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An injectable microparticle formulation provides long-term inhibition of hypothalamic ERK1/2 activity and sympathetic excitation in rats with heart failure

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Abstract

Sympathetic excitation contributes to clinical deterioration in systolic heart failure (HF). Significant inhibition of hypothalamic paraventricular nucleus (PVN) ERK1/2 signaling and a subsequent reduction of plasma norepinephrine (NE) levels in HF rats were achieved two weeks after a single subcutaneous injection of PD98059-loaded polymeric microparticles, without apparent adverse events, while blank microparticles had no effect. Similar reductions in plasma NE, a general indicator of sympathetic excitation, were previously achieved in HF rats by

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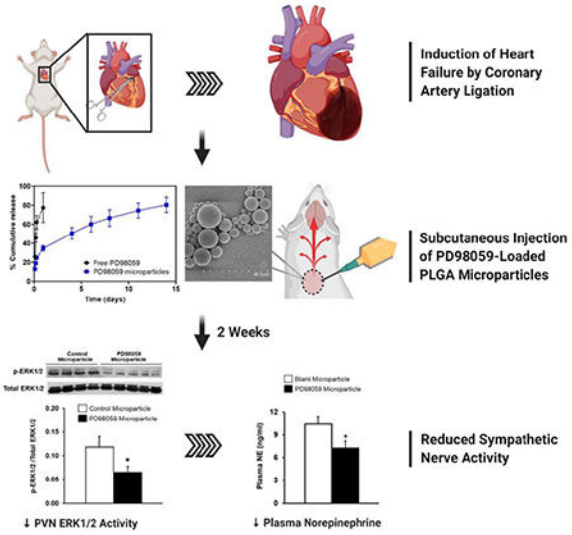
Supporting Information. Experimental methods and supplementary figures on echocardiographic assessment of left ventricular (LV) function and body weight, KIM-1, mRNA, plasma cystatin C, plasma CPK levels, and serum AST activity can be found in the supporting documents.

intracerebroventricular infusion of PD98059 or genetic knockdown of PVN ERK1/2 expression. This study presents a clinically feasible therapeutic approach to the central abnormalities contributing to HF progression.

Graphical Abstract



Keywords

Microparticles; MEK1/2 inhibitor; hypothalamic paraventricular nucleus; p-ERK1/2; heart failure; PD98059

Systolic heart failure (HF), also known as heart failure with reduced ejection fraction, is characterized by exaggerated sympathetic nerve activity (SNA) that contributes to increased preload (volume accumulation), afterload (peripheral vasoconstriction), cardiac remodeling with further deterioration in cardiac performance, and an increased vulnerability to life-threatening ventricular arrhythmias [1–6]. The hypothalamic paraventricular nucleus (PVN), a forebrain region rich in presympathetic neurons that regulate SNA [7–9], has been implicated as a source of the exaggerated SNA in HF. In a rat model of HF induced by myocardial infarction, mimicking a common cause of HF in humans, the neurochemical milieu in the PVN is deranged, with increased brain renin-angiotensin system (RAS) activity, the new expression of proinflammatory cytokines (PIC), and the induction of endoplasmic reticulum (ER) stress [10–13]. Central interventions that interfere with these neurochemical abnormalities in the PVN reduce SNA and improve the peripheral manifestations of HF in this rat model.

Our research over the past decade has provided compelling evidence that the Ras-Raf-MEK-ERK pathway in the PVN plays a fundamental role in mediating the sympathetic excitation that accompanies, and ultimately aggravates, HF in rats [10, 11, 13–17]. In rats with established HF, ERK1/2 activity is increased in the PVN, along with PVN neuronal excitation [15–17], and short-term inhibition of ERK1/2 activity with a 1-hour

intracerebroventricular (ICV) infusion of PD98059, a specific MEK1/2 inhibitor, reduces PVN neuronal excitation and renal sympathetic nerve activity [16]. A long-term (4-week) ICV infusion of PD98059 beginning 24 hours after induction of HF [14], or selective downregulation of ERK1/2 expression in the PVN prior to the induction of HF [17], results in lower levels of plasma norepinephrine (NE), a general indicator of SNA that predicts adverse outcomes in humans with HF [18, 19].

While these findings suggest a novel therapeutic approach, targeting the central abnormalities driving the excess SNA in HF, the translation of these experimental observations to the clinical arena is clearly problematic. We previously reported [20] that intravenously administered PD98059 can reach the brain, raising the possibility that systemic administration of this MEK1/2 inhibitor might be a therapeutic option. However, the short half-life of PD98059, and its reversible MEK1/2 inhibitory activity, are major hurdles that would have to be overcome. We found that the elimination half-life following IV injection of a solution of the PD98059 in rats was approximately 70 min [20], and Dudley et al. reported that the in vitro activity of MEK was fully restored once PD98059 was removed from the medium [21].

In the present study, we tested the hypothesis that subcutaneous (SC) administration of a poly (lactide-co-glycolide) (PLGA) microparticle preparation might provide sustained plasma levels of PD98059 in concentrations sufficient to facilitate a continuous therapeutic reduction of ERK1/2 activity in the PVN of rats with HF.

The PLGA microparticles were prepared as previously described [22, 23]. The microparticles were spherical and had smooth surfaces, as can be seen in the scanning electron microscopy image (Figure 1A). Their average particle size was approximately 4 μm and drug loading was 13.22 ± 1.24 μg PD98059 per mg microparticles (Figure 1B). A detailed experimental procedure for microparticle preparation and characterization can be found in the supplementary section. In vitro, the microparticles exhibited slow drug release, as approximately 60% of the loaded drug was released in the first week, and approximately 80% was released after two weeks (Figure 1C). Meanwhile, it took only 24 hours for about 80% of the unencapsulated drug to dissolve in the release medium. This shows the ability of the microparticles to control the drug release in vitro.

HF was induced in rats by ligating the left coronary artery, as previously described [11, 12, 14–17, 24]. Rats with left ventricular (LV) ejection fraction less than 40 % (normal, ~80%) by echocardiography performed within 24 hours of coronary artery ligation received a SC injection of PLGA microparticles containing 400 μg PD98059 in 1 ml of Dulbecco's phosphate buffer saline (DPBS, pH 7.4, Thermo Fisher, Waltham, MA), or a SC injection of empty PLGA microparticles. Two weeks later, the rats underwent repeat echocardiographic assessment of LV function and were sacrificed to collect tissues and plasma for molecular and biochemical studies.

Previous studies from our laboratory have revealed substantial increases in phosphorylated (p-) ERK1/2 levels in the PVN of HF rats, compared with sham-operated or normal control rats [11, 14, 15, 25]. The present study examined the effect of SC administration of

PD98059-loaded microparticles on these increased p-ERK1/2 levels. PVN p-ERK1/2 levels in the HF rats treated with the drug-loaded microparticles were about half those in the HF rats that received blank microparticles ($p < 0.05$, Fig. 2), while total ERK1/2 was unaffected. This finding demonstrated for the first time that PD98059 released from a peripherally administered sustained release microparticle preparation can inhibit ERK1/2 activity in the PVN.

Plasma NE levels are high in this rat model of HF, compared with sham-operated or normal control rats [14, 11, 25], consistent with their augmented SNA. Notably, an ELISA assay revealed that HF rats treated with the PD98059-loaded microparticles had significantly lower plasma NE levels than HF rats treated with blank PLGA microparticles (Fig. 3). These results, demonstrating a dependence of sympathetic activation on brain ERK1/2 signaling, are similar to our prior results showing a significant reduction of plasma NE levels in HF rats following a prolonged ICV infusion of PD98059 [14] or a selective knockdown of ERK1/2 expression in the PVN [17].

ERK1/2 is not the only MAPK protein in the PVN likely to contribute to sympathetic excitation in HF rats. Increased levels of phosphorylated p38 MAPK and c-Jun N-terminal kinase (c-JNK) are also found in the PVN of HF rats [15]. However, p-ERK1/2 appears most closely associated with sympathetic excitation in HF. Thus, chronic ICV infusion of the p38 MAPK inhibitor SB203580 was less effective than the ERK1/2 inhibitor PD98059 in reducing plasma NE in HF rats, and the c-JNK inhibitor SP600125 was ineffective [14]. Likewise, acute ICV administration of the ERK1/2 inhibitor reduced recorded SNA in HF rats, while the p38 MAPK and c-JNK inhibitors had no effect [16]. These findings suggested that PD98059 might be the MAPK inhibitor most likely to be effective if delivered via a sustained-release microparticle preparation.

We also assessed the HF rats treated with PD98059-loaded or blank microparticles for evidence of toxicity 2 weeks after the SC injection. Repeat echocardiography revealed evidence of the natural progression of HF in both groups, with an increase in left ventricular end-diastolic volume, but there was no echocardiographic evidence of an adverse (or beneficial) effect of PD98059 (Fig. S1). Interventions in the central neurochemical abnormalities in this HF model rarely affect echocardiographic indices of heart function, despite beneficial effects on cardiac function related to improved volume regulation (reduced left ventricular end-diastolic pressure and pulmonary congestion) and reduced cardiac remodeling [14, 17].

ERK1/2 signaling is a ubiquitous intracellular process regulating normal cellular functions throughout the body. However, the dramatic increases in p-ERK1/2 in brain regions contributing to the pathogenesis in HF suggest that excessive ERK1/2 activity might be modulated without compromising its normal functions. We found that treatment of HF rats with PD98059 microparticles had no effects on body weight (Fig. S2A), kidney injury molecule-1 (KIM-1) mRNA (Fig. S2B), plasma cystatin C levels (Fig. S2C), and plasma creatine phosphokinase (CPK) levels (Fig. S2D), compared with treatment with blank microparticles or no treatment, suggesting no evidence of renal or muscle injury. Drug-loaded and blank microparticles also had no effect on serum aspartate aminotransferase

(AST) levels in HF rats, compared to untreated HF rats (Fig. S2E), suggesting no liver toxicity. However, the limitations associated with the early time point and the small groups of animals used in this assessment are acknowledged. A more comprehensive assessment will be required, including an examination of the effects of PD98059-loaded microparticles on cardiac hemodynamics and cardiac remodeling, in addition to a broader evaluation of safety and toxicity of this proposed treatment.

Peripheral edema and vasoconstriction are common manifestations of HF, and might be expected to adversely affect drug absorption from a subcutaneous site [26–29]. The present data suggest that the microparticles may be able to provide therapeutic brain levels of PD98059 despite the poor peripheral perfusion characteristic of HF. In this regard, we previously reported that brain levels of PD98059 were detectable 2 weeks after SC injection of PD98059-loaded microparticles in HF rats [20].

There are no echocardiographic differences between control and drug-loaded microparticles. We rarely see any significant change in echocardiographic indices of heart function following central interventions that reduce SNA, mainly due to the permanent damage elicited by coronary artery ligation. As such and as expected, there was no difference in LVEDV progression between rats treated with drug-loaded or blank microparticles.

We previously administered PD98059 by continuous ICV infusion for 4 weeks to HF rats. The dose given was 0.25 μl/h of 0.6 mM solution of PD98059 for 4 weeks [14], which is equivalent to approximately 40 ng/h delivered to the brain. We have recently shown brain PD98059 levels following SC injection of the microparticles in HF rats were fluctuating, but they reached values close to 40 ng/g, which is equal to about 60-80 ng delivered to the brain [20]. The levels declined in later time points. These concentrations are perceived to be effective (based on the ICV data) although further studies are necessary to identify the optimal dose.

PLGA is listed by the US FDA as a GRAS (generally regarded as safe) material [30]. It is a nontoxic material with no pharmacological activity [31]. High biocompatibility with low, or even no, local irritation have been reported with commercial products containing PLGA as an excipient [32], and thus, we used blank PLGA microparticles as a vehicle control.

In summary, we report, for the first time, the use of a systemically administered sustained-release ERK1/2 inhibitor formulation to mitigate the central neural mechanisms driving augmented sympathetic excitation in HF rats. Our in vitro studies revealed that microparticles loaded with the MEK1/2 inhibitor PD98059 released the drug slowly over 2 weeks. A single SC injection of this preparation within 24 hours of coronary ligation to induce HF resulted in reduced ERK1/2 signaling in the PVN when measured 2 weeks later, along with reduced sympathetic excitation as evidenced by reduced plasma NE levels. These findings are consistent with our previous studies showing that a reduction in ERK1/2 signaling in the brain, and specifically in the PVN, reduces SNA in this model of HF [14, 15, 17], and suggest that the reduction in PVN ERK1/2 signaling accounts for the drug-induced reduction in NE levels in this study. This novel microparticle formulation effectively

addresses the 2 major problems that have stymied the clinical usefulness of PD98059, its short half-life and its reversible activity.

Figure 4 illustrates the contribution of ERK1/2 activity in the PVN to sympathetic excitation, and highlights the proposed therapeutic benefit of a PD98059-loaded microparticle preparation. HF induced by coronary artery ligation, mimicking HF in humans following myocardial infarction, initiates renin-angiotensin system (RAS) activity and inflammation both peripherally and in cardiovascular regions of the brain including the PVN. Angiotensin II (Ang II) and pro-inflammatory cytokines (PIC) activate MAPK signaling in the PVN (and other brain regions), amplifying the production of these excitatory mediators that act upon pre-sympathetic neurons in the PVN to augment SNA. The increased SNA, in turn, contributes to worsening HF by increasing renal sodium reabsorption (leading to increased preload) and peripheral vasoconstriction (leading to increased afterload), increasing renal renin release (leading to increase systemic RAS activity) and stimulating cardiac remodeling. Increased SNA also renders the heart more susceptible to serious ventricular arrhythmias and sudden death.

We propose that subcutaneous administration of PD98059-loaded microparticles has the potential to interrupt this devastating central feed-forward process by reducing ERK1/2 signaling at the PVN level, where it contributes to the production of RAS elements and PIC that drive pre-sympathetic neurons. However, fully achieving that potential will require additional steps to optimize the microparticle preparation to provide sustained plasma levels of PD98059 sufficient to continuously inhibit phosphorylation of brain ERK1/2, ideally for prolonged intervals of weeks to months, and to fully characterize its safety profile. This approach has clear translational potential, as a single therapy or an adjuvant therapy with other peripheral treatments (e.g. angiotensin converting enzyme inhibitors, and β-blockers) to control increased sympathetic activity in HF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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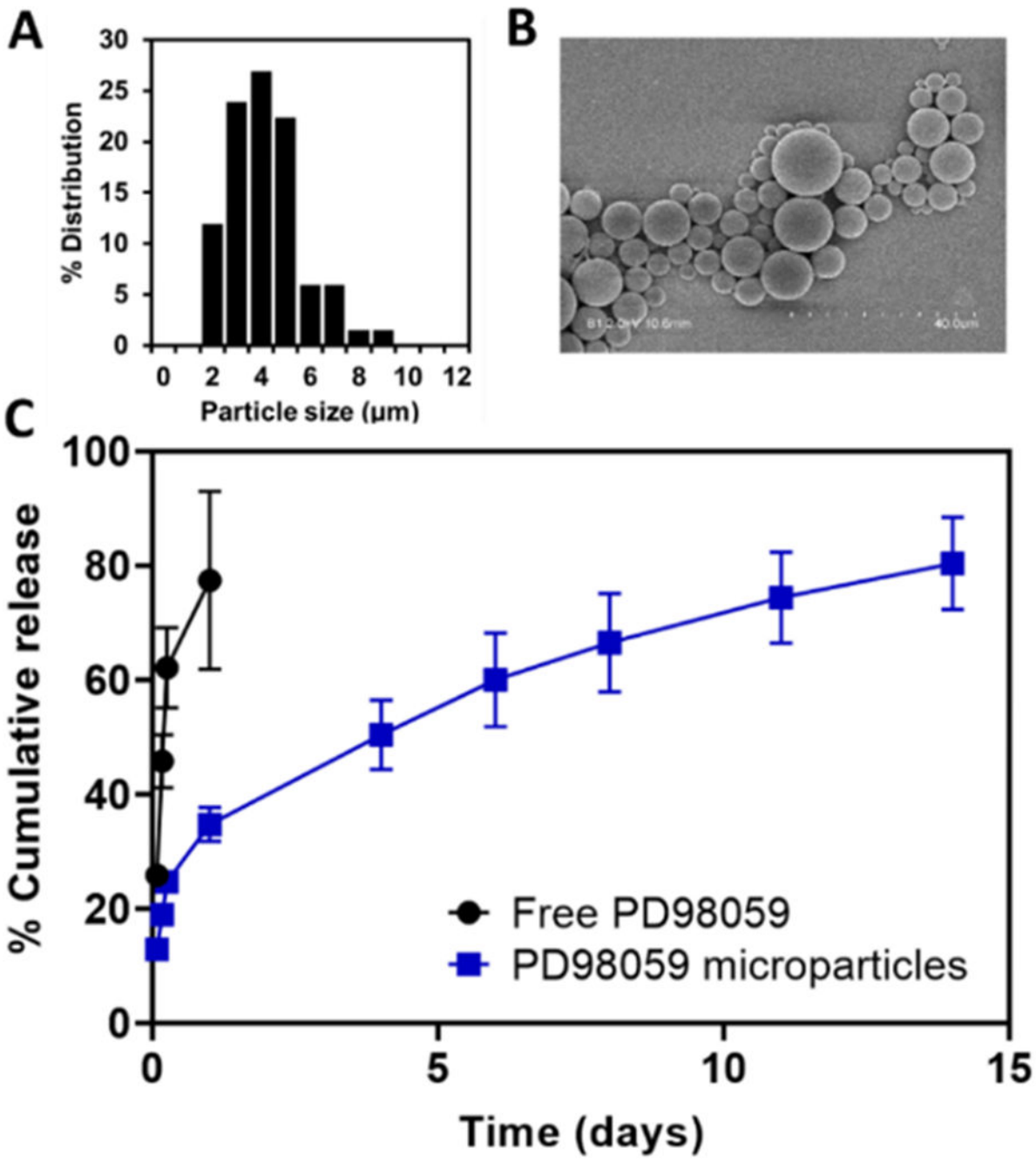

Figure 1:
In vitro characterization of PD98059-loaded PLGA microparticles. **A**. Particle size distribution. **B**. Morphology of the microparticles by SEM. **C**. Cumulative release of PD98059 from the microparticles compared to dissolution of PD98059 powder in the release medium (n = 3, data represented are mean ± SD).

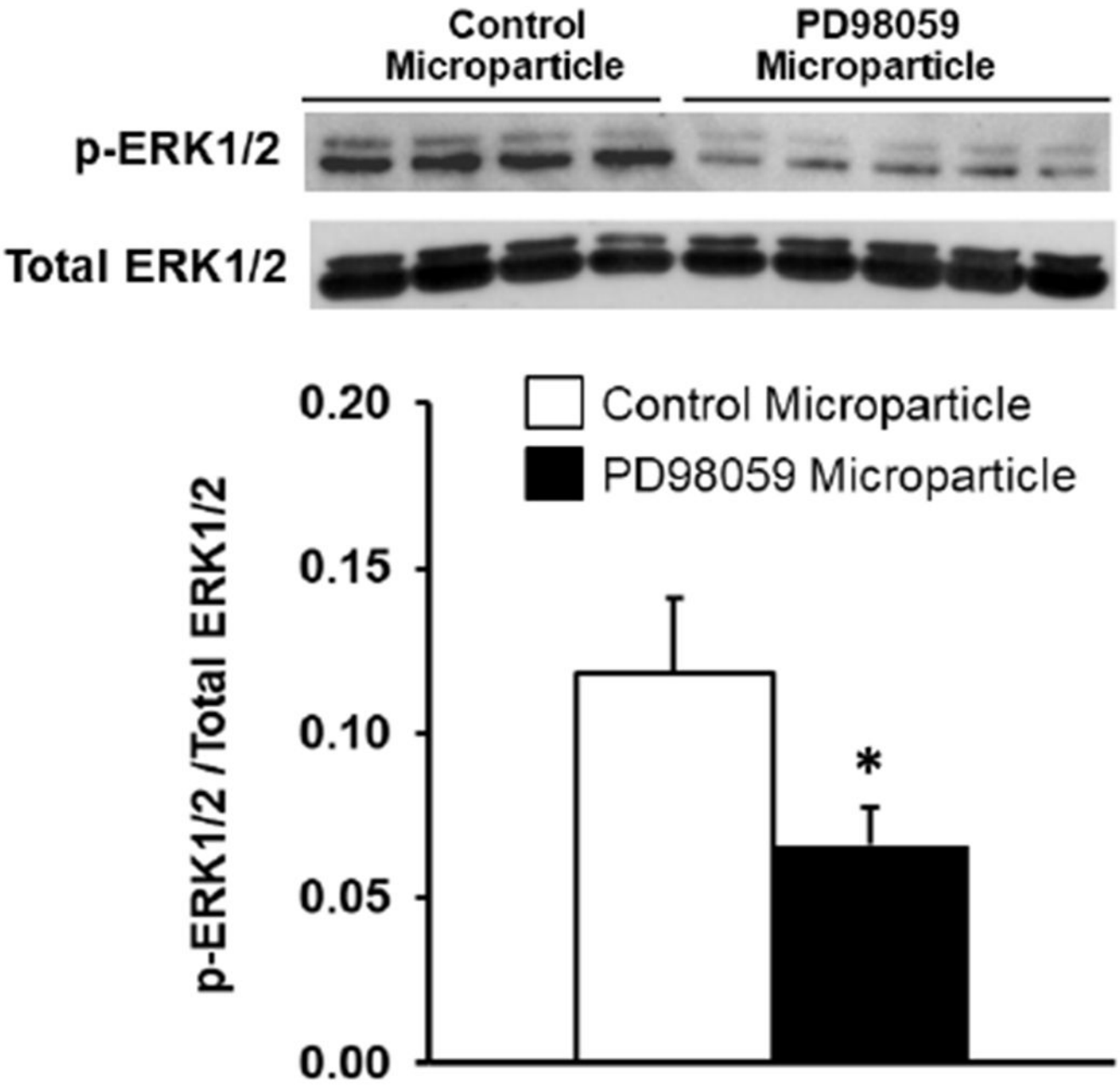

Figure 2:

Western blot analysis of p-ERK1/2 levels in the PVN of HF rats two weeks after the SC injection of PD98059-loaded PLGA microparticles or blank microparticles. **Top panel**: Western blot bands. Each lane represents one rat of each group. **Bottom panel**: values are normalized to total ERK1/2 and represented as means ± SEM and (n=4-5/group, * $p < 0.05$).

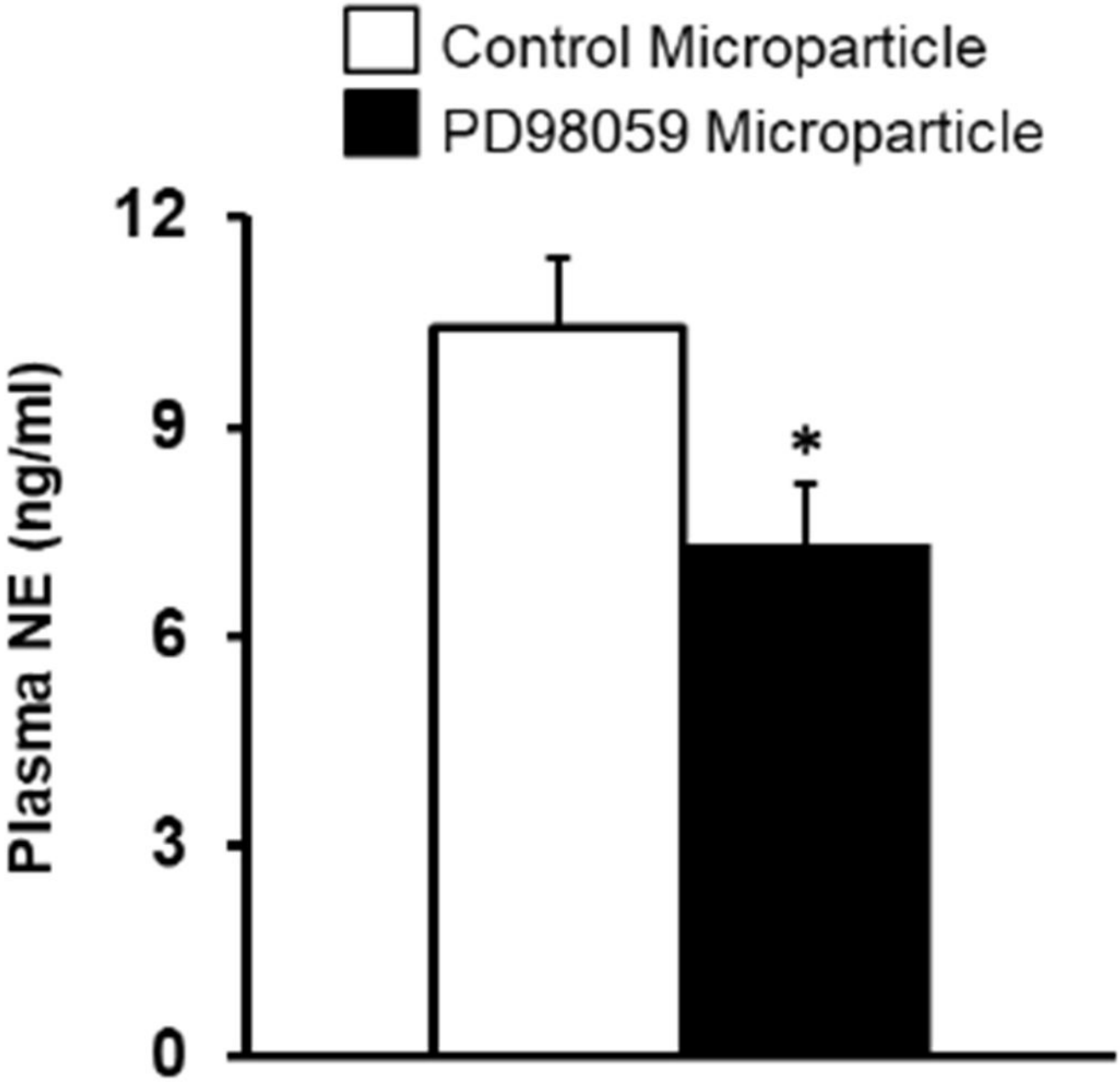

Figure 3:
Plasma norepinephrine levels in HF rats two weeks after the SC injection of PD98059-loaded PLGA microparticles or blank microparticles. Values are represented as means ± SEM (n=4-5/group, * $p < 0.05$).

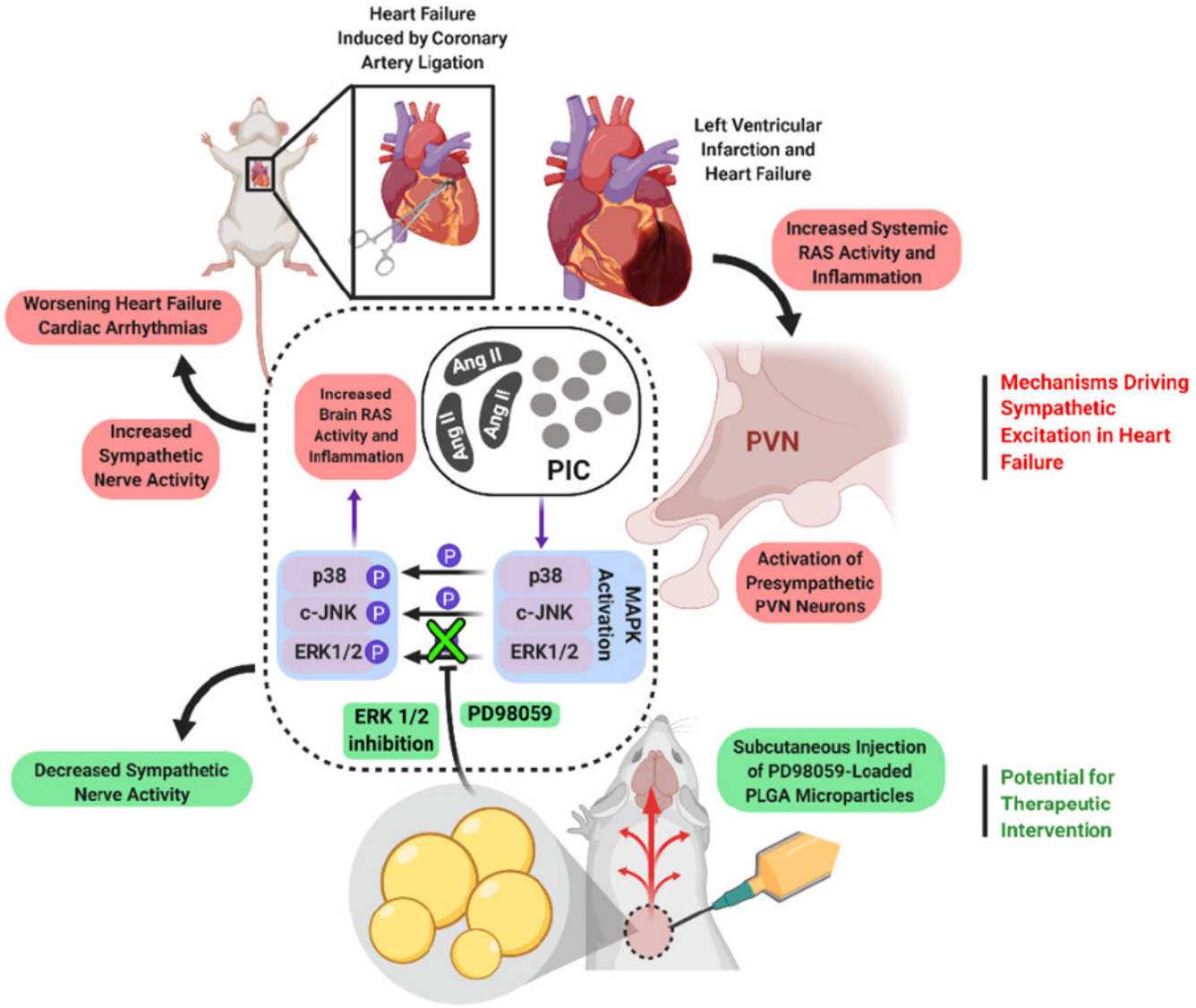

Figure 4:

Schematic illustrating the putative effect of subcutaneously administered PD98059-loaded microparticles to mitigate neurochemical abnormalities in the PVN that contribute to the progression of heart failure (HF). In rats with coronary artery ligation-induced HF without treatment, increased systemic renin-angiotensin system (RAS) activity and inflammation upregulate levels of angiotensin II (Ang II) and proinflammatory cytokines (PIC) in the PVN, increasing phosphorylation (P) of ERK1/2. The resulting augmentation of brain RAS activity and inflammation increases sympathetic nerve activity, further compromising cardiac performance. We propose that the subcutaneously injected PD98059-loaded microparticles will provide sustained plasma levels of PD98059, resulting in a prolonged inhibition of ERK1/2 phosphorylation. Other mitogen-activated protein kinase (MAPK) signaling pathways (p38 MAPK and c-JNK) are unaffected by treatment with this selective MEK1/2 inhibitor. This treatment strategy significantly lowered plasma NE levels compared

to vehicle-treated rats as an indication of reduced sympathetic nerve activity, which sets it out as a potential therapeutic intervention.