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C1q/TNF-Related Protein-9, a Novel Adipocyte-Derived Cytokine, Attenuates Adverse Remodeling in the Ischemic Mouse Heart via PKA Activation

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Abstract

Background—CTRP9 is a newly identified adiponectin paralog with established metabolic-regulatory properties. However, the role of CTRP9 in post-myocardial infarction (post-MI) remodeling remains completely unknown. This study determined whether C1q/TNF-related protein-9 (CTRP9) may regulate cardiac remodeling following acute myocardial infarction (AMI), and elucidated the underlying mechanisms.

Methods and Results—Male adult mice were subject to AMI by left anterior descending coronary artery (LAD) ligation or sham surgery, and treated with saline (vehicle) or globular CTRP9 via peritoneal implant osmotic-pumps for 6 weeks. H9C2 cardiac cell lines were utilized in vitro for determining underlying mechanisms. Adipocyte CTRP9 expression and plasma CTRP9 levels were both significantly reduced after AMI. Compared to vehicle, CTRP9 treatment improved animal survival rate ($P<0.05$), restored cardiac function ($P<0.05$), attenuated adverse remodeling ($P<0.01$), and ameliorated cardiomyocyte apoptosis and fibrosis following AMI ($P<0.01$). Among multiple anti-remodeling molecules determined, AMP-activated protein kinase (AMPK), protein kinase-A (PKA), and Akt were significantly activated in CTRP9-treated heart. Surprisingly, CTRP9 remains cardioprotective in cardiac-specific AMPK-DN mice. Additional in vitro experiments demonstrated that administration of either PKA inhibitor or PKA-specific siRNA virtually abolished CTRP9's anti-apoptotic effect ($P<0.05$), whereas inhibition of Akt is less effective in blocking CTRP9 cardioprotection. Finally, CTRP9 phosphorylates BAD at its multiple anti-apoptotic sites, an effect blocked by PKA inhibitor.

Conclusions—We demonstrate that adipokine CTRP9 attenuates adverse cardiac remodeling following AMI, largely via a PKA-dependent pathway.

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Conflict of Interest Disclosures: None

Keywords

adipokine; remodeling; apoptosis; myocardial infarction

Ischemic heart disease (IHD) remains a leading cause of morbidity and mortality in the developed world. Despite improved reperfusion technologies enhancing patient survival post-acute myocardial infarction (AMI), both incidence and prevalence of adverse cardiac remodeling leading to heart failure have increased[1]. Following AMI, the left ventricle undergoes complex anatomic remodeling, a pathological transformation involving molecular maladaptation, cellular dysfunction, matrix reorganization, and gross geometric volume and wall mass abnormalities[2]. Despite pharmacological advances (e.g., β-blockers, renin–angiotensin–aldosterone system inhibitors), post-MI mortality remains high (5-year survival rate 30–70%). An efficacious therapeutic preventing post-MI ventricular remodeling is therefore invaluable.

Obese individuals are more vulnerable to heart failure. The relationship between adipokines and obesity-linked pathologies, including heart failure, has been the subject of intense study. The circulating adipokine (signaling proteins of adipocyte origin) profile in the obese is often deranged. Leptin, tumor necrosis factor α (TNF-α), plasminogen activator inhibitor type 1(PAI-1), transforming growth factor β, and resistin all contribute to maladaptive cardiac remodeling in vivo[3]. However, whether there exist adipokines that may positively influence cardiac remodeling post-MI remains unknown.

Adiponectin (APN), an abundant adipokine predominantly secreted by adipocytes, has numerous functions, including regulation of metabolism, anti-inflammation, protection against endothelial dysfunction, and protection against ischemic injury. The C1q tumor necrosis factor (TNF)-related proteins (CTRPs) are a newly discovered highly conserved family of APN paralogs, containing 13 members (CTRP1 to CTRP13). The protein family exhibits similar structure as APN, consisting of four distinct domains including a N-terminal signal peptide, a short variable domain, a collagen-like domain, and a C-terminal C1q-like globular domain[4, 5]. Of all CTRPs identified to date, CTRP9 shares the greatest amino acid overlap with APN in its globular C1q domain. More importantly, CTRP9 is also predominantly expressed in adipose tissue, forms heterotrimers with APN, and reduces mouse serum glucose similarly to APN[6]. We recently demonstrated that CTRP9 is an endothelium-dependent vasodilator and CTRP9 treatment attenuates diabetes-induced endothelial dysfunction[7]. Moreover, others and we have recently demonstrated that CTRP9 protects the heart from acute ischemic/reperfusion injury[8, 9]. However, whether CTRP9 functions as a mediator or inhibitor of post-ischemic remodeling, the critical process underlying progression to heart failure, is unknown.

Thus, the aims of this study were 1) to detect whether CTRP9 levels change after AMI; 2) to determine the effects of exogenous CTRP9 supplementation upon post-ischemic cardiac remodeling and dysfunction; and 3) to elucidate the mechanisms responsible for CTRP's effects upon cardiac function.

Methods

All study experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals, and were approved by the Thomas Jefferson University Committee on Animal Care. All authors had full access to, and take full responsibility for data integrity. All authors have read and agree to the manuscript as written.

Animal model and cardiac function

All in vivo experiments were performed upon adult (8-week old) male C57BL/6 mice. LAD occlusion was performed as previously described[10]. 4 hours post-MI, animals were randomized to one of the following groups: Sham AMI, AMI+Vehicle, or AMI+CTRP9 (0.25μg/g/day intra-peritoneal infusion via mini-osmotic pump)[11]. CTRP9 was produced as we recently reported[7]. The dosage of CTRP9 effectively restoring plasma CTRP9 to control levels after AMI was determined in a pilot experiment. Sham operation animals were subject to identical surgical procedures without LAD ligation. After 2 or 6 weeks, mice were sacrificed. Immunohistological, biochemical, and Western blot assays were performed. Echocardiography was performed at baseline, 1 day, and 2, 4, and 6 weeks thereafter. Hemodynamic data was obtained via invasive left ventricular catheterization after 2 and 6 weeks, as previously described[10].

Kinase activity assay, quantitative real time PCR, Western blot analysis and histology

Zymography determining gelatinase activity (Bio-Rad) and PepTag®Non-Radioactive Protein Kinase-A Assay (Promega) were performed per manufacturer's instructions. LDH release and caspase-3 activity assays were performed as previously described[12]. CTRP9 mRNA levels were determined by quantitative real time PCR[6]. Western blot analysis was performed as previously described[7]. Cardiac collagen content and infarct size were assessed via Masson trichrome staining. Cardiac sections were immunohistochemically stained, via primary antibodies to CD-31 (BD Biosciences), actinin (Sigma), and TUNEL (Roche). Infarct border zone regions were specifically examined.

Statistical analysis

All values in the text and figures are presented as the mean±SEM of n independent experiments. Data were analyzed with Graph Pad Prism-6 statistic software (La Jolla, CA). ANOVA across all investigated groups was conducted first. Post hoc pairwise tests for certain group pairs with assessment of statistical significance were performed after Bonferroni correction of the overall significance level. While Bonferroni-adjustment was performed across groups, it was not performed across variables. Probabilities less than or equal to 0.05 (two-sided) were considered statistically significant. More specific information regarding group comparison is provided in the figure ledged for each figure.

Results and Discussion

AMI reduces CTRP9 expression and production

To determine whether AMI may inhibit CTRP9 expression/production, adipose CTRP9 mRNA expression level and plasma CTRP9 were determined during the first two weeks of AMI. As illustrated in Figure 1, adipose CTRP9 mRNA and plasma CTRP9 levels both began to decline 1 day after AMI, reaching nadir 3 days post-MI, and gradually increasing to baseline thereafter. These data demonstrate for the first time that AMI inhibits CTRP9 at both the mRNA and protein levels.

Restoring plasma CTRP9 in AMI mice improves survival and enhances left ventricular cardiac function post AMI

To determine the pathological significance of AMI inhibition of CTRP9 expression/ production, the following experiments were performed. Since neither CTRP9 knockout nor CTRP9 transgenic mice are currently available, we employed a pharmacological restorative approach, administering recombinant CTRP9 at a dose regiment maintaining plasma CTRP9 at control levels. 42 days after AMI, the effect of CTRP9 upon survival rate and cardiac function was determined. CTRP9 administration significantly improved post-MI survival

rate (Figure 2A). Moreover, exogenous CTRP9 significantly augmented left ventricular ejection fraction (LVEF), increased dP/dt_{max}, and decreased LV end diastolic pressure (LVEDP) (Figure 2B–2D). Taken together, these data demonstrate CTRP9 improves survival rate, and restores both LV systolic and diastolic function post-MI.

CTRP9 attenuates left ventricular hypertrophy and dilation post-MI

Having demonstrated that restoring CTRP9 plasma level after AMI improves cardiac function and survival rate, we further investigated whether CTRP9 may attenuate pathological remodeling, the primary cause of post-ischemic cardiac dysfunction and heart failure. Over the course of 6 weeks, compared to vehicle, CTRP9 administration reduced cardiac size, decreased HW/BW by 24.2% ($P<0.01$, Figure 3A), and reduced LW/BW by 21.9% ($P<0.01$, Figure 3B). Moreover, CTRP9 treatment preserved LV end-diastolic dimension (LVEDD) and LV mass (Figure 3C–3E). Taken together, these data are the first evidence that CTRP9 attenuates adverse cardiac heart failure remodeling.

CTRP9 supplementation has no significant effect upon angiogenesis but inhibits cardiomyocyte apoptosis and fibrosis post-MI

To determine the cellular mechanisms responsible for CTRP9's anti-remodeling effect, the potential angiogenic effect of CTRP9 (a mechanism known to contribute to the cardioprotective actions of APN) was first assessed by CD31 immunohistochemical staining. Surprisingly, CTRP9 treatment did not significantly increase CD31-positive microvessels in the border regions of post-MI hearts compared to vehicle (Figure 4A), suggesting that mechanisms other than pro-angiogenesis are responsible for CTRP9's anti-remodeling effect. We then determined extent of apoptotic cell death and interstitial fibrosis in the infarct border zone, two significant etiologies of infarct expansion and adverse remodeling post-MI. CTRP9 treatment dramatically attenuated apoptotic cell death (61.3% reduction versus vehicle, $P<0.01$, Figure 4B) and significantly suppressed interstitial fibrosis by 56.1% (Figure 4C). Finally, CTRP9 treatment significantly decreased MMP2/MMP9 activity and TGF-β1 production, the two most significant mechanisms contributing to post-MI fibrosis (Figures 4D and 4E). Collectively, these results demonstrate CTRP9 prevents LV remodeling by reducing apoptosis and fibrosis.

CTRP9 supplementation activates multiple pro-survival signaling molecules

To further determine the mechanisms responsible for CTRP9-mediated attenuated post-MI remodeling at a molecular level, several pro-survival signaling molecules were determined. Among multiple cardioprotective molecules screened, AMPK, Akt, and PKA were identified as the most significantly activated molecules by CTRP9. As summarized in Figure 5, although CTRP9 had no significant effect upon AMPK, Akt, PKA, and eNOS expression, continuous administration of CTRP9 (via osmotic pump) maintained phosphorylated levels of AMPK, Akt, PKA, and eNOS at significantly greater levels than vehicle-treated animals 2 and 6 weeks after MI.

Cardioprotective effect of CTRP9 is attenuated but not lost when AMPK is inhibited

It is well-recognized that AMPK plays a critical role in APN biological function[13, 14]. Moreover, a recent study demonstrated that, in an acute myocardial ischemia/reperfusion model, CTRP9 protects against myocardial injury through an AMPK-dependent mechanism[8]. As CTRP9 administration significantly activated AMPK in our chronic permanent ischemia model, we first determined the role of AMPK in CTRP9-mediated cardioprotection. Adult male mice selectively overexpressing a dominant negative α_2 subunit (D157A) of AMPK (AMPK-DN) specifically in cardiomyocytes[15] were subjected to AMI, and treated with either vehicle or CTRP9 for 2 or 6 weeks. The phosphorylation of

Acetyl-CoA carboxylase, a downstream molecule mediating AMPK's metabolic function, by CTRP9 was completely blocked in AMPK-DN animals (data not shown). Compared to WT control, cardiac apoptosis and interstitial fibrosis were both increased in AMPK-DN mice (Figure 6). However, CTRP9 remains effective in improving post-MI survival rate ($P<0.05$, Figure 6A) and attenuating post-MI remodeling (Figure 6B) in these transgenic animals. At the cellular level, CTRP9 significantly inhibited cardiac apoptosis (Figure 6C) and reduced interstitial fibrosis (Figure 6D) in AMPK-DN animals, albeit to lesser degree than observed in WT animals (apoptosis: 61% reduction in WT vs. 36% reduction in AMPK-DN; fibrosis: 56% reduction in WT vs. 35% reduction in AMPK-DN). Taken together, these results demonstrate that although AMPK is significantly activated by CTRP9 and partially mediates its cardioprotective effects, other existent signaling systems contribute to CTRP's cardioprotective effect against chronic myocardial ischemia-induced cardiac remodeling.

Cardioprotective actions of CTRP9 were blocked by PKA inhibition

Our results presented in Figure 5 demonstrate that, in addition to AMPK, activation of Akt and PKA also occurs during CTRP9 administration. Cardiac specific Akt and PKA knockout mice are currently unavailable. Whole body Akt and PKA knockout has profound systemic metabolic alterations that may not be suitable for specifically determining their role in CTRP9-mediated cardioprotection. To obtain the most reliable evidence identifying the role of Akt and PKA in CTRP9 cardioprotection, we employed a cardiomyocyte line (H9C2) utilizing pharmacologic and genetic manipulation of Akt and PKA signaling. H9C2 cells were subjected to hypoxia (2% O_2) for 12 hours. H89 (a PKA inhibitor) and LY294002 (a PI3 Kinase inhibitor) were administered 1 hour before hypoxia. Similar to our in vivo experimental results demonstrating CTRP9 is cardioprotective, CTRP9 treatment of H9C2 cells attenuated hypoxia-induced LDH release and caspase-3 activity. Most importantly, pre-treatment with H89 virtually abolished the cardioprotective effect of CTRP9, whereas LY294002 pre-treatment only partially attenuated CTRP9-mediated cardioprotection (Figure 7A and 7B). These data suggest that PKA-initiated signaling plays a significant role in CTRP9 cardioprotection. To obtain more evidence supporting this notion, H9C2 cells were subjected to PKA-specific siRNA or scramble siRNA 48 hours before hypoxia. PKA siRNA successfully inhibited PKA activity (Figure 7C). Most importantly, the cardioprotective effect of CTRP9 in H9C2 cells determined by LDH release and caspase-3 activation was blocked in H9C2 cells pre-treated with PKA-specific siRNA (Figure 7D–E).

CTRP9 caused PKA-dependent BAD phosphorylation at multiple residues

In a final attempt to determine how CTRP9-activated PKA may reduce post-ischemic myocardial apoptosis, the effect of CTRP9 upon multiple apoptotic regulatory proteins were investigated. Significant phosphorylation of BAD (a post-translational modification inhibiting BAD's pro-apoptotic activity) at multiple phosphorylation sites, including Ser112, Ser136, as well as Ser155, was observed in CTRP9-treated cells (Figure 7F). Moreover, PKA inhibitor H89 blocked CTRP9-mediated phosphorylation of BAD at both Ser155 and Ser136, and partially blunted Ser112 phosphorylation. In contrast, PI-3K/Akt inhibitor LY294002 only significantly blocked CTRP9-induced BAD phosphorylation at Ser136 (Figure 7F).

BAD plays a central regulatory role in mitochondrial apoptosis pathway. It induces apoptosis by inhibiting antiapoptotic Bcl-2-family members (e.g., BCL-x, Bcl-2) thereby allowing two other pro-apoptotic proteins, BAK and BAX, to aggregate and induce release of cytochrome c. Among five phosphorylation sites identified on BAD, phosphorylation of Ser112, Ser136 and Ser155 inactivates BAD and inhibits apoptosis. BAD phosphorylation at Ser112 and 136 may function as the initial decoupling step between BAD and Bcl-2

proteins, whereas BAD phosphorylation at Ser155 exacerbates and completes the dissociation of Bcl-2 from BAD[16]. Previous studies have demonstrated that the pro-apoptotic effects of BAD are inhibited by PKA-mediated phosphorylation at Ser155[17–19]. Our current study demonstrated that CTRP9 caused BAD phosphorylation at all three sites. Moreover, our inhibitory experiments suggest that CTRP9 phosphorylates Ser136 through both PI3K/Akt and PKA signalings, whereas CTRP9-induced Ser155 phosphorylation is largely PKA dependent. Finally, the anti-apoptotic effect of CTRP9 is virtually abolished when PKA signaling is blocked, but only slightly blunted when Akt is inhibited, suggesting that simultaneous BAD phosphorylation at multiple sites are required to achieve CTRP9's anti-apoptotic and cardioprotective effects (Figure 8).

A recent study demonstrated that CTRP9 protects the heart from reperfusion injury largely via AMPK signaling[8]. However, our current study provides clear evidence that although AMPK is activated after CTRP9 administration, AMPK activation is not the primary signaling responsible for CTRP9-mediated protective effect against post-MI remodeling. This observation is important because it suggests that although CTRP9 protects the heart from both acute and chronic cardiac injury, different signaling mechanisms are involved. A likely explanation concerns the ischemic/reperfused model, where cardiac cells that are protected by CTRP9 are the cells subjected to acute ischemia followed by reperfusion. AMPK may play an important role in such cells, as demonstrated by the recently published work by Ouchi and colleagues. In contrast, in the post-MI remodeling model employed in the current study, the cardiac cells protected by CTRP9 are those adjacent to and remote from the ischemic zone, but never subjected to ischemia/reperfusion. Different intracellular signaling systems are activated and are responsible for CTRP9-mediated cardioprotection in these cells. Deeper molecular mechanisms, such as the differential impact of ischemia/reperfusion versus permanent ischemia upon CTRP binding protein expression, will be investigated in our future experiments. CTRP9 expression is significantly reduced after myocardial infarction. Administration of CTRP9 protects against acute reperfusion injury, as well as chronic post-MI remodeling. These results suggest that preventing post-MI CTRP9 inhibition or CTRP9 supplementation may be a promising therapeutic avenue protecting the heart from ischemic injury.

In summary, we demonstrate that administration of adipokine CTRP9 shortly after ischemia attenuated adverse cardiac remodeling post-MI, largely via a PKA-dependent pathway. It would be very interesting to evaluate whether CTRP9 administration at a more delayed interval (e.g., 7 days) may be cardioprotective, as such an experiment would clarify whether CTRP9 only terminates (as we have shown), or may also reverse pathological remodeling after adverse remodeling has already developed. Such experiments will be performed in our future studies.

Acknowledgments

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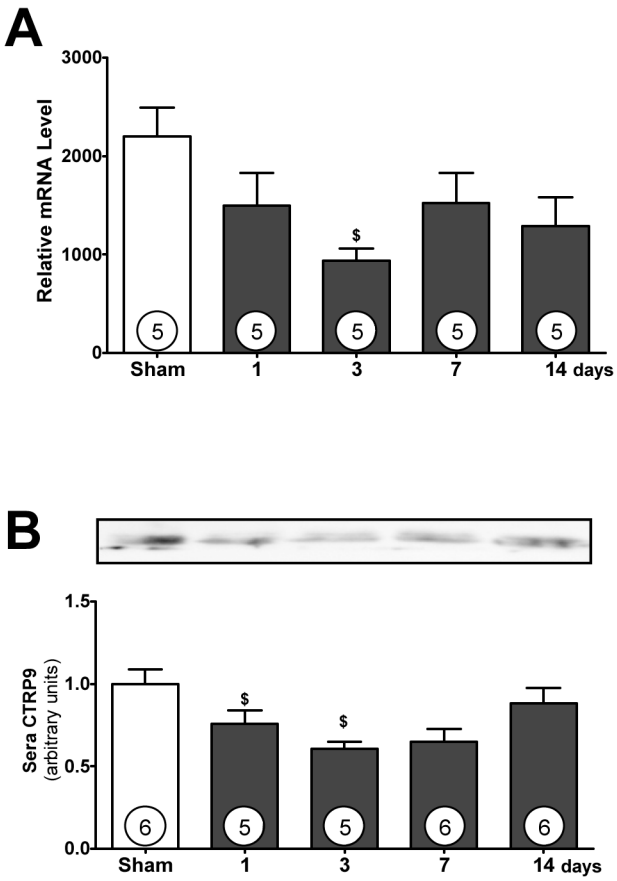

Figure 1.

AMI reduces CTRP9 expression and production in adult mice. Adipose CTRP9 expression determined by **(A)** quantitative real time PCR and **(B)** circulating CTRP9 levels determined by Western blot in adult male mice subject to AMI up to 14 days. $^{\$}$Bonferroni-adjusted $P<0.05$ vs. Sham group.

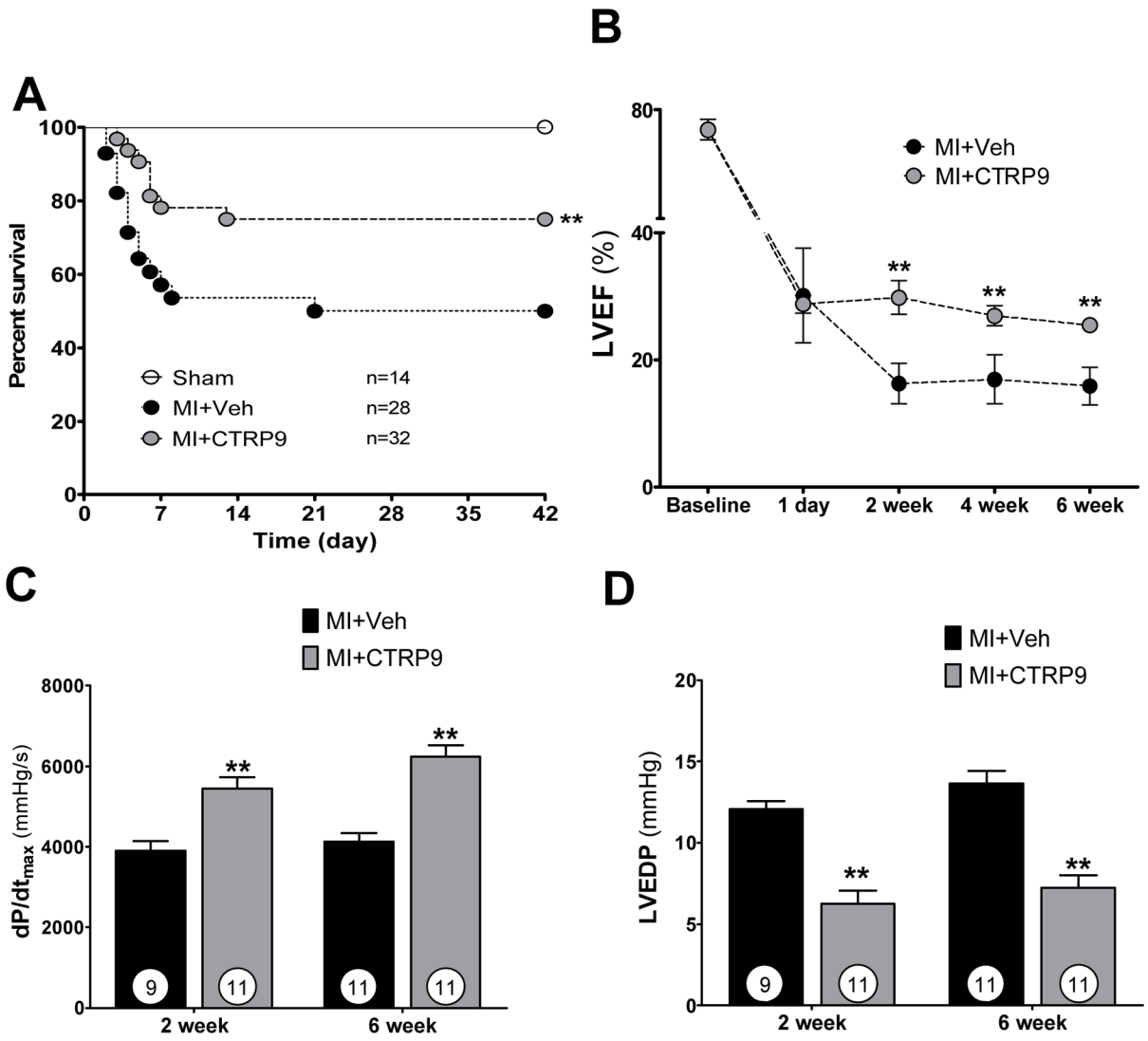

Figure 2.

CTRP9 improves survival and restores left ventricular cardiac function after AMI. The effects of CTRP9 treatment upon survival and LV function were determined up to 6 weeks post operation. **(A)** Survival curves; **(B)** Echocardiographic analyses; **(C, D)** Hemodynamic analyses. Abbreviations: LVEF, Left Ventricular Ejection Fraction; LVEDP, Left Ventricular End-Diastolic Pressure. *P<0.05; **P<0.01 vs. MI+Vehicle group. P-value comparing the two groups at each time point adjusted for Bonferroni across the time points.

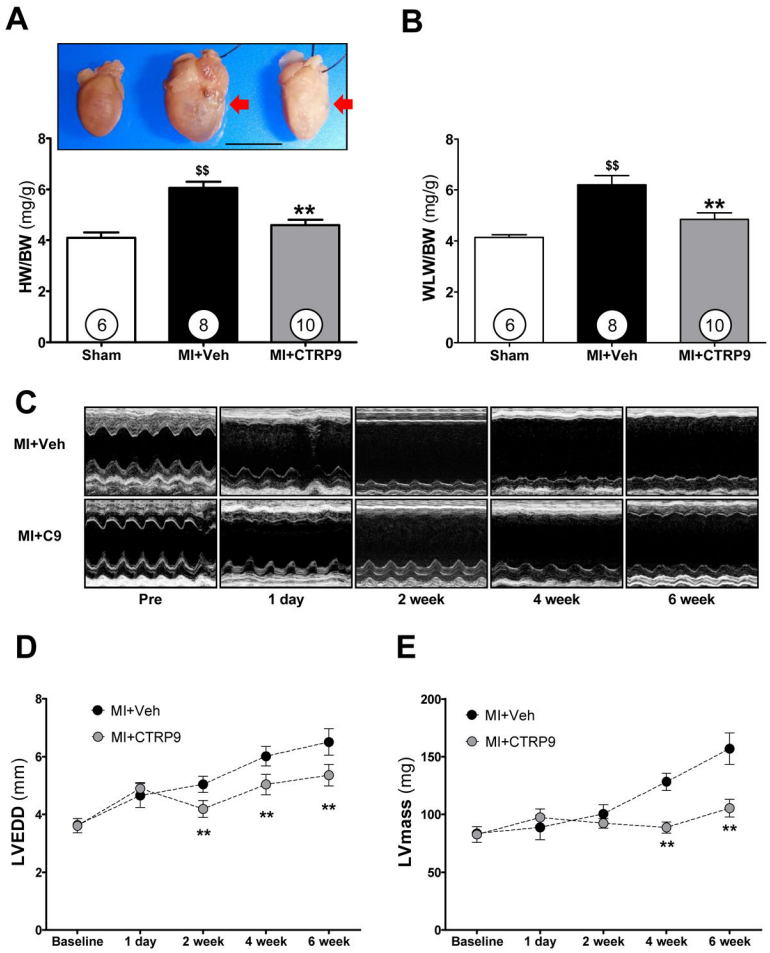

Figure 3.

CTRP9 prevents left ventricular cardiac remodeling after AMI. **(A)** Representative gross images and statistics of heart weight/body weight (HW/BW); **(B)** Wet lung weight to body weight ratio; **(C)** Representative echocardiographic images; **(D,E)** LVEDD and LVESD, from echocardiographic analysis. Abbreviations: LVEDD, Left Ventricular End-Diastolic Diameter; LVESD, Left Ventricular End-Systolic Diameter. $^{\$\$}$P<0.01 vs. Sham group, **P<0.01 vs. MI+Vehicle group. P-value comparing the two groups at each time point adjusted for Bonferroni across the time points.

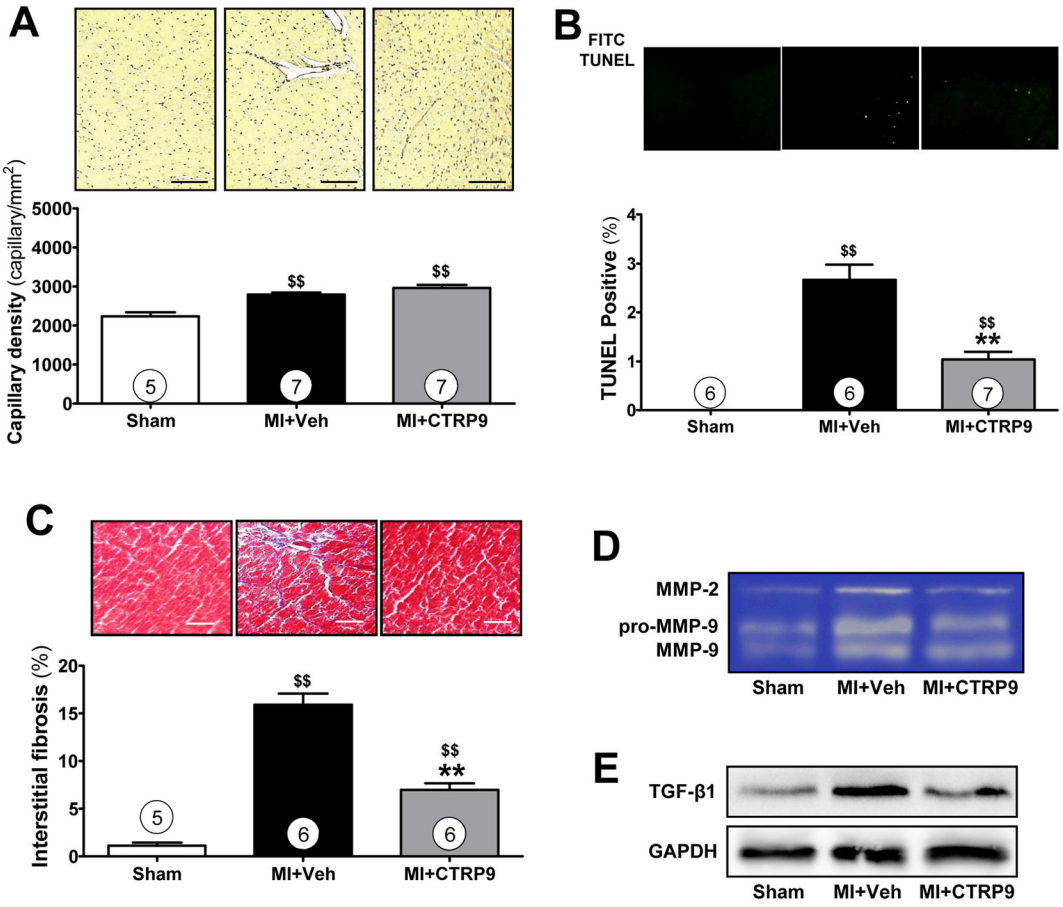

Figure 4.

Effect of CTRP9 on angiogenesis, apoptosis, and fibrosis. **(A)** Immunohistochemical staining for CD31 (brown) in the border region (AMI group) or LV free wall (Sham). Scale bars denote 200 μm; **(B)** Cardiomyocyte apoptosis determined by TUNEL staining. TUNEL positivity is green, nuclear staining is blue, and α-actinin staining is red. Scale bars: 200 μm. **(C)** Interstitial fibrosis. Scale bars denote 100 μm; **(D,E):**Representative gelatin zymograph and Western blot analysis of TGF-β1 from border region (AMI group) or LV free wall (Sham). $^{\$\$}$Bonferroni-adjusted P<0.01 vs. Sham group; **P<0.01 vs. MI+Vehicle group.

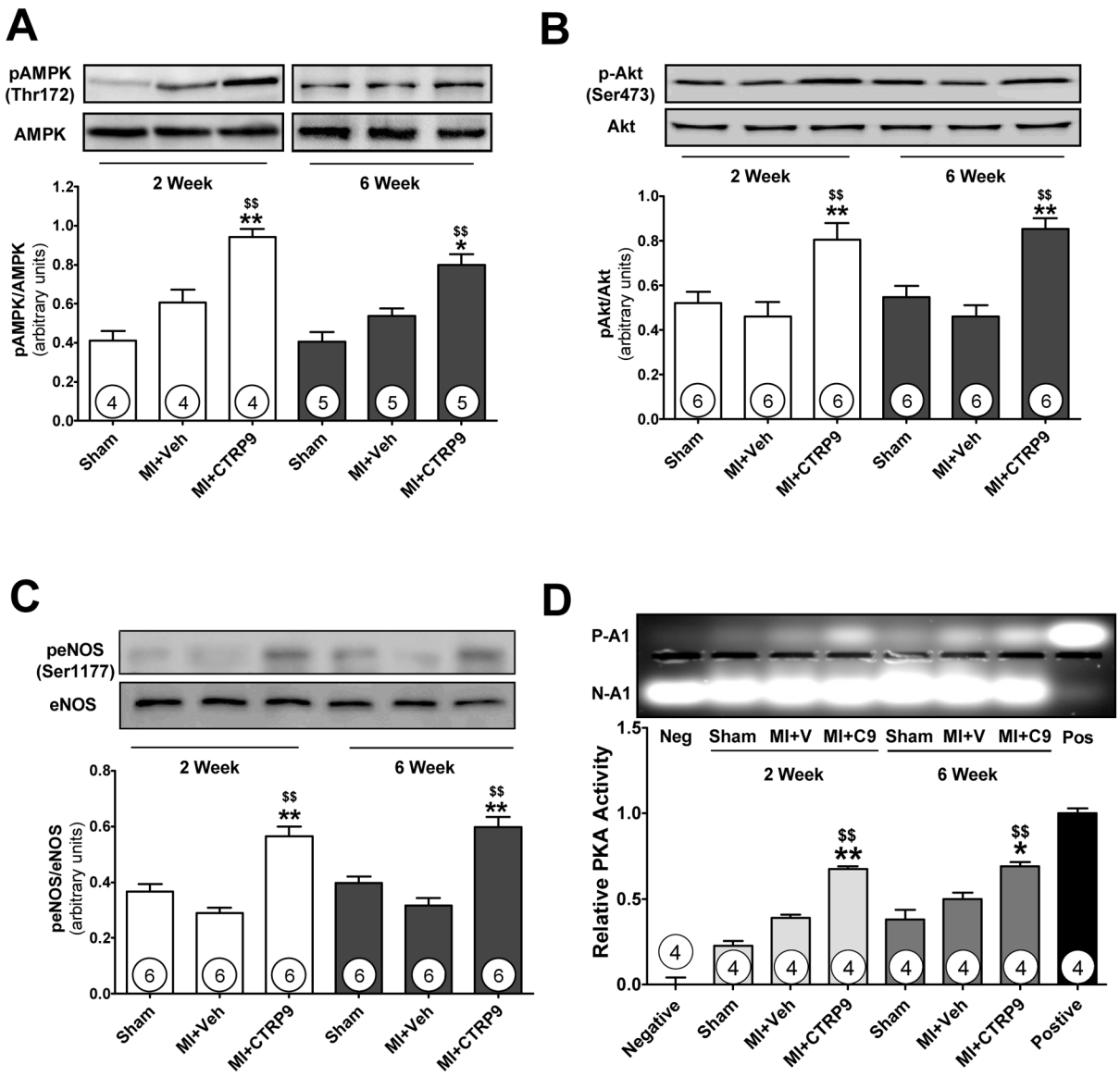

Figure 5.

CTRP9 activates multiple intracellular signaling pathways in vivo. **(A, B, C)** Western blot analysis for pAMPK(Thr172)/AMPK, pAkt(Ser473)/Akt and peNOS (Ser1177)/eNOS; **(D)** PKA activity from the border region (AMI group) or LV free wall (Sham) at 2 and 6 weeks post-MI. $^{\$\$}$Bonferroni-adjusted P<0.01 vs. Sham group; *P<0.05, **P<0.01 vs. MI+Vehicle group.

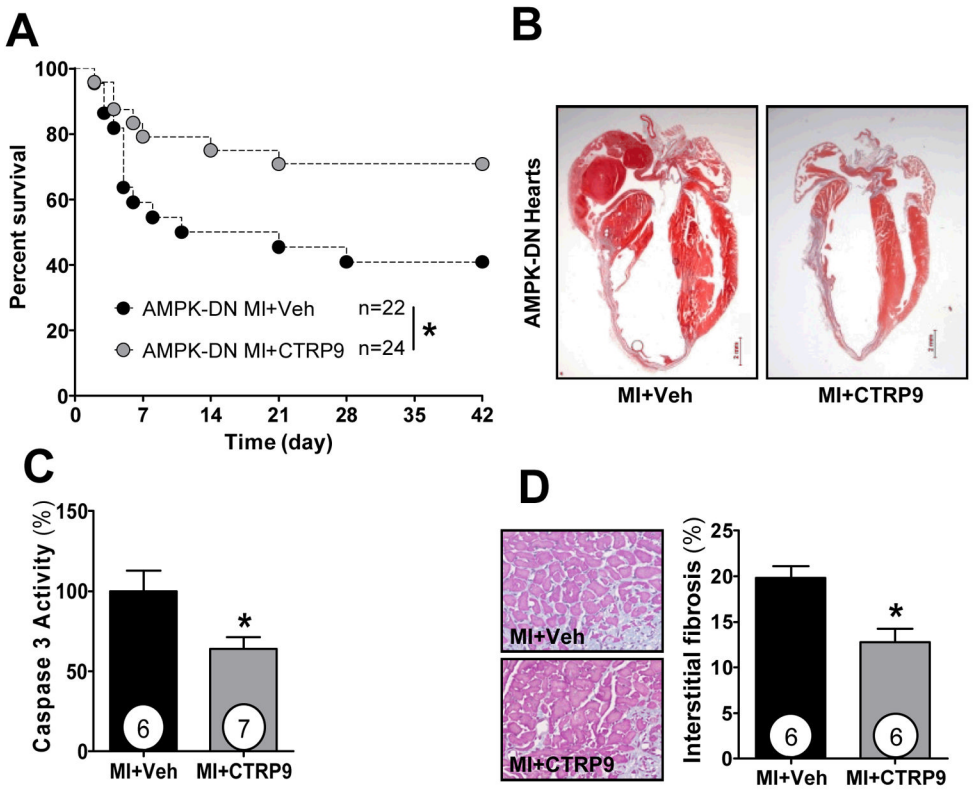

Figure 6.

Cardioprotective effects of CTRP9 are attenuated but not lost in cardiac-specific AMPK-DN mice. **(A)** Survival curves; **(B)** Representative heart images 6 weeks after AMI; **(C)** Caspase-3 activity; **(D)** Interstitial fibrosis from the border region of AMPK-DN hearts at 6 weeks. *P<0.05 vs. MI+Vehicle group.

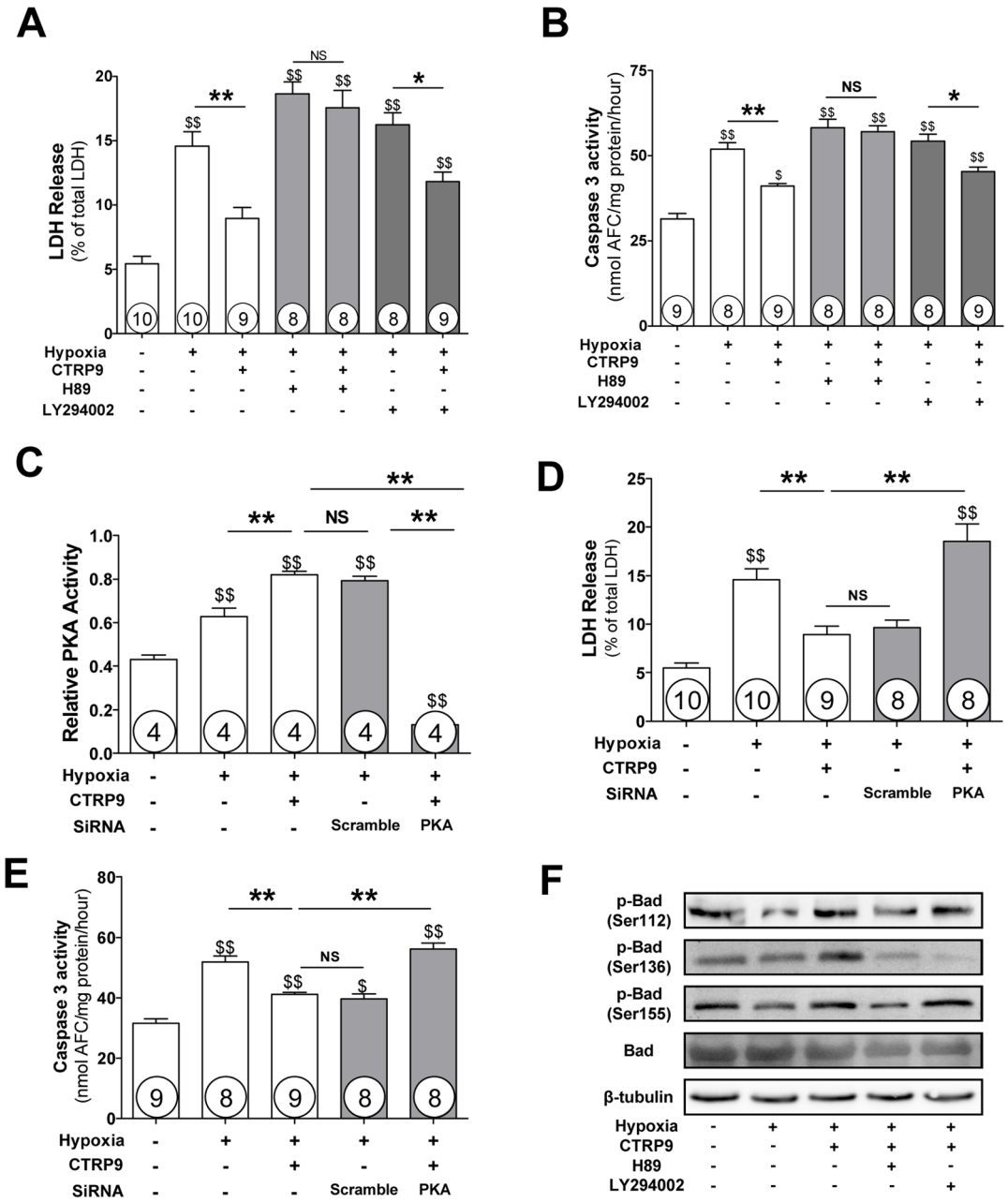

Figure 7.

Effect of PKA and Akt inhibition upon CTRP9's anti-apoptotic action. H9C2 cells were subjected to hypoxia or normoxia for 12 hours with or without CTRP9 (3 μg/ml) treatment. In some experiments, specific inhibitors of PKA (H89) or Akt (LY294002) were added in conjunction with CTRP9. **(A)** %LDH release; **(B)** Caspase-3 activity. Pretreatment with PKA-specific siRNA or control (scramble siRNA) for 48 hours commenced before hypoxia; **(C)** siRNA efficiency of PKA activity knockdown**; (D)** % LDH release; **(E)** Caspase-3 activity; **(F)** Western blot analysis for phospho-BAD at Ser112, Ser136, and Ser155 (1 hour post-hypoxia and CTRP9 treatment). Representative blots from at least 5 repeated experiments are shown. $Bonferroni-adjusted P<0.05, $$P<0.01 vs. Sham group (no hypoxia, no treatment); **P<0.01 between the two groups connected by the line.

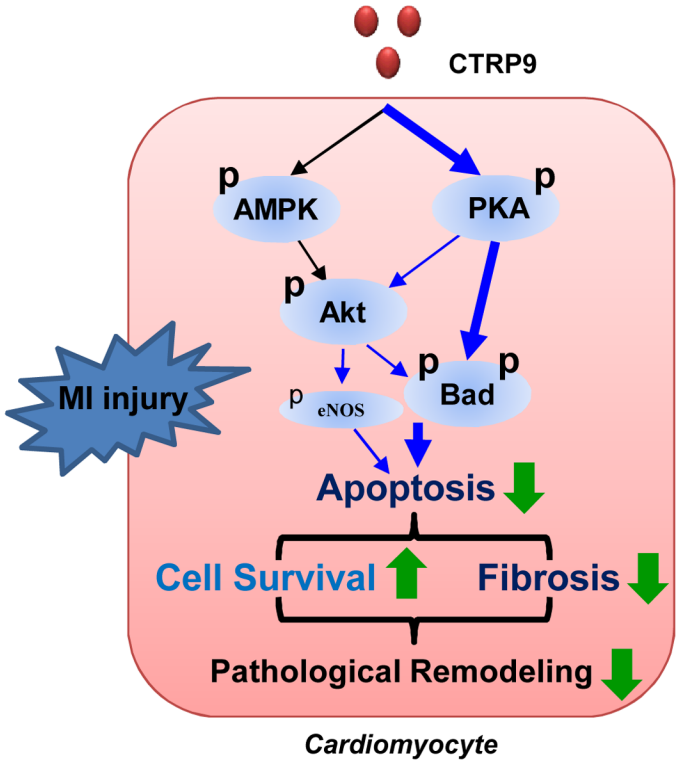

Figure 8.
Proposed mechanisms responsible for the cardioprotective actions of CTRP9 against adverse remodeling post-MI. Mechanisms denoted by thicker lines are emphasized for importance.