP35 abolishes the elevation of systemic blood pressure in normotensive rats in response to a bolus injection of big-endothelin-1 (36). Hemodynamic responses to ET-1, angiotensin I, and angiotensin II remained unchanged. The oral form of this ECE inhibitor, substance PP36, (N^{a}-((RS)-1-carboxy-2-benzylaminocarbonyl-ethyl)-L-Leucyl-L-tryptophan), was derived from PP35. Our previous studies in conscious rats with AHF demonstrated that the oral ECE inhibitor, PP36, corrected ischemia-induced myocardial function measured by maximal left ventricular systolic pressure ($LVSP_{max}$) and $+dP/dt_{max}$ in conscious rats (37). In particular, we observed that ECE inhibition preserved i) both basal cardiac contractility (as measured by $LVSP_{max}$ and $+dP/dt_{max}$) and mean arterial pressure; ii) $LVSP_{max}$ and $+dP/dt_{max}$ responses to dobutamine stimulation; and iii) both the elevation of mean arterial pressure and the elevation of $LVSP_{max}$ in response to two pharmacological tests designed to suppress nitric oxide production. One of the tests used was L-NAME, which is a non-specific inhibitor of all NOS isoforms. Another test was performed with aminoguanidine, a specific at low doses inhibitor of inducible NOS. These data suggest that modulation of the endothelin system by ECE inhibition with PP36 can affect the nitric oxide system during AHF development.

In this study, our goal was to investigate the cardioprotective effect of PP36 on myocardial relaxation, heart pump function, and neurohumoral activation in rats subjected to an acute myocardial ischemia due to a microsphere embolization of the coronary microcirculation. Observed beneficial results may be linked to an adaptive mechanism involving the modulation of the cardiac norepinephrine release and eNOS expression.

Materials and Methods

Animals. A total of 126 male Wistar rats (400–500 g) were used in the study. Animals were housed individually under standard conditions with free access to food and water. The rats were kept in a temperature-controlled room (20–22°C) under a 12 hour light–dark cycle. All procedures involving animals in this study adhered to standards published in the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, U.S.A.).

Acute Heart Failure Model. Experimental myocardial injury was induced by coronary vessel microembolization described previously (38, 39). Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally). A polyethylene catheter (PE-10, inner diameter = 0.28 mm, outer diameter = 0.61 mm; Clay Adams, Parsippany, NJ, U.S.A.), containing saline solution with heparin (50 units/ml) was placed in the left ventricle via the right carotid artery. A suspension of approximately 100,000 to 150,000 microspheres with diameter 15 μm in saline solution containing 0.05% Tween-80 was injected into the left ventricle during seven to 10 seconds occlusion of the ascending aorta, which is the straight portion of the vessel between the heart's left ventricular and right carotid artery bifurcation. Occlusion was performed by pressing the aorta to the vertebral column as proximal to the heart as possible, using the L-shaped wire, inserted through a needle-made opening between the second and fourth ribs. The procedure was repeated at 10- to 15-minute intervals up to three times until the left ventricular maximal pressure in response to aortic occlusion decreased from the basal level by at least 25%. After injection of the microspheres, the occlusion device and left ventricular catheter were removed, and the skin incisions were closed with sutures. The sham-operated rats (control group) were subjected to the same procedures except for the injection of the microspheres. The number of microspheres accumulating in the heart following coronary embolization was 100,003 ± 4,334 microspheres per gram of the left ventricular (39). Two days after the microembolization of the coronary arteries, focal necrosis due to microvascular microspheres accumulation was observed globally, but mostly on the epicardial side of the heart. Twenty-one days after the model induction, the animals developed multiple focal fibrosis in myocardial tissue accompanied by signs of chronic heart failure (38). No significant necrosis and scar formation were observed in other tissues including the kidney, brain, skeletal muscle, and liver.

Experimental Protocols Designed to Measure Hemodynamic Variables. To estimate the efficiency of ECE inhibition by PP36 we performed a preliminary study involving a total of eight animals. In these initial experiments, two groups of four rats were instrumented under pentobarbital anesthesia with chronic arterial (to measure systemic blood pressure) and venous (to administrate vehicle or big-ET-1) catheters placed in femoral vessels. Distal parts of the catheters were externalized in the neck area to be connected to the MacLab system (Maclab, Australia) to record and analyze arterial blood pressure. Two days after surgery, we randomly selected rats to receive *per os* PP36 (3.5×10^{-5} M/kg, $n = 4$) or vehicle ($n = 4$) for the next four days. Both groups' blood pressure responses to big-ET-1 were analyzed.

According to our previous findings from the AHF model, mortality was highest during the first 48 hours after embolization (39). The most significant and rapid changes of neurohormonal status also took place during the first two days post-infarction. Additionally, systemic and regional hemodynamic changes were the most dramatic at this early stage of heart failure development. Thus, we aimed to analyze the cardioprotective potential of ECE inhibition during that critical time frame. However, our preliminary data demonstrated that four days of oral PP36 treatment led to a significant decrease of blood pressure response to intravenous administration of big-ET-1. Big-ET-1 is supposed to be cleaved by ECE, generating vasoconstrictive ET-1 evoking systemic hypertensive reaction. Thus, two days before coronary embolization, we randomly selected rats for treatment with PP36 (3.5×10^{-5} M/kg, orally, once a day; PP36 group) or with a vehicle (vehicle group). Application of PP36 continued for two days post-embolization. The experiments described below involved a total of 118 rats. Twenty-nine rats were assigned to the control group, 50 rats were assigned to the AHF group, and 39 rats were assigned to the AHF group with continuous PP36 consumption. Differences in the number of animals per group reflect adjustments for mortality after coronary vessels embolization.

Protocol 1. After two days of PP36 or vehicle consumption, embolization or a sham operation was performed. At the end of induction of myocardial ischemia, a polyethylene catheter was placed into the abdominal aorta via the femoral artery to measure arterial blood pressure. Two days after induction of myocardial ischemia, arterial blood pressure was measured in conscious rats from three groups: control ($n = 17$), ischemic ($n = 15$), and ischemic with PP36 treatment ($n = 11$). The arterial catheter was connected to a blood pressure measuring system (Maclab, Australia). Animals were allowed a minimum 45-minute adaptation period. Then the mean blood pressure was recorded for another 30 minutes. At the end of the blood pressure recording, blood samples were collected from arterial catheters for ET-1 concentration and ACE activity measurements. At the end of Protocol 1, animals were euthanized by an overdose of sodium pentobarbital (100 mg/kg).

Protocol 2. To analyze cardiac output and left ventricular pressure two days after embolization or the sham operation, an acute experiment was performed in rats anesthetized with urethane (1.5 g/kg, intraperitoneally) from control ($n = 7$), AHF ($n = 7$), and AHF with ECE inhibition ($n = 6$) groups. The animals were placed on a heating pad to maintain body temperature at 37°C. Polyethylene catheters were placed into the abdominal aorta via the femoral artery to measure arterial blood pressure and into the femoral vein for saline infusion. A fluid-filled PE-10 catheter was inserted into the right carotid artery and advanced retrogradely into the left ventricle to measure left ventricular end-diastolic pressure (LVEDP). LVEDP signals were recorded at 1,000 Hz on a beat-to-beat basis. Contractile function was studied by monitoring the LVSP signal and dP/dt, which was calculated on-line as the first derivate of LVSP. After LVEDP recording, the catheter was removed from the left ventricle. The trachea was intubated for lung ventilation. The left chest was opened between the second and third ribs, and an ultrasound flow probe (Transonic Systems, Inc., Ithaca, NY, U.S.A.) was placed around the ascending aorta. At the end of instrumentation, the chest was closed, and the animal was allowed to stabilize for 15 to 20 minutes. After hemodynamic parameters reached the steady state, recording continued for another 20 minutes, followed by euthanization by an overdose of sodium pentobarbital (100 mg/kg).

ET-1 Plasma Concentration Measurement. Plasma from the control group ($n = 7$), the ischemic group ($n = 7$), and the ischemic with PP36 treatment group ($n = 6$) were collected at the end of Protocol 1 to measure ET-1. Blood (~3 ml) was rapidly drawn from the arterial catheter directly into plastic tubes with 60 µl of 7% EDTA solution. The samples were mixed immediately, and centrifuged at 3,000 *g* for 20 minutes at 4°C. Supernatants of plasma (1.2 ml) were stored at –20°C until an analysis could be conducted using an I^{125}-based ET-1/2 detection kit (Amersham Pharmacia Biotech, U.K.) according to manufacturer's instructions.

Angiotensin Converting Enzyme Activity Measurement. ACE activity was measured in the serum of the control group ($n = 6$), the ischemic group ($n = 5$), and the ischemic with PP36 treatment group ($n = 5$) at the end of Protocol 1. Blood (1–1.5 ml) was drawn directly into tubes from the arterial catheter. The activity of ACE was measured in 200 µl of serum as the rate of enzymatic degradation the purine-histidine-leucine per one minute. The amount of emerging histidine-leucine formed a fluorescent compound when combined with orthostalic aldehyde in an alkaline solution. The fluorescence was measured by a spectrophotometer (Hitachi, Japan) at Ext 360 nm, Em 500 nm. The protein concentration in the samples was measured by the Lowry method (40). Relative

ACE activity was calculated per mg of protein in the sample.

Microdialysis of Myocardium *In Vivo* and Catecholamine Measurement. Data from the three groups of rats, the sham group ($n = 3$), the AHF vehicle group ($n = 4$), and the AHF PP36 group ($n = 5$), were used to detect catecholamine in heart and adrenal gland tissues 48 hours after coronary vessel embolization. A microdialysis probe was implanted into the left ventricular myocardium in nearly the same area of the myocardium in all groups of rats anesthetized with sodium pentobarbital (40 mg/kg, intraperitoneally) as described previously (41). Microdialysis probes were constructed with a single dialysis fiber (Cordis Dow, Brussels, Belgium; 0.25 mm outer diameter, molecular weight cutoff 5,000). Each end of the fiber was inserted into outflow and inflow silicon tubes and sealed in place with cyanoacrylic glue. The dialysis fiber length was 6 to 8 mm. The inflow silicon tube was connected to a glass syringe of a perfusion pump (CMA 100, Carnegie Medicine AB, Stockholm, Sweden). After implantation, the microdialysis probe was perfused for 60 minutes with Ringer solution (containing 147 mM NaCl, 4.0 mM KCl, and 2.3 mM $CaCl_2$) at a rate 3 μl/min. Then two 20-minute dialysate samples were collected to analyze the release of norepinephrine (NE). At the end of the experiment both adrenal glands were extracted. The amounts of epinephrine (E) and norepinephrine were analyzed as markers of catecholamine store, reflecting the severity of the stress response to acute myocardial ischemia (42). After partial purification by absorption of alumina, samples were analyzed for NE and E using high-performance liquid chromatography with electrochemical detection (43, 44).

Endothelial Nitric Oxide Synthase (eNOS) Protein Analysis in Cardiac Tissue. Data from the three groups of rats, the sham group ($n = 3$), the AHF vehicle group ($n = 3$), and the AHF PP36 group ($n = 2$), were used for eNOS protein level detection in the heart 48 hours after coronary vessels embolization. The apical part of the left ventricular (900 μg) was removed and homogenized in liquid nitrogen. Powdered tissue was further homogenized in a glass-tephlon homogenizer with a buffer (1:4 weight/volume ratio) containing 83 mM Tris-HCl, pH 6.8, 2.7% sodium dodecil sulphate, 13.3% glycerol, 0.47 M 2-mercaptoethanol, 0.033% bromphenol blue. After additional incubation for 15 minutes at 56°C, it was centrifuged for two minutes, 16 000 *g*, and supernatant was stored at –20°C until all samples were collected. Samples were equalized for protein and subjected to SDS-PAGE followed by Western blotting analysis. After blocking non-specific binding with 5% milk, primary rabbit anti-eNOS antibodies (10 μg/ml, a generous gift from Dr. Bernd Mayer, Karl-Franzens-University, Graz, Austria) were incubated with the membrane for one hour at 37°C. A secondary antibody was diluted 1:2000 and incubated as above. The enhanced chemiluminescence detection system kit was supplied by Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

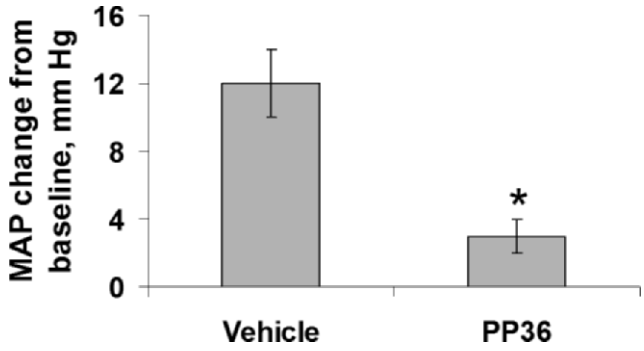

Figure 1. Mean arterial pressure (MAP) response to bolus injection of big-Endothelin-1 after four days of vehicle ($n = 4$) or PP36 ($n = 4$) treatment in conscious healthy rats. Endothelin-converting enzyme inhibition with PP36 led to a significant ($P < 0.05$, marked by *) decrease of MAP response compared to the vehicle control group.

The BCA protein assay kit was from Pierce (Rockford, IL, U.S.A.). Western blot data were subjected to a densitometry evaluation using ImageJ 1.36b software (Wayne Rasband, National Institutes of Health, U.S.A.) and then expressed as relative units and compared to the control group.

Statistics. Data are reported as means ± SEM. Statistical analyses were performed with one-way ANOVA analysis of variance followed by Student-Newman-Keuls or Dunn's methods for multiple comparisons using SigmaStat 3.5 software (Systat Software Inc., San Jose, CA, U.S.A.). Values were considered significantly different at $P < 0.05$.

Results

Four Days of PP36 Treatment Reduced the Mean Blood Pressure Response to Big-ET-1 in Conscious Rats. After four days of PP36 or vehicle administration, the baseline mean arterial blood pressure (MAP) was similar between the treated group (102 ± 4 mm Hg, $n = 4$) and the untreated control group (97 ± 5 mm Hg, $n = 4$). In the control group maximal MAP elevation in response to intravenous bolus administration of big-ET-1 (5×10^{-5} M/kg) was 12 ± 2 mm Hg compared to the baseline level (Fig. 1). Four days of PP36 treatment led to a significant ($P < 0.05$) decrease of MAP response, which was 3 ± 1 mm Hg compared to the baseline.

PP36 Had No Effect on Developed $LVSP_{max}$ During AHF Induction. Two days after beginning PP36 treatment in anesthetized animals, the sham operation or coronary vessels embolization took place and was evaluated by examining the $LVSP_{max}$ decrease from the basal level (see Methods for more details). In agreement with our previously described model (39), the sham operation resulted in a 4.2 ± 1.7% reduction of developed $LVSP_{max}$ (Fig. 2). Coronary embolization was associated with a significant (27.8 ± 1.6%) decrease from the basal level of developed $LVSP_{max}$ compared to the control group (Fig. 2). PP36 pretreatment did not affect the embolization-induced decline of developed $LVSP_{max}$ which was a 26.6 ± 2.6% reduction from the basal level (Fig. 2).

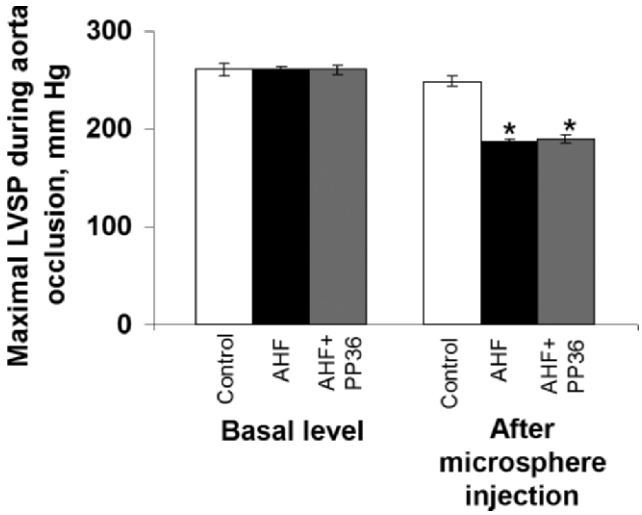

Figure 2. Quantification of global heart dysfunction due to coronary embolization during AHF induction (see Methods for details) in anesthetized rats. Sham control animals ($n = 15$) were subjected to coronary saline injection. AHF, animals received plastic microspheres embolizing the coronary vessels ($n = 27$); AHF+PP36, animals were subjected to a coronary embolization and endothelin-converting enzyme inhibition with PP36 ($n = 21$). Coronary embolization significantly ($P < 0.05$, marked by *) reduced the development of left ventricular systolic pressure (LVSP) in response to aorta occlusion by 27.8 ± 1.6% from the basal level in the AHF group. PP36 pretreatment did not affect this decline, making it 26.6 ± 2.6%.

PP36 Supported Systolic and Diastolic Cardiac Function Two Days After AHF Induction. In the study none of the control animals died. After AHF induction mortality was 46% in both embolized groups, which corresponds to 27 surviving rats out of 50 in the AHF group and 21 surviving out of 39 in the AHF with PP36 treatment. Thus, PP36 pretreatment did not change the mortality rate during AHF development.

Baseline measurements of hemodynamic variables in conscious and anesthetized animals, and left ventricular function in the control group, the acute ischemic heart failure group, and the acute ischemic heart failure treated with PP36 rats are summarized in Table 1. Without anesthesia the heart rate was similar between control rats (378 ± 13 beat/min) and both ischemic heart failure rat groups with and without PP36 treatment (411 ± 17 and 377 ± 10 beat/min, respectively). MAP was significantly reduced in the AHF group (84 ± 2 mm Hg) compared to the control group (97 ± 4 mm Hg), and the PP36-treated group (97 ± 4 mm Hg). In anesthetized animals, the heart rate and MAP were markedly reduced in both AHF groups with and without PP36 application by 22% and 34%, respectively, compared to the control rats. Left ventricular contractility as measured by $LVSP_{max}$ and $+dP/dt_{max}$ was significantly decreased in the AHF groups (by 28% and by 56%, correspondently) and indicated partial restoration (no statistically detectable difference compared with the control group) after PP36 treatment. Both cardiac end-diastolic and lusitropic functions were impaired in rats with ischemic heart failure without PP36 application. $-dP/dt_{max}$ was significantly decreased (by 65% compared to the control group and by 58% compared to the PP36 treated group) and LVEDP and the relaxation constant τ were significantly higher (both by about 2.3-fold compared to the control and PP36 treated groups) in rats with acute ischemic heart failure than in the control rats and animals after PP36 treatment. Cardiac output, stroke volume, and total peripheral resistance were similar in both acute ischemic heart failure groups. Statistical analysis did not reveal any significant difference between AHF groups and the control

Table 1. Basal Cardiovascular Parameters in Rats Two Days After Heart Ischemia Induction or Sham Operation[a]

	Control	AHF	AHF+PP36
Conscious	$n = 17$	$n = 15$	$n = 11$
MAP, mm Hg	97 ± 4	84 ± 2*,#	97 ± 4
HR, beat/min	378 ± 13	377 ± 10	411 ± 17
Anesthetized	$n = 6$	$n = 5$	$n = 5$
MAP, mm Hg	99 ± 6	65 ± 7*	77 ± 4*
HR, beat/min	418 ± 12	346 ± 7*	356 ± 19*
LVSPmax, mm Hg	142 ± 11	102 ± 8*	127 ± 2
+dP/dtmax, mm Hg/msec	10.4 ± 1.9	4.6 ± 1.2*	7.6 ± 1.1
-dP/dtmax, mm Hg/msec	5.2 ± 0.9	1.8 ± 0.2*,#	4.3 ± 0.6
LVEDP, mm Hg	14.9 ± 2.5	34.9 ± 6.6*,#	16.3 ± 3.2
τ	4.6 ± 1.0	10.8 ± 1.2*,#	4.7 ± 1.0
CO, ml/min	69 ± 6	43 ± 6	47 ± 12
SV, μl	166 ± 16	122 ± 17	129 ± 26
TPR, mm Hg/ml/min	5.05 ± 0.65	5.69 ± 0.74	6.55 ± 1.11

[a] MAP, mean arterial pressure; HR, heart rate; LVSPmax, maximal left ventricular systolic pressure; +dP/dt, velocity of pressure increase in heart left ventricular during systolic contraction; -dP/dt, velocity of pressure decrease in heart left ventricular during diastolic relaxation; LVEDP, left ventricular end diastolic pressure; τ, constant of left ventricular active relaxation; CO, cardiac output; SV, stroke volume; TPR, total peripheral resistance; Control, sham operated rat group; AHF, coronary vessels embolized group; AHF+PP36, coronary vessels embolized group treated with PP36; *n*, number rats per group; mm Hg, millimeters mercury; min, minute; msec, millisecond.
* $P < 0.05$ compared to Control; # $P < 0.05$ compared to AHF+PP36.

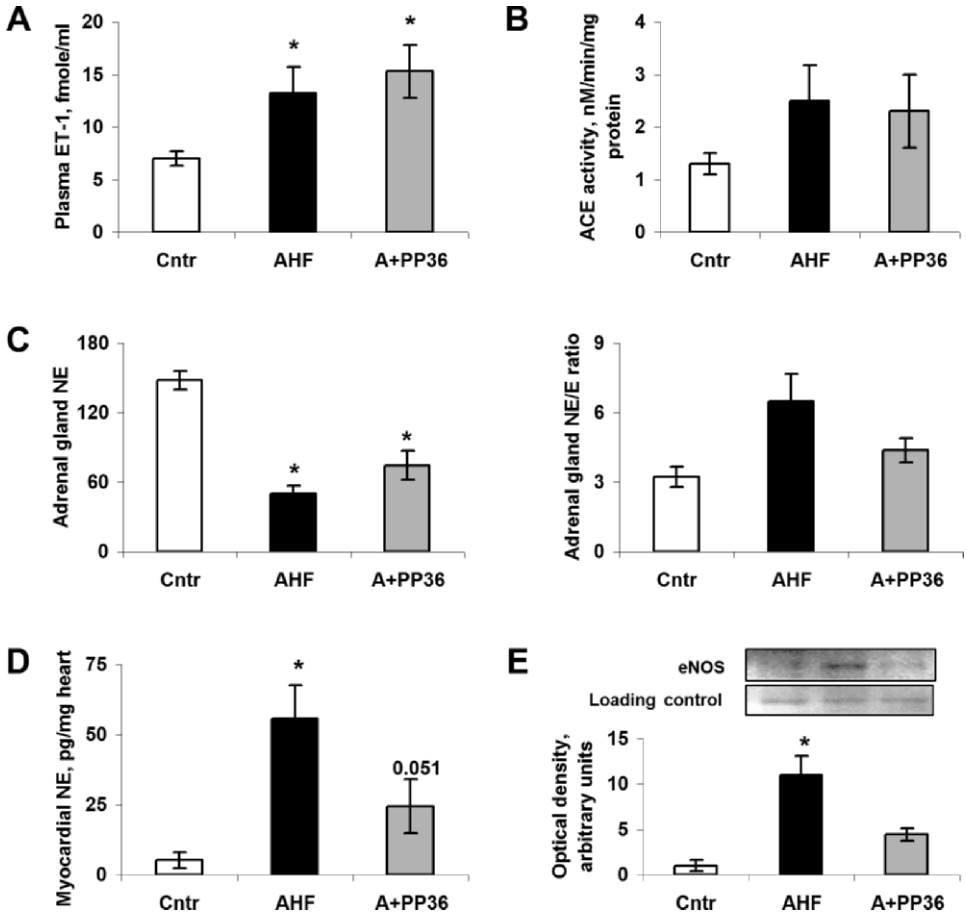

Figure 3. A. Plasma Endothelin-1 concentration significantly ($P < 0.05$) increased after AHF induction compared to the sham control group ($n = 7$). No differences were detected between the PP36 treated ($n = 6$) group and the untreated ($n = 7$) AHF group. Cntr, sham control animals were subjected to a coronary saline injection; AHF, animals received plastic microspheres embolizing the coronary vessels; A+PP36, animals were subjected to a coronary embolization and endothelin-converting enzyme inhibition with PP36. B. Angiotensin converting enzyme (ACE) activity in the serum of the control group ($n = 6$), the ischemic group ($n = 5$), and the ischemic with PP36 treatment group ($n = 5$) two days after model induction. No significant differences between experimental groups were detected. C. Adrenal gland contents of norepinephrin (NE) and epinephrine (E) two days after acute heart failure induction or the sham operation. The NE amount was significantly reduced in both AHF groups with ($n = 4$) and without PP36 ($n = 5$) treatment compared to the sham control group ($n = 3$). No difference between ischemic groups was observed. NE to E ratio was similar in all three groups. D. Myocardial norepinephrine (NE) release as detected by microdialysis *in vivo* (see Methods for details) was significantly increased in the acute heart failure ($n = 7$) group compared to the sham control group ($n = 4$). PP36 treated animals ($n = 4$) demonstrated a 56% reduction compared to the AHF group's myocardial NE release. The difference with the control group became marginal ($P = 0.051$). E. Myocardial endothelial nitric oxide synthase (eNOS) protein expression in rats two days after acute heart failure induction or a sham procedure. The myocardial tissues were homogenized and resolved by SDS-PAGE followed by a Western blotting technique described in Methods. Representative results of three samples of the control group, three samples of the AHF group, and two samples of the AHF+PP36 group are demonstrated on the upper part of the figure. The graph represents the densitometry analysis of all samples used for the eNOS expression study. After AHF induction eNOS expression significantly increased in myocardial tissue ($P < 0.05$, marked as * on the graph) compared to the control group. PP36 treatment reduced eNOS overexpression by about 60%, and the difference with the control group was statistically insignificant.

rats regarding heart pump function characteristics and vascular resistance.

PP36 Partially Attenuated Myocardial NE Release and Cardiac eNOS Overexpression After Acute Myocardial Ischemia. Two days after myocardial ischemia induction, plasma ET-1 concentration, the serum activity of ACE, cardiac tissue catecholamine release, adrenal gland norepinephrine and epinephrine pools, and the level of eNOS protein in the heart muscle were measured. Plasma ET-1 concentration significantly increased in both groups with ischemic heart injury compared to the control group (Fig. 3A). There was no significant difference observed in plasma ET-1 concentration between AHF and AHF with PP36 application. The serum ACE activity was similar between the three experimental groups (Fig. 3B). The adrenal gland NE pool significantly decreased in both AHF groups compared to the control group. PP36 did not have any major effect on that decline. NE to E ratio was similar in all three groups (Fig. 3C). Myocardial NE release (Fig. 3D) significantly increased in the AHF group compared to the control rats, and demonstrated only a tendency ($P = 0.051$) to increase in the PP36 treated group. Myocardial eNOS protein expression was increased after heart embolization (Fig. 3E). PP36 application partially

reduced eNOS overexpression in the AHF+PP36 group, compared to the AHF group (Fig. 3E).

Discussion

In this study, we used a rat model to study the relationship between the inhibition of endogenous ECE activity with the PP36 compound, and the development of acute ischemic heart failure. Our study indicated that administration of PP36 can: i) improve systemic blood pressure; ii) preserve contractile and lusitropic cardiac function; and iii) be associated with partial attenuation of the enhanced cardiac NE release and eNOS overexpression two days after induction of acute myocardial ischemia. These responses to ECE inhibition were observed even though plasma ET-1 was not reduced.

Our AHF model is characterized by extensive myocardial hypoxia due to embolization of the coronary arteries. The experimental injury was most likely associated with an increased ET-1 level in cardiac muscle and secondary in plasma as was detected in our study. Despite a similar concentration of ET-1 in the blood of rats with and without four days of PP36 treatment, we detected functional differences in response to big-ET injections between treated and untreated groups. Additionally, two weeks of similar ECE inhibition led to significant decreases of ET-1 concentration in plasma (45), suggesting that changes in tissue peptide levels appear earlier than in blood. Increased plasma levels of ET-1 have been noted in association with acute myocardial infarction, but conflicting results regarding myocardial effects were reported, which possibly reflect a complex mixture of cardiac and coronary effects of ET-1 (46–48). Recently, using load-independent analysis, it was demonstrated that ET-1 elicits positive inotropic and negative lusitropic myocardial effects in a pig model, possibly resulting from ETA and ETB receptor activation, respectively (49). PP36 may counteract the heart-specific activities of endothelin-1, which include inhibition of myocardial relaxation and coronary vasoconstriction, which contribute to decreased cardiac contractility in ischemic heart failure.

Despite potential therapeutic interest, little is known about ECE inhibition in acute heart failure. In mice models of acute myocardial infarction, the inhibition of ECE was shown to increase the survival rate up to 80% compared to 20% in an untreated group (18). In acute myocardial infarction of rabbits subjected to coronary occlusion and reperfusion, ECE inhibition reduced the infarct size, reduced the elevated serum concentration of ET-1, and the serum activity of creatinine phosphokinase (19). However, the role of ECE inhibition in cardiac function, system hemodynamic variables, or neurohormonal activation was not extensively studied. Previously, we demonstrated that ECE inhibitor PP36 can preserve basal cardiac contractility and left ventricle response to β-adrenergic agonist (as measured by $LVSP_{max}$ and $+dP/dt_{max}$) in conscious rats with experimental AHF (37). In the current study under anesthesia conditions, PP36 not only preserved cardiac contractility, but also restored relaxation and diastolic myocardial properties as evaluated by LVEDP, $-dP/dt_{max}$, and the relaxation constant. Although, no significant changes in heart pump function or total peripheral resistance were observed in the three experimental groups, taking into account the altered heart rate and blood pressure, plus the reduced contractile indices, it seems that heart pump function was depressed in the AHF animals. There was no marked effect of PP36 application observed on cardiac output, stroke volume, or total peripheral resistance.

As we reported previously (37), systemic blood pressure was restored after ECE inhibition in conscious rats. However, this effect disappeared in anesthetized animals. These results suggest that endogenous ET-1 is important for the regulation of neuronal mechanisms supporting systemic blood pressure. Our finding of a partially restored myocardial NE discharge after PP36 application *in vivo* is in agreement with a recently published observation that ECE inhibition suppressed excessive norepinephrine overflow in the coronary effluent from the post-ischemic isolated hearts perfused according to the Langendorff technique (22). However, despite known ET-1 effects on catecholamine release from sympathetic terminals (21, 50), we did not detect any difference between PP36-treated and untreated AHF groups regarding adrenal gland contents of NE and E. Similarly, a mixed endothelin-A and -B receptor antagonist, PD-142893, did not significantly change the stress-induced increase in adrenal catecholamine secretion (51). Both of these findings suggest a minimal influence of endogenous ET-1 on the total catecholamine store.

Additionally, a hypoxic condition induces transcription factors such as hypoxia-inducible factors (HIF-1 and HIF-2), which were shown to be involved in increasing the expression of eNOS and inducible NOS (iNOS) within minutes of reduced oxygen availability (52). In hypoxic conditions, impaired eNOS enzymatic function in concert with iNOS overproduction of nitric oxide (53), as well as an increased level of ET-1, can contribute to an enhanced level of oxidative stress and compromised heart contractility and relaxation (54). In agreement with our observation of increased eNOS expression after microembolization of coronary vessels, eNOS significantly increased in diabetic cardiomyopathy and was greatly inhibited by the treatment with the ET antagonist (55). Interestingly, eNOS up-regulation was observed in endothelial cells of brain microvessels two to 48 hours after ischemia due to 48 μm diameter microsphere embolism (56). Considered together those reports indicate the association of eNOS over-expression with structural and/or functional vascular disorder. Thus, in the case of heart eNOS analysis, there is a balance between the ET-1 and NO complex interplay in coronary vessels and cardiomyocites. On one hand, endothelin can stimulate vascular endothelial nitric oxide

release, counteracting unnecessary vasoconstriction in coronary arteries and veins. On the other hand, NO overproduction in cardiac cells causes the depression of heart contractility due to damaging NO effects during organ ischemia. The major mechanism causing cell injury due to excessive NO likely involved the reaction of NO with $O_2^{\bullet-}$ to generate cytotoxic peroxynitrite $ONOO^-$ followed by a reactive nitrogen species (52). These highly reactive agents can lead to the oxidization of proteins, membrane lipid, and DNA, or the nitration of proteins, resulting in their loss of function, disruption of myocardial energy regulation, and the initiation of cell death signaling (52). In support of the suggested mechanism, two days after brain microvessels embolization, eNOS overexpression was closely associated with protein tyrosine nitration, a marker of the generation of cytotoxic peroxynitrite (56). The inhibition of ECE might abolish the negative consequences of excessive NO production in ischemic myocardium via the modulation of the endothelin-dependent eNOS expression.

We believe that our data support the hypothesis that in the model of myocardial ischemic injury by coronary embolization with microspheres, endothelin-converting enzyme inhibition correlated with a reduction in the increase of eNOS overexpression in response to ischemic injury. Indeed, cardiac ischemic injury led to an 11-fold increase in the eNOS protein level compared to the sham operated control group. After PP36 treatment, the total level of eNOS expression was 4.4-fold higher compared to the sham operated control group. At the same time, after endothelin-converting enzyme inhibition, myocardial eNOS expression diminished by 60% compared to the untreated ischemic group. In addition, after acute heart ischemia induction, we observed i) decreased systemic blood pressure and $LVSP_{max}$ in conscious and anesthetized rats; ii) enhanced systemic blood pressure and $LVSP_{max}$ responses to a non-selective blockade of nitric oxide production with L-NAME and to selective inhibition of inducible NOS isoform with aminoguanidine (37); and iii) reduced basal myocardial contractility and relaxation, as well as contractility response to beta-adrenergic stimulation. PP36 treatment ameliorated these effects during the development of myocardial ischemia. The effects of NOS inhibition-dependent acute hypotension and impaired cardiac contractility can be explained by NO overproduction. The NO overproduction is the result of increased iNOS in macrophages, and/or vascular smooth muscle cells, or excessive activation of eNOS in coronary, and peripheral vascular endothelium, as reviewed by Manukhina *et al*. (52). Acute myocardial infarction-induced NO-overproduction was accompanied by excessive endothelium-dependent relaxation of isolated blood vessels, which was inversely correlated with blood pressure. This suggests that eNOS plays an important role in NO-induced acute hypotension (52). Furthermore, as was recently published by Ryou *et al*. (57), a decrease in myocardial eNOS content by 30% was associated with a 60% reduction of enzyme activity and suppressed NO formation upon reperfusion of coronary arteries. Thus, it is plausible that reducing the increase of eNOS expression after ECE inhibition, as observed in our study, may be associated with a decrease of excessive NO generation during the development of myocardial ischemia in an employed model of coronary microembolization. However, since we did not directly measure eNOS-mediated NO production we cannot exclude the possibility that even with suppressed eNOS synthesis and abolished cardiac injury-derived hypotension the improved heart function will have no connection with decreased eNOS overexpression. Alternative explanations may involve changes in iNOS activity/expression or NO-independent mechanisms.

In conclusion, our results demonstrate that PP36 improves the recovery from acute heart ischemia, preserving myocardial contractility and relaxation in an *in vivo* animal model. This cardioprotection correlated to the partially attenuated myocardial NE overflow and reduced eNOS overexpression in ischemic myocardial tissue after PP36 treatment. The detailed mechanisms by which PP36 may be involved in eNOS expression regulation in acute heart ischemia remain to be studied. Endothelin-converting enzyme inhibitors such as PP36 may hold promising therapeutic potential for the treatment of acute heart insufficiency.

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