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C1q/tumor necrosis factor-related protein-3, a newly identified adipokine, is a novel antiapoptotic, proangiogenic, and cardioprotective molecule in the ischemic mouse heart.

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C1q/Tumor Necrosis Factor-Related Protein-3, a Newly Identified Adipokine, Is a Novel Antiapoptotic, Proangiogenic, and Cardioprotective Molecule in the Ischemic Mouse Heart

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Background—Obesity and diabetes mellitus adversely affect postischemic heart remodeling via incompletely understood mechanisms. C1q/tumor necrosis factor–related protein-3 (CTRP3) is a newly identified adipokine exerting beneficial metabolic regulation, similar to adiponectin. The aim of the present study was to determine whether CTRP3 may regulate postischemic cardiac remodeling and cardiac dysfunction, and, if so, to elucidate the underlying mechanisms.

Methods and Results—Male adult mice were subjected to myocardial infarction (MI) via left anterior descending coronary artery occlusion. Both the effect of MI on endogenous CTRP3 expression/production and the effect of exogenous CTRP3 (adenovirus or recombinant CTRP3) replenishment on MI injury were investigated. MI significantly inhibited adipocyte CTRP3 expression and reduced the plasma CTRP3 level, reaching a nadir 3 days after MI. CTRP3 replenishment improved survival rate ($P<0.05$), restored cardiac function, attenuated cardiomyocyte apoptosis, increased revascularization, and dramatically reduced interstitial fibrosis (all $P<0.01$). CTRP3 replenishment had no significant effect on cardiac AMP-activated protein kinase phosphorylation but significantly increased Akt phosphorylation and expression of hypoxia inducing factor-1α and vascular endothelial growth factor. Surprisingly, treatment of human umbilical vascular endothelial cells with CTRP3 did not directly affect nitric oxide production or tube formation. However, preconditioned medium from CTRP3-treated cardiomyocytes significantly enhanced human umbilical vascular endothelial cell tube formation, an effect blocked by either pretreatment of cardiomyocytes with a PI3K inhibitor or pretreatment of human umbilical vascular endothelial cells with a vascular endothelial growth factor inhibitor. Finally, the protective effect of adipocyte-conditioned medium against hypoxia-induced cardiomyocyte injury is significantly blunted when CTRP3 is knocked down.

Conclusion—CTRP3 is a novel antiapoptotic, proangiogenic, and cardioprotective adipokine, the expression of which is significantly inhibited after MI. (***Circulation***. 2012;125:3159-3169.)

Key Words: adiponectin ■ myocardial infarction ■ revascularization ■ signal transduction

Cardiovascular disease remains a leading cause of mortality worldwide. Although improved reperfusion strategies have led to declined death rates after acute myocardial infarction (MI), both the incidence and prevalence of post-MI heart failure have continually increased in recent years.[1] Despite pharmacological advances (eg, β-blockers, renin-angiotensin-aldosterone system inhibitors), mortality after MI remains very high, with 5-year rates being 30% to 70%. Left ventricular (LV) remodeling, which includes ventricular dilatation and increased interstitial fibrosis, is the critical process underlying the progression to heart failure. A novel approach preventing LV remodeling after MI is greatly needed.

Editorial see p 3066
Clinical Perspective on p 3169

As a result of the increased incidence of diabetes mellitus and its association with heart failure, there is now great

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interest in elucidating the underlying molecular mechanisms linking these pathologies. Since their original discovery, the important regulatory role of adipose-derived hormones and cytokines (adipokines) in myocardial function has gained recognition. Considerable evidence exists that the majority of adipokines (including tumor necrosis factor [TNF]-α, leptin, plasminogen activator inhibitor type 1, transforming growth factor-β, and resistin) adversely regulate myocardial metabolism, cardiomyocyte hypertrophy, extracellular matrix structure and composition, and cell death.[2] Despite having been consistently shown to increase cardiomyocyte glucose uptake, to stimulate fatty acid oxidation, and to protect against acute myocardial ischemia/reperfusion injury,[3] the effect of adiponectin on chronic cardiac remodeling and heart failure development remains controversial.[4] Whether any adipokine exerts beneficial regulatory effect on postischemic cardiac remodeling, improving cardiac function, remains unknown.

Recently, a highly conserved family of adiponectin paralogs, designated C1q/TNF-related proteins (CTRPs), was discovered. Each of the 10 known members (CTRP1–CTRP10) consists of 4 distinct domains, including an N-terminal signal peptide, a short variable domain, a collagen-like domain, and a C-terminal C1q-like globular domain.[5,6] Both CTRPs and adiponectin belong to the C1q/TNF protein superfamily, which continues to grow as more C1q domain proteins are discovered.[7] Investigated for its structural similarity to adiponectin, the CTRP family members exhibit broadly diverse functions. CTRP3 (also known as cartducin) is a growth plate secretory protein of primarily cartilaginous origin but has also been reported in monocytic and osteosarcoma cells.[8] Initially identified during a search for genes responsible for chondrocyte differentiation induction, CTRP3 also stimulates adiponectin and resistin release and therefore serves as a regulator of adiponectin secretion from adipocytes.[9,10] Most important, CTRP3 is the first and only CTRP for which the in vivo biological function (as a metabolic regulator of glucose homeostasis) has been recently established.[11]

Interestingly, CTRP3 has been reported to stimulate in vitro endothelial cell proliferation and migration.[12] However, whether CTRP3 might promote in vivo revascularization in the infarct border zones after MI is unknown. Moreover, whether CTRP3, a key member of the newest adipokine family, may function as a mediator or inhibitor of post-MI remodeling has never been previously investigated. Therefore, the aims of this study were (1) to determine the effect of MI on CTRP3 expression/production, (2) to investigate the effect of CTRP3 replenishment on postischemic cardiac remodeling and dysfunction, (3) to determine whether CTRP3 administration might promote revascularization after MI, and (4) to elucidate the mechanisms responsible for the cardiac biological actions of CTRP3.

Methods

All 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.

Experimental Protocols

Adult male C57/BL6 mice were anesthetized with 2% isoflurane, and MI was induced via left anterior descending coronary artery ligation as previously described.[13] Sham-operated control mice (sham) underwent the same surgical procedures except that the suture placed under the left anterior descending was not tied. After full recovery from cardiac surgery (4 hours after MI), animals were randomized to one of the following groups: sham+vehicle, sham+CTRP3, MI+vehicle, MI+CTRP3 (0.25 μg $\cdot$ g^{-1} $\cdot$ d^{-1} intraperitoneal infusion via mini-osmotic pump, ALZET; DURECT Corp, Cupertino, CA). After 2 weeks, mice were euthanized. Immunohistological, biochemical, and Western blot assays were performed as previously reported[14] and described in detail in the online-only Data Supplement.

Construct and Expression of Globular CTRP3

Globular domain of mouse CTRP3 gene was generated by polymerase chain reaction and cloned into the prokaryotic protein expression vector pET45b (Novagen, Merck, USA). The construct was verified by DNA sequencing. Globular CTRP3 prokaryotic expression vector was transferred into BL21 (DE3) bacterium protein expression host, grown in LB medium, and shaken overnight at 37°C. Protein expression inducer IPTG (isopropyl-β-D-thio-galactoside) was added to the medium (final concentration, 1 mmol/L). The solution was shaken for 4 to 5 hours and subjected to 5000-rpm centrifugation. Proteins were purified in native condition by Ni-NTA resin per the manufacturer's instructions (Novagen, No. 70666–3, Merck, USA). Endotoxin was removed by an endotoxin-removing column (Acti-CleanEtoc resin, Sterogene, Carlsbad, CA). Proteins were desalted and concentrated by centrifugation (Millipore-Centricon Plus-20). Purified proteins were examined by Western blot analysis (Figure IA and IB in the online-only Data Supplement), and the endotoxin activity was determined by use of the LAL assay kit (BioWhittaker, Walkersville, MD) according to the manufacturer's recommendation (27.6±3.9 EU/mg protein).

Adenoviral Vector Production and Transfection

Adenovirus expressing human full-length CTRP3 was constructed by SinoGenoMax Co, Ltd (Beijing, China). Briefly, pShuttle-GFP-CTRP3 was constructed by cloning the target gene CTRP3 into pShuttle-GFP-CMV. After sequence confirmation, pShuttle-GFP-CTRP3 was transferred into the recombinant adenovirus frame vector of pADxsi. The pADxsi-GFP-CTRP3 adenovirus vector was then amplified in 293 cells, and the viral titer was measured. Control vectors (*Ad.Null*) not expressing CTRP3 but β-galactosidase were constructed and produced concomitantly. Then, 2×10^8 plaque-forming units of *Ad.CTRP3* or *Ad.Null* was injected into the jugular vein of mice 3 days before MI.

Statistical Analysis

Data were analyzed with GraphPad Prism-5 statistic software (La Jolla, CA). All values in the text and figures are presented as the mean±SEM of n independent experiments. One-way ANOVA was conducted across all investigated groups first. Post hoc tests were then performed with Bonferroni correction, and all 2-group comparisons were made. Data presented in Figure 5A were determined by 2-way ANOVA followed by post hoc tests with Holm adjustment. Survival data were analyzed by the Kaplan-Meier method followed by the log rank test. Western blot densities were analyzed by the Kruskal-Wallis test followed by the Dunn post hoc test. Values of $P \leq 0.05$ were considered statistically significant.

All authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

MI Inhibits Adipocyte CTRP mRNA Expression and Decreases Plasma CTRP3 Level

Clinical and experimental studies have demonstrated that plasma adiponectin levels are significantly reduced after MI. However,
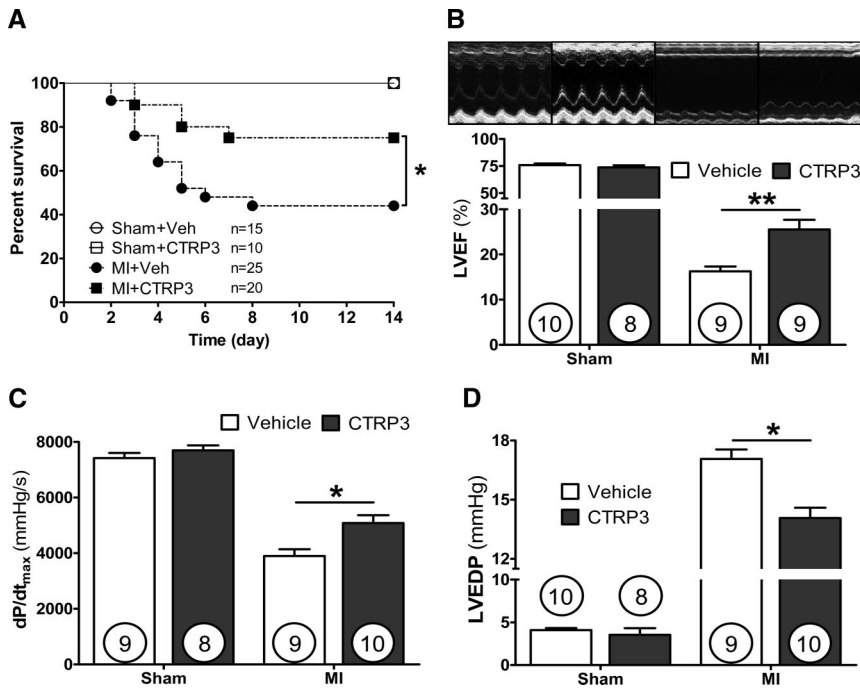
Figure 1. C1q/tumor necrosis factor–related protein-3 (CTRP3) improves survival and restores left ventricular cardiac function after myocardial infarction (MI). The effects of CTRP3 treatment on survival and left ventricular function were determined 14 days after operation. Wild-type mice were subjected to MI or sham operation (Sham) and treated with CTRP3 or saline as vehicle (Veh). **A**, Survival curve. **B**, Echocardiographic analysis. **C** and **D**, Hemodynamic analysis. LVEF indicates left ventricular ejection fraction; LVEDP, LV end-diastolic pressure. *$P<0.05$, **$P<0.01$.

the effect of MI on CTRP expression has not previously been determined. Adipocyte CTRP3 mRNA expression significantly decreased 1 day after MI and gradually recovered thereafter (Figure IIA in the online-only Data Supplement). Consequently, plasma CTRP3 levels (determined by Western blot densitometry analysis) were significantly decreased after MI, reaching a nadir 3 days after MI and gradually recovering thereafter (Figure II in the online-only Data Supplement, solid line). These results demonstrate that CTRP3 expression/production is regulated during post-MI cardiac remodeling.

Replenishment of CTRP3 Improves Survival and Restores LV Cardiac Function After MI

Having demonstrated that adipocyte CTRP3 expression is inhibited and that plasma CTRP3 levels are reduced in MI animals, we attempted to determine whether post-MI CTRP3 reduction is pathologically relevant and whether supplementation of exogenous CTRP3 may protect the heart against post-MI remodeling. As illustrated in Figure IIB in the online-only Data Supplement, administration of recombinant CTRP3 via intraperitoneal osmotic pump (dose, 0.25 $\mu g \cdot g^{-1} \cdot d^{-1}$) caused an $\approx$1.5-fold CTRP3 increase in sham-operated mice (open triangles connected with dashed lines) and prevented MI-induced plasma CTRP3 decline (open circles connected with dashed lines). Administration of CTRP3 in sham-operated mice had no effect on cardiac function assessed by echocardiography or ventricular catheterization. However, CTRP3 administration significantly improved the post-MI survival rate (Figure 1A), augmented the LV ejection fraction (Figure 1B), increased dP/dtmax (Figure 1C), and decreased the LV end-diastolic pressure (Figure 1D). These data demonstrate that pharmacological restoration of plasma CTRP3 to physiological levels improved both LV systolic and diastolic function in MI animals and increased the survival rate.

CTRP3 Prevents LV Cardiac Remodeling After MI

Because pathological remodeling plays a critical role in postischemic cardiac dysfunction, we determined indexes of remodeling by gross anatomy, echocardiography, and Masson trichrome staining 14 days after MI. Compared with MI+vehicle, CTRP3 administration reduced heart size and mass (Figure 2A and 2B) and preserved LV end-diastolic and LV end-systolic dimension (Figure 2C and 2D). Most noticeably, CTRP3 treatment dramatically increased the ratio of myocytes to fibrotic cells in the ischemic zone (Figure 3A), significantly attenuated interstitial fibrosis (Figure 3B), reduced transforming growth factor-β1 expression, and decreased cardiomyocyte size in remote nonischemic zone (Figure 3C and 3D). Together, these results suggest that endogenous CTRP3 may play a significant role in regulating cardiac remodeling in post-MI hearts and that recombinant CTRP3 may have therapeutic potential against MI injury.

CTRP3 Promotes Angiogenesis and Activates the Akt–Hypoxia-Inducing Factor-1α–Vascular Endothelial Growth Factor Axis in Infarct Border Zone

Angiogenesis is a critical step initiating cardiac repair after MI. Having demonstrated that CTRP3 is a novel adipokine potently inhibiting fibrosis and strongly augmenting cardiomyocyte survival, we assessed the angiogenic effects of CTRP3 as a potential underlying mechanism. As illustrated in Figure 4A, CTRP3 treatment markedly increased the number of CD31-positive capillary vessels and increased α-smooth muscle active–positive arterial density (Figure 4B) in the border zone 14 days after MI, suggesting that CTRP3 induces mature vessel formation after MI.

To further determine the molecular signaling mechanisms responsible for increased angiogenesis after CTRP3 treatment,
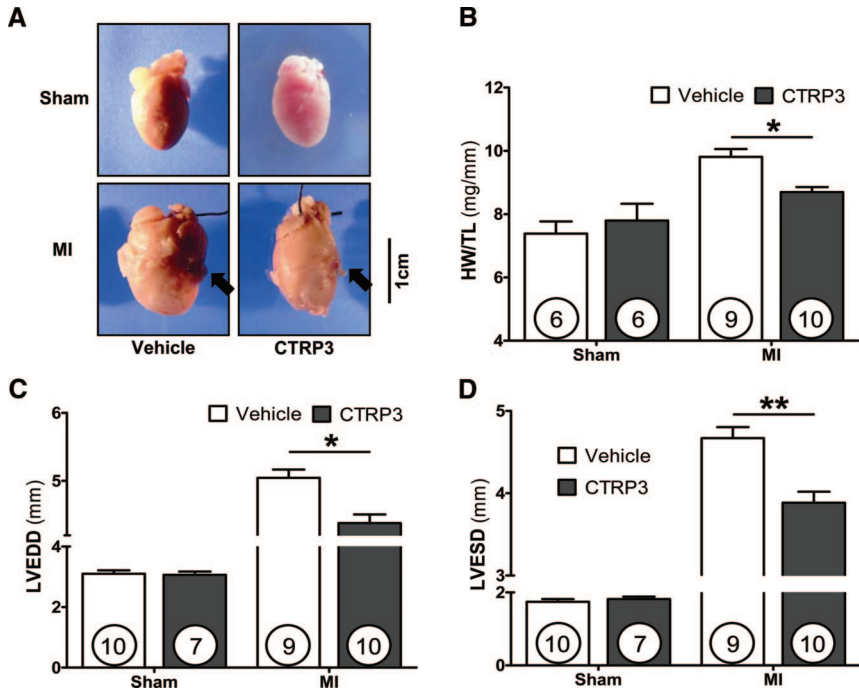
Figure 2. C1q/tumor necrosis factor–related protein-3 (CTRP3) prevents left ventricular (LV) cardiac remodeling after myocardial infarction (MI). **A,** Gross observation. **B,** Ratio of heart weight to tibia length (HW/TL). **C** and **D,** LV end-diastolic and end-systolic dimensions (LVEDD and LVESD) from echocardiographic analysis. *$P<0.05$, **$P<0.01$.

several critical mediators and cytokines requisite for angiogenesis were assessed. CTRP3 had no significant effect on AMP-activated protein kinase phosphorylation but significantly enhanced Akt phosphorylation and increased hypoxia-inducing factor-1α (HIF1α) and vascular endothelial growth factor (VEGF) expression (Figure 4C– 4F). These results suggest that CTRP3 promotes angiogenesis after MI possibly through the Akt-HIF1α-VEGF axis.
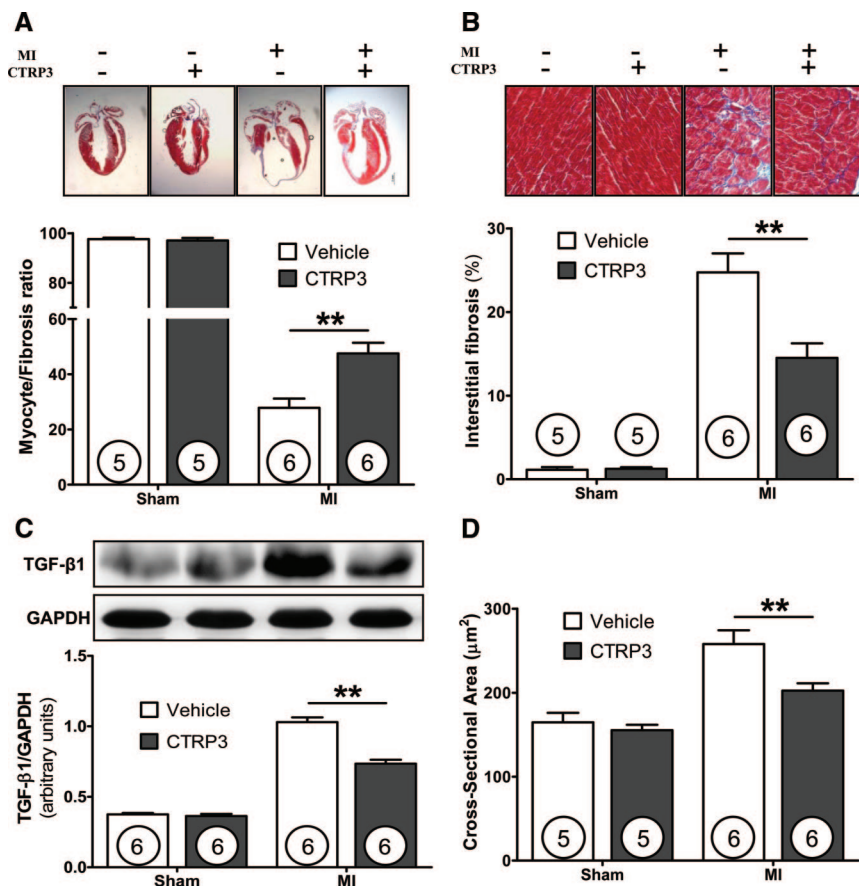

Figure 3. C1q/tumor necrosis factor–related protein-3 (CTRP3) attenuates left ventricular cardiac fibrosis after myocardial infarction (MI). **A,** Cardiac Masson trichrome staining. **B,** Interstitial fibrosis. **C,** Western blot analysis for transforming growth factor-β1 (TGF-β1) expression. **D,** Cross-sectional area of cardiomyocytes in the remote area. **$P<0.01$.
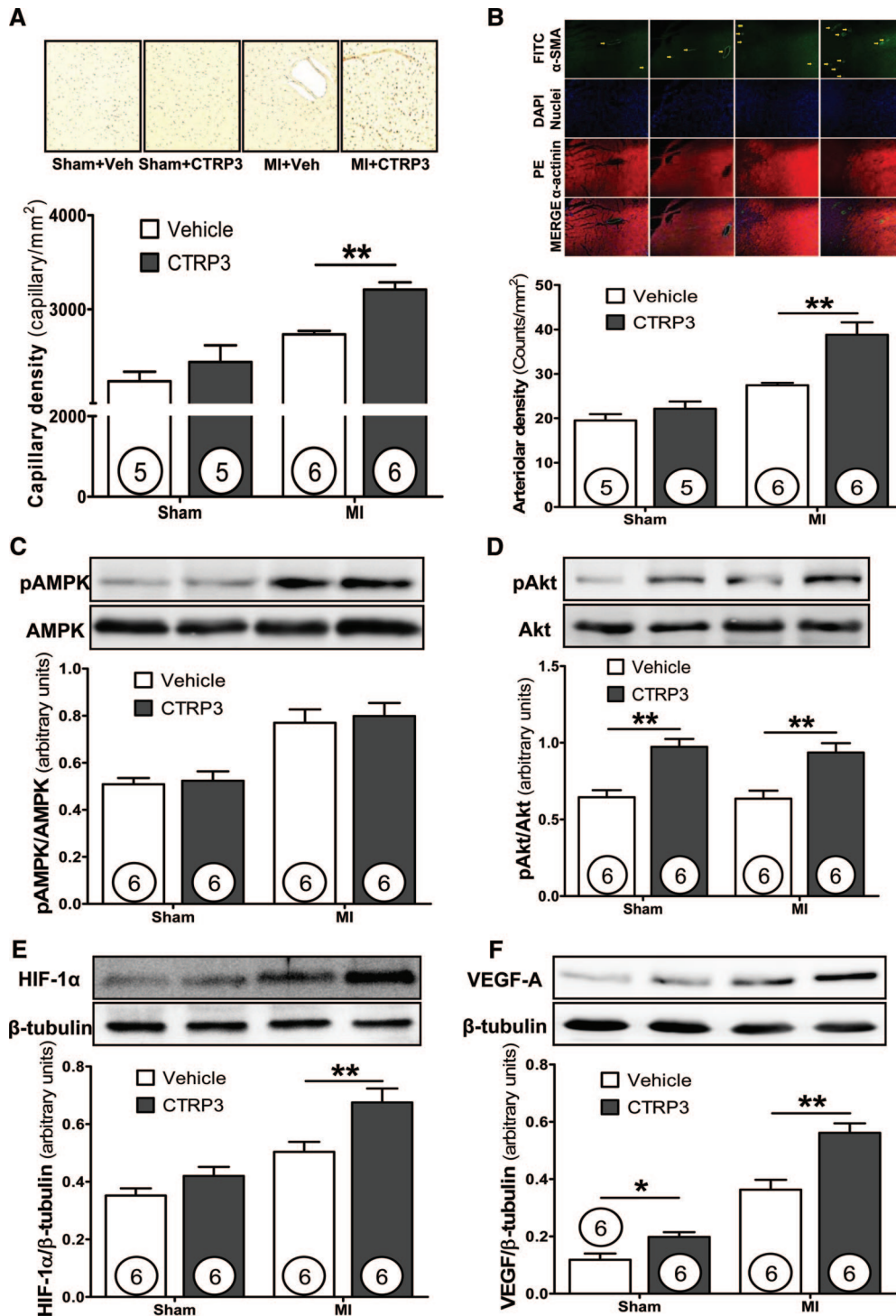
Figure 4. C1q/tumor necrosis factor–related protein-3 (CTRP3) promotes angiogenesis and activates the Akt-hypoxia-inducible factor-1α–vascular endothelial growth factor (HIF1α-VEGF) axis in the infarct border zone. (**A**) Capillary density measured by immunohisto-chemical staining for CD31 (brown) in the border region (myocardial infarction [MI] group) or left ventricular (LV) free wall (sham group). **B**, Immunostaining for α-smooth muscle actin (α-SMA; green) and actinin (red) shown together with DAPI (blue) staining in the border region (MI group) or LV free wall (sham group) 14 days after operation. Quantity of α-SMA–positive vessels within the infarct border zone is summarized by the bar graph. **P<0.01. Western blot analysis for phosphorylated (p) AMP-activated protein kinase (AMPK) (Thr172)/AMPK (**C**), pAkt(Ser473)/Akt (**D**), HIF1α (**E**), and VEGF-A (**F**). *P<0.05, **P<0.01.

Adenoviral Supplementation of CTRP3 Significantly Attenuates LV Cardiac Remodeling

Recognizing that the globular domain of CTRP3 induced angiogenesis and prevented LV cardiac remodeling, we further determined the effect of adenoviral production of full-length CTRP3 on adverse LV cardiac remodeling. CTRP3-expressing adenovirus infection resulted in an ≈1.9-fold increase in plasma CTRP3 before MI and maintained plasma CTRP3 at significantly higher levels than seen in *Ad.Null*-treated animals throughout the observation period
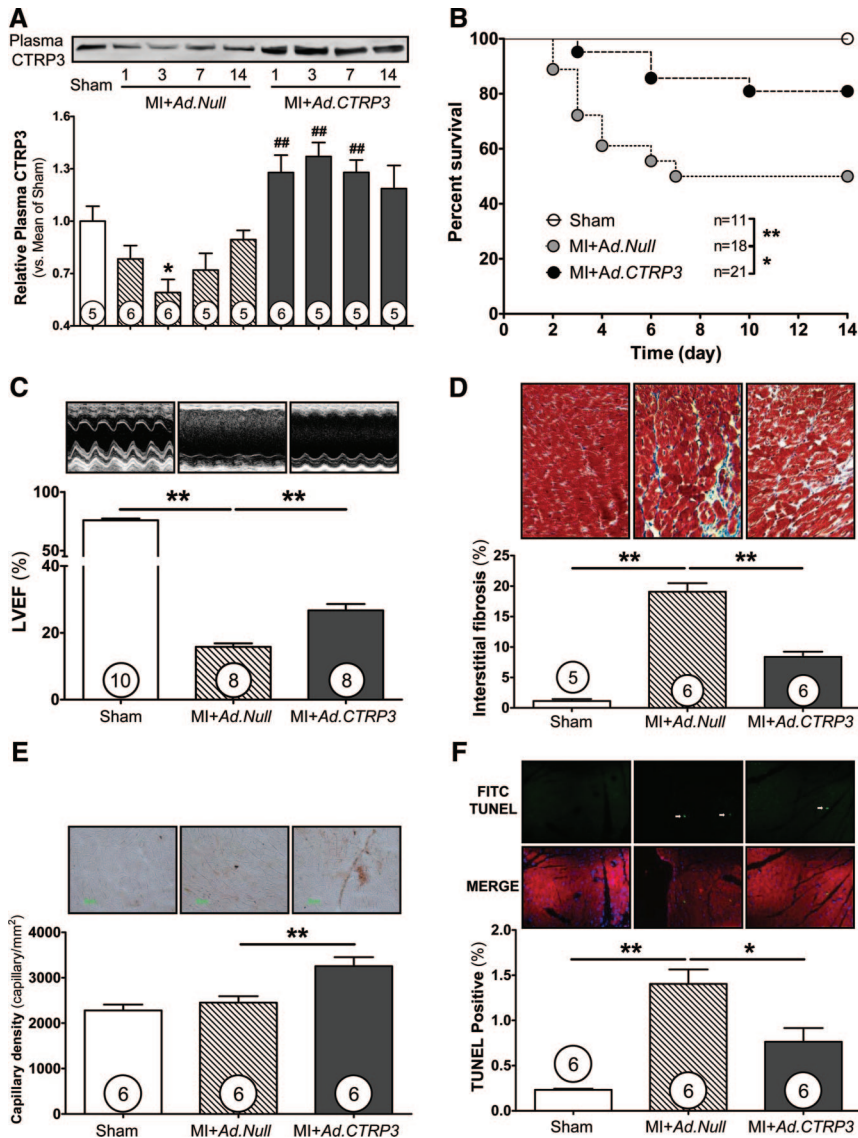
Figure 5. C1q/tumor necrosis factor–related protein-3 (CTRP3) adenovirus improves survival, restores cardiac function, and attenuates left ventricular (LV) cardiac remodeling. Ad.CTRP3 or Ad.Null was injected into the jugular vein of mice 3 days before myocardial infarction (MI). Effects of adenoviral CTRP3 supplement on plasma CTRP3 level by Western analysis (**A**), survival rate by survival curve analysis (**B**), LV function by echocardiographic analysis (**C**), and cardiac remodeling by interstitial fibrosis (**D**), angiogenesis (**E**), and cardiomyocyte apoptosis (**F**) were determined 14 days after MI. LVEF indicated LV ejection fraction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling. $*P<0.05$, $**P<0.01$.

(Figure 5A). CTRP3 overproduction markedly improved survival rate (Figure 5B) and restored left cardiac function (Figure 5C). Moreover, CTRP3-expressing adenovirus significantly attenuated interstitial fibrosis in the remote area (Figure 5D), increased CD31-positive capillary density (Figure 5E), and ameliorated terminal deoxynucleotidyl transferase dUTP nick-end labeling and α-actinin double-positive apoptotic cardiomyocyte death in the border zone (Figure 5F). Finally, transfection of neonatal cardiomyocytes with adenoviral CTRP3 activated Akt and upregulated HIF1α expression in vitro (Figure III in the online-only Data Supplement).

CTRP3 Does Not Significantly Promote Angiogenesis in Cultured Human Umbilical Vascular Endothelial Cells

Endothelial cells are essential in angiogenesis and postischemic cardiac repair. To directly investigate the effect of CTRP3 on endothelial cells, we determined the effect of CTRP3 on tube formation in cultured human umbilical vascular endothelial cells (HUVECs) with VEGF as a posi-

tive control. Somewhat to our surprise, although in vivo administration of CTRP3 significantly stimulated angiogenesis, direct HUVEC treatment with CTRP3 (both gCTRP3 and full-length CTRP3) failed to significantly promote tube formation (Figure 6A) or stimulate nitric oxide (determined as previously described[14]) production (Figure 6B). Moreover, although in vivo CTRP3 treatment significantly increased Akt phosphorylation and increased HIF1α and VEGF expression in the ischemic heart, in vitro treatment of HUVECs with CTRP3 did not increase Akt phosphorylation or HIF1α or VEGF expression (Figure 6C–6G). These results indicate that the proangiogenic effect of CTRP3 observed in vivo cannot be attributed to its direct effect on endothelial cells.

CTRP3 Promotes Angiogenesis via a Cardiomyocyte-Initiated, Akt-Dependent Pathway

Recognizing that CTRP3 induced angiogenesis in vivo but not in endothelial cells in vitro, we considered the hypothesis that cardiomyocytes, in response to CTRP3 stimulation, might secrete factors promoting angiogenesis in endothelial cells (cardiomyocyte–endothelial cell communication). To
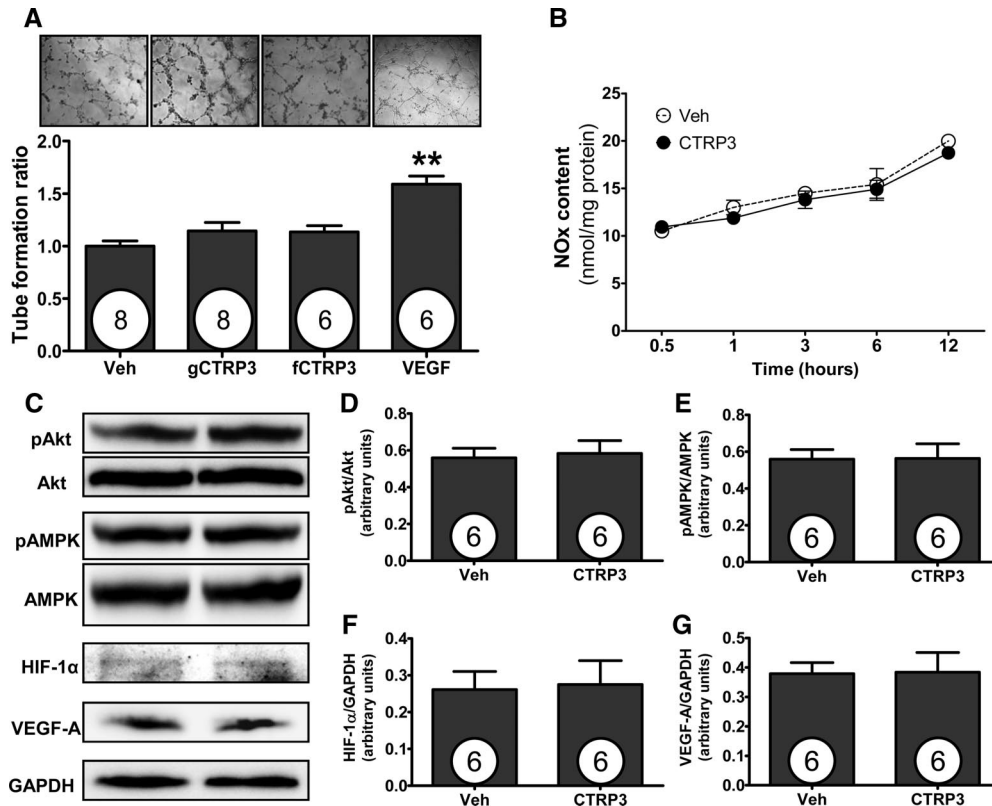
Figure 6. C1q/tumor necrosis factor–related protein-3 (CTRP3) does not significantly promote angiogenesis in cultured human umbilical vascular endothelial cells (HUVECs). **A**, Tube formation assay. HUVECs were treated with globular domain of CTRP3 (gCTRP3; 3 μg/mL), full length CTRP3 (fCTRP3; 30 μg/mL), vehicle, or vascular endothelial growth factor (VEGF; positive control) for 6 hours. Representative experiment data are shown (n=6 per condition). **P<0.01 vs vehicle. **B**, Nitric oxide (NOx) formation assay. HUVECs were treated with CTRP3 (3 μg/mL) or vehicle for 12 hours. NOx formation was analyzed after 0.5, 1, 3, 6, and 12 hours as described in our previous studies. **C** through **G**, Western blot analysis for pAkt(Ser473)/Akt, phosphorylated (p) AMP-activated protein kinase (AMPK) (Thr172)/AMPK, hypoxia-inducible factor-1α (HIF1α), and VEGF from cultured HUVECs treated with CTRP3 directly.

test this hypothesis, adult mouse cardiomyocytes were isolated as described in our previous study[15] and treated with CTRP3 in vitro for various periods (6, 12, and 24 hours). The conditioned medium from CTRP3-treated cardiomyocytes was then administered to cultured HUVECs for 6 hours. As illustrated in Figure 7A, HUVEC treatment with conditioned medium significantly enhanced HUVEC tube formation, suggesting that CTRP3 invokes an angiogenic response by inducing cardiomyocyte-secreted paracrine factors. Furthermore, CTRP3 upregulated levels of phosphorylated Akt, HIF1α, and VEGF in cultured cardiomyocytes in both a time-dependent (Figure 7B and 7C) and dose-dependent (Figure 7D) manner. These effects were not inhibited by pretreatment with polymixin B (Figure IV in the online-only Data Supplement). Additionally, blocking cardiomyocyte Akt activation with a PI3-kinase inhibitor (LY294002) abolished CTRP3-induced HIF1α and VEGF upregulation (Figure 7E) and eliminated HUVEC tube formation induced by the conditioned medium (Figure 7A, third bar). Finally, HUVEC tube formation induced by the conditioned medium from CTRP3-treated cardiomyocytes was significantly suppressed by the VEGF receptor antagonist CBO-P11 (12 μmol/L, Calbiochem) administered directly to HUVECs (Figure 7A, last bar).

The in vitro experimental results presented above demonstrate that CTRP3 enhances HUVEC tube formation by

promoting cardiomyocyte/endothelial cell communication involving Akt-HIF1α-VEGF signaling. To determine whether this signaling pathway is also responsible for CTRP3 cardioprotection observed in vivo, LY294002 (to inhibit PI3-kinase Akt axis) or CBO-P11 (to inhibit VEGF) was administered together with CTRP3 via a mini-osmotic pump, and cardiac function and capillary density were determined 14 days after MI. As summarized in Figure 7F, CTRP3-induced capillary formation was completely abolished when either Akt or VEGF was inhibited. However, the cardiac protective effect of CTRP3 (determined by LV ejection fraction) was completely blocked by LY294002 and partially blocked by CBO-P11 (Figure 7G). These results suggest that although the cardioprotective effect of CTRP3 is mediated largely by Akt-HIF1α-VEGF signaling–induced angiogenesis, other mechanisms also contribute to the cardioprotection of CTRP3 against MI.

Endogenous CTRP3 Produced Under Physiological Conditions Is Cardioprotective

In a final attempt to obtain more evidence that CTRP3 is an endogenous cardioprotective adipokine, we performed an additional in vitro experiment as recently reported.[16] 3T3-L1 fibroblasts (ATCC, Manassas, VA) were maintained per the manufacturer's protocol and transfected with CTRP3 siRNA or scramble siRNA. Forty-eight hours after transfection, cells
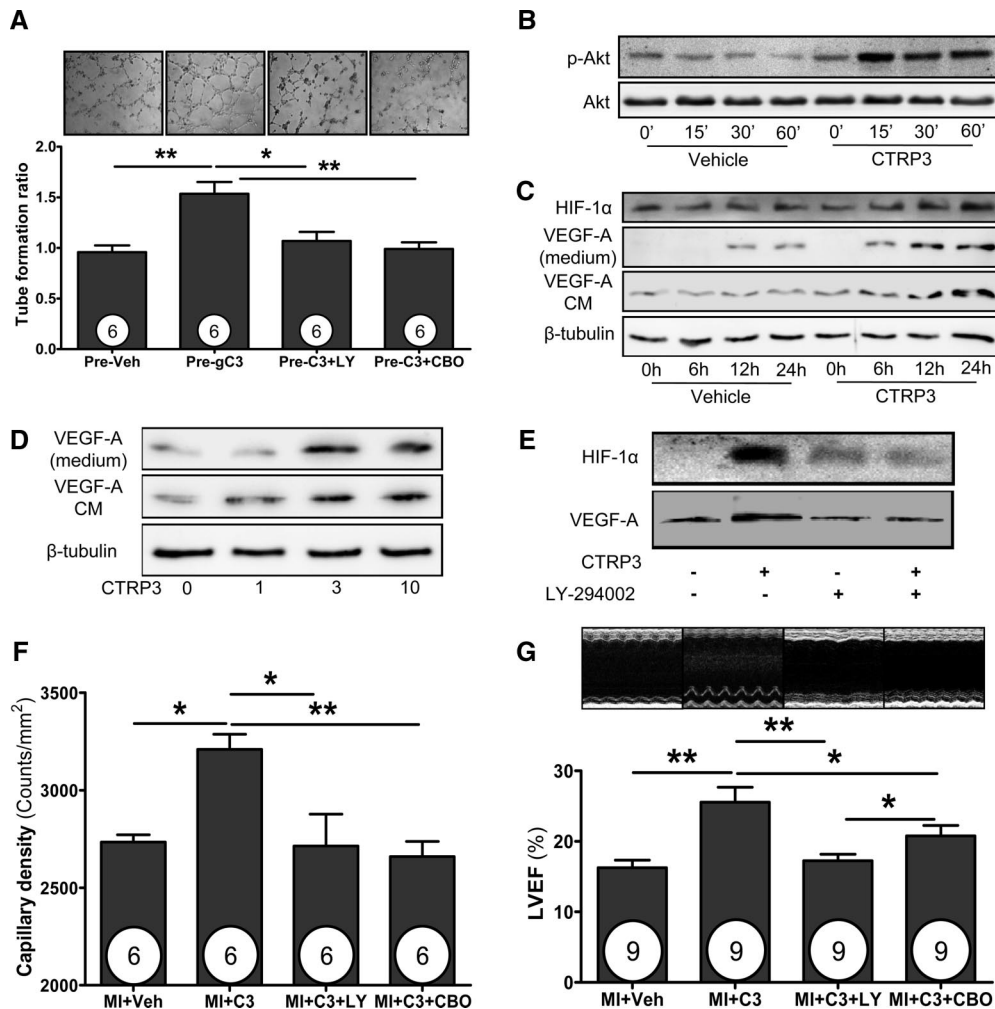
Figure 7. C1q/tumor necrosis factor–related protein-3 (CTRP3) promotes angiogenesis via a cardiomyocyte-mediated, Akt-dependent signaling mechanism in vitro. **A**, Tube formation assay. Isolated adult cardiomyocytes were pretreated with CTRP3 (3 μg/mL) or saline as vehicle for 12 hours. Human umbilical vascular endothelial cells (HUVECs) were treated with CTRP3-pretreated conditioned medium (Pre-C3) or saline-pretreated medium (Preveh). Specific inhibitors of PI3-K (LY294002) and vascular endothelial growth factor (VEGF; CBO-P11) were added to culture medium of cardiomyocytes or HUVECs, respectively. Western blot analysis showing time-dependent activation of cardiac Akt (**B**) and cardiac expression of hypoxia-inducible factor-1α (HIF1α) and VEGF in both cardiomyocytes (CMs) and culture medium (**C**) after treatment with CTRP3 or vehicle. **D**, Western blot analysis showing a dose-dependent increase in VEGF in both CMs and culture medium (n=3). **E**, Western blot analysis showing that CTRP3-induced cardiomyocyte HIF1α and VEGF expression was blocked by LY294002. **F**, Capillary density measured by CD31 immunohistochemical staining in the border region. Results show that CTRP3-induced capillary formation in vivo is abolished by coadministration of LY294002 or CBO-P11. **G**, Echocardiographic analysis showing that cardiac functional improvement by CTRP3 is completely blocked by LY294002 (PI3-kinase/Akt signaling inhibition) and significantly inhibited by VEGF inhibition. LVEF indicates left ventricular ejection fraction. *$P<0.05$, **$P<0.01$.

were washed and cultured for an additional 6 hours. Conditioned medium from 3T3-L1 cells was then collected. Adult cardiomyocytes were isolated as described previously[15]; incubated with regular control culture medium or conditioned medium from control (no siRNA), scramble, or CTRP3 siRNA pretreated 3T3-L1 cells; and exposed to normoxic (20% oxygen) or hypoxic (2% oxygen) conditions. Lactate dehydrogenase concentration in culture medium and caspase-3 activity in cardiomyocytes were determined 12 hours after normoxia/hypoxia incubation. Compared with regular control medium, hypoxia-induced lactate dehydrogenase release (Figure 8A) and caspase-3 activation (Figure 8B) were significantly attenuated in cardiomyocytes cultured with conditioned medium from 3T3-L1 cells (second versus fourth bar). Pretreatment of 3T3-L1 cells with scramble siRNA did

not alter the protective effect offered by adipocyte conditioned medium (fourth versus fifth bar in Figure 8A and 8B). However, the protective effect conferred by adipocyte-conditioned medium was significantly blunted (last bars in Figure 8A and 8B) when cardiomyocytes were cultured with conditioned medium from CTRP3-knockdown 3T3-L1 cells ($72\pm2.2\%$ reduced CTRP3 protein expression; Figure IC in the online-only Data Supplement). These results indicate that CTRP3 deficiency tilts the balance between cytoprotective and cytotoxic molecules produced by 3T3-L1 cells, indicating that CTRP3 is an endogenous cardioprotective adipokine.

Discussion

Our study presents several important observations. To the best of our knowledge, we demonstrate for the first time that
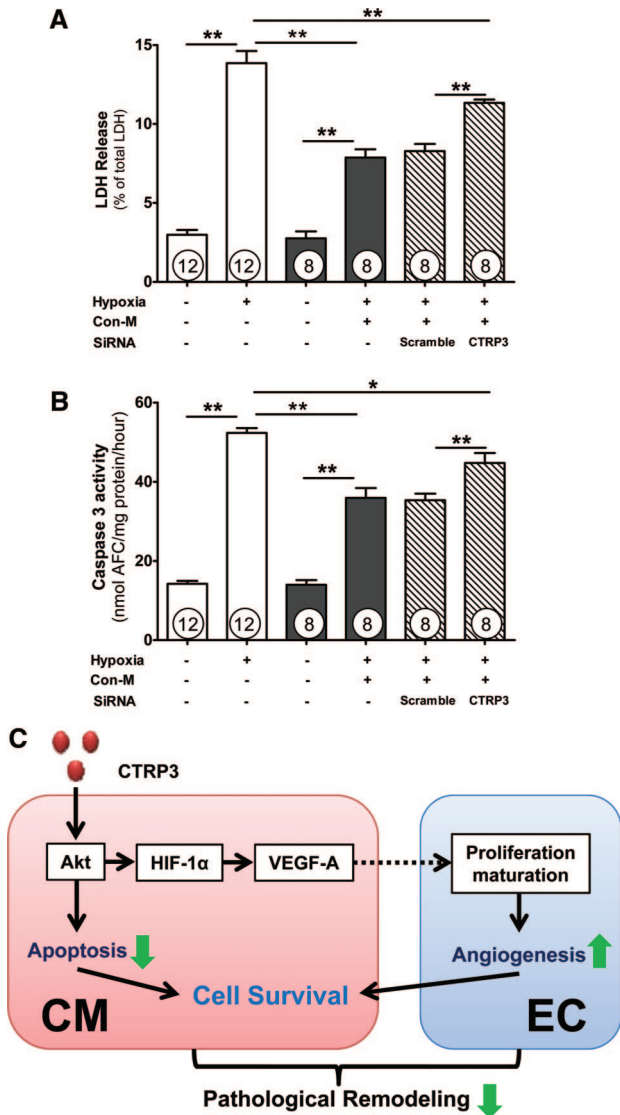
Figure 8. Endogenous C1q/tumor necrosis factor–related protein-3 (CTRP3) produced under physiological conditions is cardioprotective. Lactate dehydrogenase (LDH) release (**A**) and caspase-3 activity (**B**) from adult cardiomyocytes (cultured with regular culture medium, adipocyte-conditioned medium with or without CTRP3 knockdown) exposed to normoxia or hypoxia (2% O_2 for 12 hours). Con-M indicates conditioned medium; EC, endothelial cell. *$P<0.05$, **$P<0.01$. **C**, Proposed mechanism responsible for the cardioprotective effects of CTRP3 after myocardial infarction (MI).

expression and production of CTRP3, a key member of a newly identified adipokine family, are significantly reduced after MI; (2) that replenishment of CTRP3 improves survival and restores cardiac function after MI; (3) that CTRP3 attenuates postischemic pathological remodeling, as evidenced by reduced heart size, preserved LV chamber dimensions, decreased remote-area interstitial fibrosis, increased ischemic regional cardiomyocyte survival/regeneration, and enhanced infarct border-zone revascularization and reduced apoptosis; and (4) that CTRP3 exerts its angiogenic effect by promoting cardiomyocyte–endothelial cell communication involving Akt-HIF1α-VEGF signaling.

The extent of tissue loss in the acute phase after MI is a major determinant of the resultant cardiac dysfunction de-

gree. However, chronic processes such as extracellular matrix turnover, fibrosis, and inflammation stimulate adverse remodeling and are crucial determinants in the transition from compensatory cardiac hypertrophy to decompensatory heart failure.[17] Many cytokines have been reported either to mediate post-MI adverse remodeling or to prevent cardiac remodeling and dysfunction. It is thus widely accepted that the degree of post-MI cardiac remodeling is critically dependent on the balance between detrimental and protective cytokines.[18] As a result of the increased incidence of diabetes mellitus and its association with heart failure, the role of adipocyte-derived cytokines (adipokines) in the development of postischemic remodeling has attracted great attention.[2] The effects of leptin and adiponectin, among many adipokines examined, on cardiac remodeling have been investigated extensively in recent years. The majority of published studies demonstrate the opposing effects of these adipokines on postischemic remodeling, and the balance between detrimental leptin and protective adiponectin is critically influential on post-MI heart failure development.[19,20] In patients with type 2 diabetes mellitus, the balance is disturbed, with markedly increased plasma leptin levels and significantly reduced plasma adiponectin levels favoring adverse cardiac remodeling.[2] Therefore, identifying cardioprotective adipokines and augmenting their production are scientifically and clinically imperative. In the present study, we have provided the first direct evidence that CTRP3 is an adipokine possessing strong antiremodeling and cardioprotective properties, suggesting that CTRP3 might be a novel therapeutic target attenuating postischemic cardiac remodeling, thereby improving cardiac function.

Angiogenesis represents an excellent therapeutic modality for ischemic heart disease treatment. Many preclinical studies have been performed with gene-, cell-, and protein-based therapies.[17] Gene therapy, once regarded as a potential advanced treatment of choice for cardiovascular disease, remains impractical for regular clinical application because of many serious, unsolved problems. Cell-based proangiogenic therapies linger in early research stages, hindered by numerous unresolved questions regarding best usable cell types and dosages. The failure of gene- or cell-based therapeutics to deliver, as of yet, a suitable treatment choice for diseases stemming from poor blood flow has led to a resurgence of interest in returning to protein-based therapies stimulating angiogenesis.

Among many different growth factors implicated in angiogenesis, VEGF and fibroblast growth factor are the 2 most intensively investigated growth factors. Numerous animal model experimental studies have demonstrated that VEGF or fibroblast growth factor treatment stimulates angiogenesis with great efficacy, increasing capillary quantity and alleviating postischemic remodeling.[21] Reproducible and credible successes in these early animal studies garnered enthusiastic expectations that new therapeutic approaches could be rapidly translated for clinical benefit in millions of MI patients. Unfortunately, despite the optimistic early data of many pioneering clinical trials, recent larger and better-designed clinical trials have reported one disappointment after another.[22–24] These failures suggest incorrectly selected molec-

ular targets inducing neovascularization, improper therapeutic agent formulation or administration, or ignorance of the overall cellular microenvironmental context, affecting therapeutic utility and efficacy. Presentation of therapeutic proteins in a manner mimicking natural signaling events, accounting for concentration, spatial and temporal profiles, and their simultaneous or sequential presentation with other appropriate factors, may be requisite for salutary effects.

Our present study demonstrates that CTRP3 effectively promotes postischemic angiogenesis and attenuates adverse remodeling. More interesting, we have demonstrated that CTRP3 stimulates angiogenesis by promoting cardiomyocyte–endothelial cell communication, a process closely mimicking natural cardiac signaling events. Considerable evidence supports that communication between endothelial cells and cardiomyocytes regulates not only early cardiac development but also adult cardiomyocyte function, including the contractile state.[25] Cardiomyocytes depend on endothelial cells, not only for oxygenated blood supply but also for locally protective signals promoting cardiomyocyte organization and survival. Although endothelial cells direct cardiomyocytes, cardiomyocytes reciprocally secrete factors affecting endothelial cell function. Specifically, cardiomyocyte-specific deletion of VEGF-A results in vasculogenic/angiogenic defects and a thinned ventricular wall.[26] Additionally, erythropoietin, a cardioprotective cytokine, has recently been demonstrated to promote angiogenesis by increasing cardiomyocyte VEGF expression.[27] It is thus highly likely that interventions stimulating the ongoing molecular conversation between endothelial cells and cardiomyocytes may more closely mimic natural cardiac development events and thus exert superior cardioprotection compared with direct growth factor administration. Currently, whether CTRP3 may stimulate other mediators required for different angiogenesis stages remains unknown. However, the fact that CTRP3 is known to directly promote endothelial cell proliferation and migration, but not enhance tube formation (a process requiring complexity beyond proliferation and migration), suggests that proangiogenic factors other than VEGF might also be released by cardiomyocytes after CTRP3 treatment. This intriguing possibility will be investigated in our future studies. Moreover, although our in vitro experiments demonstrated that conditioned medium from CTRP3-treated cardiomyocytes promotes angiogenesis in cultured endothelial cells and that CTRP3 significantly promoted angiogenesis in MI heart, administration of CTRP3 in the sham-operated control heart only slightly increased capillary density. These results suggest that under normal physiological conditions (in vivo normal heart), angiogenesis is tightly controlled by a balance between proangiogenesis and antiangiogenesis factors and that the proangiogenic effect of CTRP3 cannot be observed under this condition. The important underlying mechanisms responsible for the differences between in vitro and in vivo experimental results and between normal and MI heart will be directly investigated in our future experiments.

It is worth noting that the CTRP3-treated group manifested not only significantly increased angiogenesis and dramatically reduced fibrosis but also marked preservation of intact myocytes in the infarct area at risk. The precise mechanisms responsible for this protection are likely complex and cannot be answered in a single experiment. Multiple possibilities exist. First, the potent antiapoptotic effect of CTRP3 observed in the border zone may block or attenuate infarct expansion. Second, the significant angiogenic effect of CTRP3 observed in the border zone may facilitate blood flow restoration, thus salvaging dying cardiomyocytes in infarcted regions, and/or promote endothelial cell transdifferentiation into cardiomyocytes.[28] Finally, CTRP3 may promote homing of stem cells from bone marrow and/or stimulate differentiation of resident cardiac stem cells. Such intriguing possibilities all warrant direct investigation.

Although CTRP3 is a paralog of adiponectin, our present study demonstrates distinctly different cardioprotective signaling between CTRP3 and adiponectin. It is well accepted that adiponectin promotes angiogenesis via AMP-activated protein kinase signaling activation and augments nitric oxide production.[29] However, CTRP3 had no significant effect on AMP-activated protein kinase or endothelial nitric oxide synthase phosphorylation and failed to stimulate nitric oxide production. In contrast, CTRP3 induced significant Akt phosphorylation both in vivo and in cultured cardiomyocytes. More important, inhibition of Akt phosphorylation abolished CTRP3-induced HIF1α and VEGF expression and blocked the angiogenic effect of CTRP3. These results support an Akt-dependent mechanism for angiogenic effects of CTRP3.

Conclusions

We have demonstrated that CTRP3, a key member of a newly identified adipokine family, directly prevented apoptotic death of cardiomyocytes, increased cardiomyocyte survival/regeneration, attenuated postinfarct fibrosis, and enhanced cardiomyocyte expression of angiogenic cytokines inductive of robust angiogenesis, thus attenuating post-MI remodeling and augmenting post-MI contractile function (Figure 8E). Preventing post-MI CTRP3 inhibition or CTRP3 supplementation may be a promising therapeutic avenue for creating stable and functional vessels in postinfarcted myocardium, restoring cardiac function, and mitigating the heart failure phenotype.

Disclosures

None.

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CLINICAL PERSPECTIVE

Cardiovascular disease remains a leading cause of mortality worldwide. Although improved reperfusion strategies have led to declined death rates after acute myocardial infarction, both the incidence and prevalence of post–myocardial infarction heart failure have continually increased in recent years. As a result of the increased incidence of diabetes mellitus and its association with heart failure, there is now great interest in elucidating the underlying molecular mechanisms linking these pathologies. Considerable evidence exists that the majority of adipokines (including tumor necrosis factor [TNF]-α, leptin, plasminogen activator inhibitor type 1, transforming growth factor-β, and resistin) adversely regulate myocardial metabolism, cardiomyocyte hypertrophy, extracellular matrix structure and composition, and cell death. The present study demonstrated that C1q/TNF-related protein-3 (CTRP3), a key member of a newly identified adipokine family, exerts cardiac actions opposing the majority of other adipokines and is a novel and potent cardioprotective molecule. Expression and production of CTRP3 are significantly reduced after myocardial infarction, and replenishment of CTRP3 protects the heart by attenuating postischemic pathologic remodeling and promoting cardiomyocyte–endothelial cell communication. These results suggest that the balance between cardioprotective and cardio-harmful cytokines tilts during pathological conditions such as heart failure. Interventions restoring the balance may represent a novel therapeutic strategy in the treatment of cardiovascular diseases, particularly in those patients with diabetes mellitus.

SUPPLEMENTAL MATERIAL

Methods:

Determination of cardiac function: Cardiac function was determined by echocardiography as well as left ventricular catheterization. Determination of cardiac function occurred 2 weeks after mini-osmotic pump implantation, prior to thoracotomy, as described previously.

Histological analysis: Hearts were fixed with 4% paraformaldehyde, embedded in paraffin, and either coronally (for myocyte/fibrosis ratio in left ventricular free wall) or transversely(for all other assays) sectioned (6 μm thick). Five sections from each heart were mounted upon glass slides, and stained with Masson trichrome (for fibrosis determination), wheat germ agglutinin (for myocyte size), antibody against CD31 (for capillary density), or antibody against α-smooth muscle actin (α-SMA, for arteriolar density). Slides were examined with an Olympus IX51 microscope, and 5 images from each slide were captured by a Q-Imaging camera controlled by IP Lab 4.0 software. Myocyte/fibrosis ratio (left ventricular free wall), cardiac collagen deposit (remote non-infract area), and capillary/arteriolar density (infarct border zone) were determined as previously reported[1, 2]. Results from all slides obtained in the same heart were averaged, and counted as n=1.

Western blot analysis: Proteins of interest were separated on SDS-PAGE gels, transferred to PVDF membranes, and incubated with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibody. Blots were developed via SupersignalChemiluminescence detection kit (Pierce, Rockford, Ill). Bands were visualized with a Kodak Image Station 4000 (Rochester, NY).

Tube formation assay: 100 µl of Matrigel (growth factor reduced, BD Biosciences) was added to each well of a 48-well plate, and polymerized at 37°C for 1 hour. HUVECs (1×10^4) were seeded onto Matrigel, in endothelial cell basal medium-2 with EGM-2 Bullet Kit. After 1 hour culture, gCTRP3 (3 µg/ml) or full length CTRP3 (30 µg/ml) was added. For conditioned medium experiments, endothelial cell basal medium-2 was replaced with cardiomyocyte-conditioned medium after 1 hour culture. After an additional 6 hour-culture, tube length was quantified via IP Lab 4.0 image analysis software.

Assessment of cardiomyocyte apoptosis: Cardiomyocyte apoptosis was determined via TUNEL staining and caspase-3 activity, as reported previously[3].

Measurements of endotoxin activity: The endotoxin activities of globular CTRP3 preparations were determined using the LAL assay kit (catalog No.50–648U, BioWhittaker, Walkersville, MD) according to the manufacturer's recommendation.
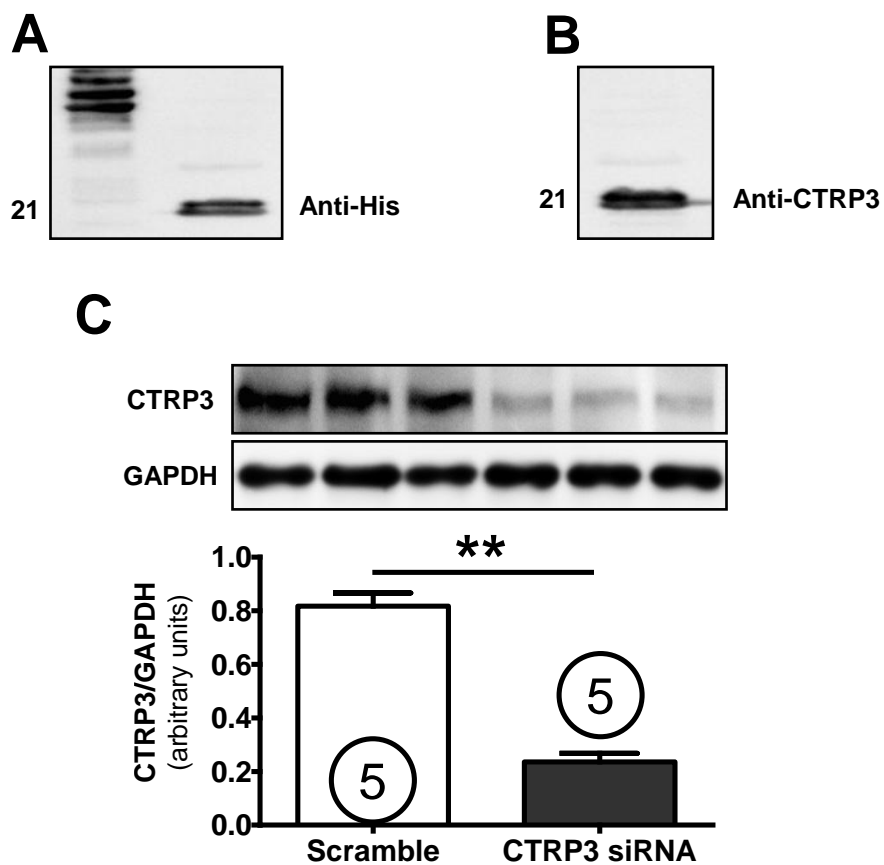
Figure S1. Western blots of recombinant globular domain of CTRP3 and CTRP3 SiRNA efficiency: Western blots of recombinant globular domain of CTRP3 with an antibody against His (A) or against CTRP3 (B); (C) Western blot of knock-down efficiency of mouse CTRP3 specific SiRNA. **$P<0.01$.
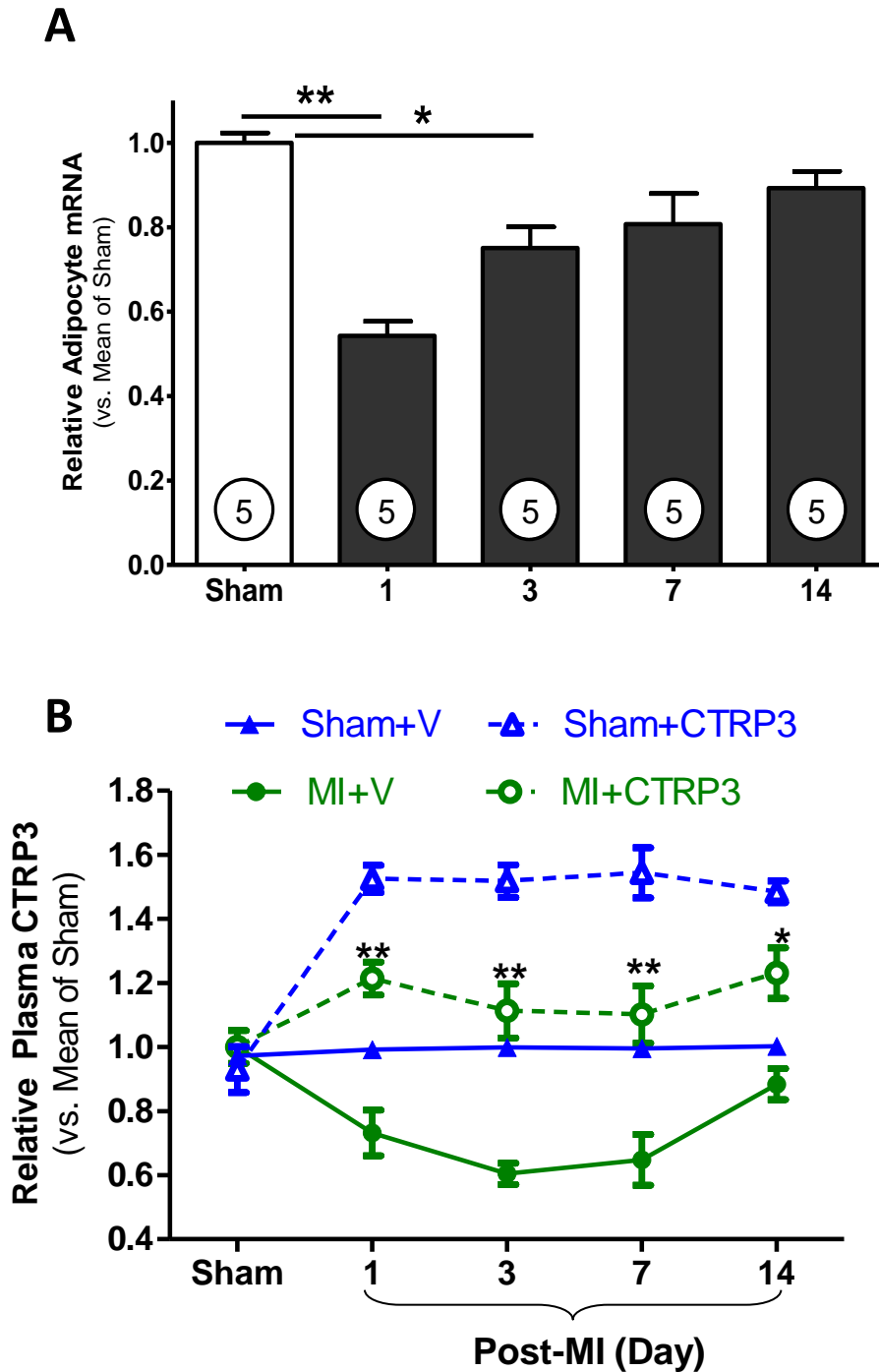
Figure S2. MI significantly inhibited adipocyte CTRP3 mRNA expression (A) and decreased serum CTRP3 levels (B, closed green circles connected with solid line). Supplementation of recombinant CTRP3 with an osmotic pump increased plasma CTRP3 levels in sham-operated mice (open blue triangles connected with dashed line) and prevented MI-induced serum CTRP3 reduction (open green circles connected with dash line). N=6/each time point; *P<0.05, **P<0.01 vs. MI+ Vehicle.
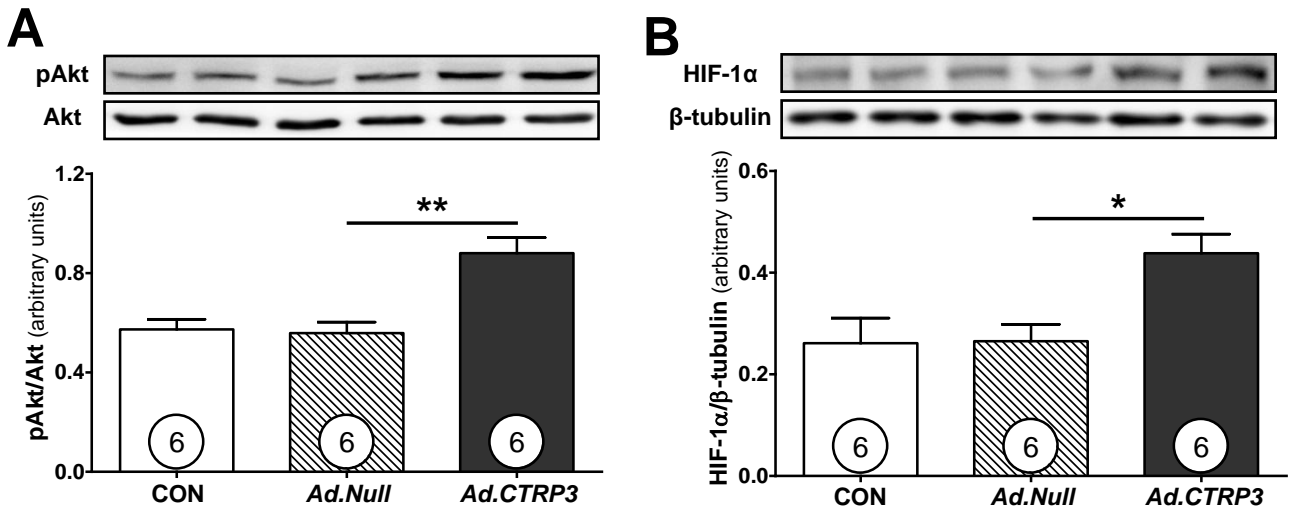
Figure S3. Adenoviral CTRP3 transfection of neonatal cardiomyocytes activated Akt (A) and upregulated HIF1α expression (B). Neonatal cardiomyocytes were transfected with Adenoviral CTRP3, and phospho-Akt (473)/Akt and HIF-1α/β-tubulin were determined 24 hours after transfection. *Ad.Null* had no significant effect upon Akt phosphorylation or HIF-1α production, but *Ad.CTRP3* significantly enhanced Akt phosphorylation, and increased HIF1-α expression. *P<0.05, **P<0.01.
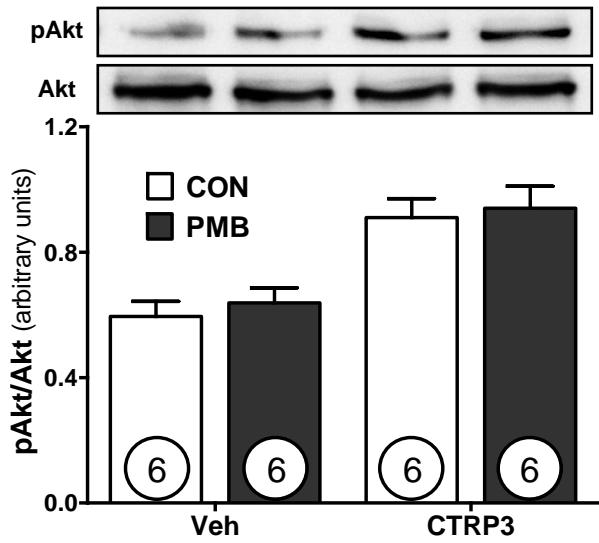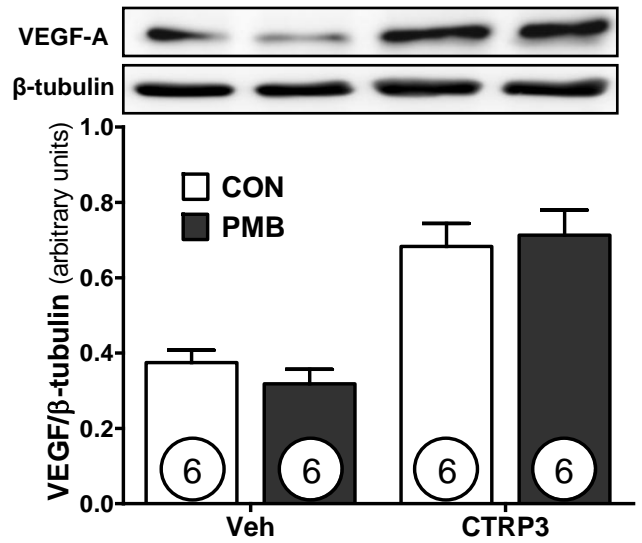
A

pAkt

Akt

B

VEGF-A

β-tubulin

Figure S4. Cardiomyocyte Akt activation (A) and VEGF expression (B) in response to globular CTRP3 treatment were not blocked by polymixin B (PMB, 30μg/mL).
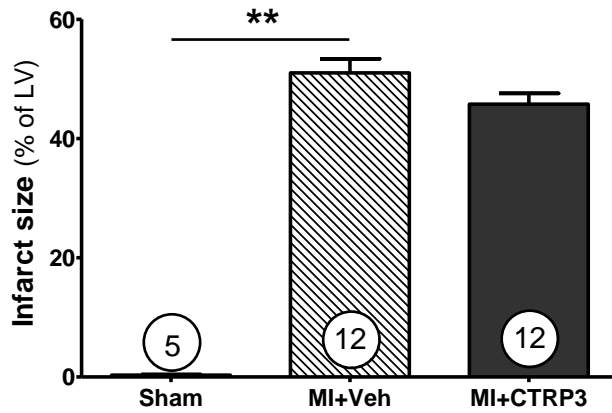
Figure S5. Treatment with CTRP slightly reduced infarct size. However, the difference is not statistically significant.