TNF-α antagonism ameliorates myocardial ischemia-reperfusion injury in mice by upregulating adiponectin

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1Department of Cardiology, Xijing Hospital, The Fourth Military Medical University, Xi’an, China; 2Department of Dermatology, Xijing Hospital, The Fourth Military Medical University, Xi’an, China; 3Department of Anesthesiology, Xijing Hospital, The Fourth Military Medical University, Xi’an, China; 4Department of Physiology, The Fourth Military Medical University, Xi’an, China; and 5Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania

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Gao C, Liu Y, Yu Q, Yang Q, Li B, Sun L, Yan W, Cai X, Gao E, Xiong L, Wang H, Tao L. TNF-α antagonism ameliorates myocardial ischemia-reperfusion injury in mice by upregulating adiponectin. Am J Physiol Heart Circ Physiol 308: H1583–H1591, 2015. First published April 17, 2015; doi:10.1152/ajpheart.00346.2014.—Tumor necrosis factor-α (TNF-α) antagonism ameliorates myocardial ischemia-reperfusion (MI/R) injury. However, the mechanisms by which the downstream mediators of TNF-α change after acute antagonism during MI/R remain unclear. Adiponectin (APN) exerts anti-ischemic effects, but it is downregulated during MI/R. This study was conducted to investigate whether TNF-α is responsible for the decrease of APN, and whether antagonizing TNF-α affects MI/R injury by increasing APN. Male adult wild-type (WT), APN knockout (APN KO) mice, and those with cardiac knockdowns of APN receptors via siRNA injection were subjected to 30 min of MI followed by reperfusion. The TNF-α antagonist etanercept or globular domain of APN (gAD) was injected 10 min before reperfusion. Etanercept ameliorated MI/R injury in WT mice as evidenced by improved cardiac function, and reduced infarct size and cardiomyocyte apoptosis. APN concentrations were augmented in response to etanercept, followed by an increase in AMP-activated protein kinase phosphorylation. Etanercept still increased cardiac function and reduced infarct size and apoptosis in both APN KO and APN receptors knockdown mice. However, its potential was significantly weakened in these mice compared with the WT mice. TNF-α is responsible for the decrease in APN during MI/R. The cardioprotective effects of TNF-α neutralization are partially due to the upregulation of APN. The results provide more insight into the TNF-α-mediated signaling effects during MI/R and support the need for clinical trials to validate the efficacy of acute TNF-α antagonism in the treatment of MI/R injury.

etanercept; globular domain of adiponectin; adiponectin knockout mice

DURING myocardial ischemia-reperfusion (MI/R), inflammatory reactions are triggered immediately after ischemia. Although chronic inflammation in the presence of long-term ischemia plays a crucial role in repairing the myocardium and restoring its function, acute inflammation triggered during reperfusion may increase infarct size and amplify contractile dysfunction (6, 28).

Tumor necrosis factor-α (TNF-α) is an important proinflammatory factor that is excessively released during MI/R and contributes to cardiac dysfunction and apoptosis (12). Most studies using pharmacological strategies to inhibit TNF-α have demonstrated improved recovery of myocardial function and a reduction in infarct size after MI/R. Drugs that can neutralize TNF-α, such as etanercept (fusion protein of the soluble TNF-α receptor 2 and the Fc end of IgG1), have been used clinically for the treatment of patients with rheumatoid arthritis, psoriasis, and other diseases. Etanercept appears promising as an anti-MI/R injury drug. However, there have been no clinical trials conducted to investigate the efficacy of etanercept or other TNF-α antagonists in the short- or long-term treatment of MI/R injury. One uncertainty regarding the utilization of TNF-α antagonists for the treatment of MI/R injury is the fact that TNF-α is a prominent cytokine in the inflammatory response. Neutralization of TNF-α may affect its multiple downstream factors. However, which downstream mediators will respond to acute TNF-α inhibition and the impact these changes will have still needs to be determined.

Adiponectin (APN) is an adipocyte-derived cytokine with high blood concentrations (0.5–30 mg/ml), comprising about 0.01% of all plasma proteins (24). APN can increase the phosphorylation of AMP-activated protein kinase (AMPK), which has vascular-protective, anti-inflammatory, and anti-ischemic effects (30). In vivo experiments have demonstrated that increasing plasma APN can protect the myocardium against MI/R injury (25, 30). However, plasma APN concentrations decrease during MI/R injury (30, 33). Previous in vitro studies have reported that TNF-α markedly inhibits APN mRNA and protein expression in adipocytes (4, 20). Clinical experiments have also shown that prolonged TNF-α antagonist therapy in patients with psoriasis can significantly increase plasma APN levels (2, 27). In our previous study, we reported that constant TNF-α inhibition prevents the decrease of APN after trauma (16). Although these experiments used prolonged anti-TNF-α therapy to increase APN levels by reversing chronic inflammation in adipose tissue, the direct evidence linking increased TNF-α expression to decreased APN concentrations during MI/R is still lacking. Whether acute anti-TNF-α therapy can reduce inflammation and increase APN levels remains unknown.

Therefore, the aims of the current study are: 1) to determine whether a single injection of etanercept (a TNF-α antagonist) during MI/R exerts cardioprotective effects; 2) to investigate whether TNF-α neutralization upregulates APN during MI/R; and, if so, 3) to delineate the potential causative relationship between the TNF-α neutralization-induced APN upregulation and the resultant cardioprotective effects.

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Materials and Methods

All experiments were performed in accordance with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Male C57BL/6 mice aged 6–8 wk, each weighing 20–25 g, were obtained from the Experimental Animal Center of Fourth Military Medical University (Xi’an, China). APN knockout mice (APN KO) (C57BL/6 background) were obtained from Prof. Du Jie’s lab from the Capital Medical University in Beijing, China. The generation, breeding, phenotypic characteristics, and genotyping of these mice have been previously described in detail (19). For MI/R surgery, the mice were anesthetized with 2% isoflurane, and heart rates were monitored. MI was induced after temporarily exteriorizing the heart via a left thoracic incision at the fourth intercostal space and subsequently tying a 6-0 silk slipknot around the left anterior descending coronary artery (7). After 30 min of ischemia, the slipknot was released. Based on previous studies and our pilot experiments, we found that etanercept has a half-life of 3 days, at least 10 min is necessary to neutralize TNF-α, and treatment with 5 mg/kg etanercept was enough to completely neutralize MIF-induced TNF-α in mice. Therefore, 10 min before reperfusion, mice were randomized to receive a vehicle (1% sucrose, 100 mM sodium chloride, 25 mM l-arginine hydrochloride, and 25 mM sodium phosphate), etanercept (TNF-α antagonist, recombinant human soluble TNF-α receptor; 5 mg/kg; Angen, US), or global domain of APN (gAD; 0.5 mg/kg; Adipobiotech, Beijing, China) via intraperitoneal injection (30). During the procedures, we used electrocardiography to confirm myocardial ischemia and reperfusion. Coronary occlusion/reperfusion resulted in a mortality rate of 3–5% in each group. Sham-operated control mice (sham MI/R) underwent the same surgical procedures except that the left coronary artery was not tied in this group.

All mice were euthanized under isoflurane anesthesia. The first group of mice was euthanized 3 h after reperfusion. Blood samples from the carotid artery were collected from these mice, and their hearts were used for immunoblotting, caspase-3 activity assay, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining (TUNEL staining). A second set of mice was euthanized 24 h after reperfusion for cardiac function assessment and infarct size determination. Because we planned to measure plasma TNF-α and APN level at 8 time points, the third group of mice was kept alive for 24 h after reperfusion, respectively. MI/R was induced after temporarily exteriorizing the heart via a left thoracic incision at the fourth intercostal space and subsequently tying a 6-0 silk slipknot around the left anterior descending coronary artery (7). After 30 min of ischemia, the slipknot was released. Based on previous studies and our pilot experiments, we found that etanercept has a half-life of 3 days, at least 10 min is necessary to neutralize TNF-α, and treatment with 5 mg/kg etanercept was enough to completely neutralize MIF-induced TNF-α in mice. Therefore, 10 min before reperfusion, mice were randomized to receive a vehicle (1% sucrose, 100 mM sodium chloride, 25 mM l-arginine hydrochloride, and 25 mM sodium phosphate), etanercept (TNF-α antagonist, recombinant human soluble TNF-α receptor; 5 mg/kg; Angen, US), or global domain of APN (gAD; 0.5 mg/kg; Adipobiotech, Beijing, China) via intraperitoneal injection (30). During the procedures, we used electrocardiography to confirm myocardial ischemia and reperfusion. Coronary occlusion/reperfusion resulted in a mortality rate of 3–5% in each group. Sham-operated control mice (sham MI/R) underwent the same surgical procedures except that the left coronary artery was not tied in this group.

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Determination of myocardial apoptosis and caspase-3 activity. Myocardial apoptosis was determined using TUNEL (Roche) and caspase-3 activity assays (Beyotime, China). Both methods were applied 3 h after reperfusion. For TUNEL staining, paraffin-embedded tissues were cut into 5-μm sections (4 slides/heart). The sections were then incubated in 50 μL of TUNEL mixture (47.5 μL of TUNEL label containing fluorescein isothiocyanate-conjugated dUTP and 2.5 μL of TUNEL enzyme) in a humidified chamber for 60 min at 37°C. Sections were photographed by a QICAM-Fast Digital Camera mounted atop an Olympus BX51 Fluorescence Microscope (Olympus America, Center Valley, PA) after incubation. Control sections were incubated with 50 μL of the TUNEL-label solution that did not have the TUNEL enzyme. Additional staining was performed using the monoclonal anti-α-sarcromeric actin (Sigma, St Louis, MO) and DAPI (Beyotime, China). All evaluated fields on each slide were photographed. Tissue sections were examined by different persons in a blinded fashion. The apoptosis index was determined by the number of TUNEL-positive nuclei divided by the total number of counted cardiomyocytes.

To assess the caspase-3 activity, myocardial tissue was homogenized in an ice-cold lysis buffer (Beyotime, China). The homogenates were centrifuged at 10,000 g for 5 min at 4°C. The supernatants were then collected, and 200 μg of protein was loaded into each well of the 96-well plate and incubated with 25 μg Ac-DEVD-pNA at 37°C for 90 min. The pNA absorbance was quantified using the SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

Quantification of plasma TNF-α and APN concentrations. Plasma TNF-α and APN concentrations were determined using ELISA kits. To measure plasma TNF-α levels, 96-well plates were coated with TNF-α antibody (Diaclone, France and SenXiong Biotech, Shanghai, China) overnight at 4°C. Next, 50 μL of each sample was added in duplicate to the plates, and 50 μL of biotinylated anti-TNF-α solution was added to each well. After incubating for 2 h at room temperature, the plates were washed 3 times, and 100 μL of streptavidin-horseradish peroxidase conjugate solution was added to each well. Color was developed utilizing OPD as a substrate, and the reaction was stopped by 2 N H2SO4 and analyzed using the SpectraMax plate reader at 492 nm.

For APN concentrations, plasma samples were diluted at 1:10,000. The APN concentration was then determined using an APN-ELISA assay.
kit (R and D Systems, Minneapolis, MN) according to manufacturer’s instructions.

**Immunohistochemical analysis.** Samples were collected 1 h after reperfusion, fixed in 4% neutral buffered paraformaldehyde, and then embedded in paraffin. Paraffin-embedded tissues were cut into 5-μm sections. The sections were deparaffinized in xylene, dehydrated in ethanol, and treated with 3% H2O2 in methanol for 10 min at room temperature to eliminate the endogenous peroxidase activity. Antigen retrieval was achieved by heat mediation. The sections were then incubated with primary antibody retrieval was achieved by heat mediation. The sections were then blocked with BSA for 1 h and then incubated with primary antibody against TNF-α (Abcam, Cambridge, MA) overnight at 4°C; anti-rabbit secondary antibody was incubated for 1 h in room temperature. Colorimetric detection was completed with diaminobenzidine for 5 min and a QICAM-Fast Digital Camera mounted atop an Olympus BX51 Fluorescence Microscope (Olympus America, Center Valley, PA) was used to photograph sections. A duplicate sample was prepared as above, but the primary antibody was omitted to control for any nonspecific signal. Based on previously published methods (11), to compare the intensity of TNF-α expression in a variety of cell types of the myocardium, relative intensity of the staining was assayed by three independent observers. TNF-α staining ranged from the lowest intensity of positive staining (scored as 1) to very intense, dark staining (scored as 3). No staining was scored as 0. Three consecutive tissue sections of each heart were assayed.

**Immunoblotting.** Homogenate proteins from the area of cardiac tissue that was considered at risk were isolated 3 h after MI/R. They were separated using SDS-PAGE gels, transferred to PVDF membranes, and incubated with monoclonal antibody against AdipoR1 and AdipoR2 (Abcam, Cambridge, MA), phosphorylated AMPK and total AMPK (Cell Signaling Technology, Danvers, MA), and β-actin (Santa Cruz Biotechnology), respectively. They were then incubated with HRP-conjugated antibody (Santa Cruz Biotechnology) for 1 h. The blot was developed with an ECL-Plus chemiluminescence reagent kit (Millipore) and visualized using the UVP Bio-Imaging Systems. Blot densities were analyzed with Vision Works LS Acquisition and Analysis software.

**Statistical analysis.** All values in the text and figures are presented as means ± SE of the sample of independent experiments. All data, except the Western blot densities, were subjected to one-way ANOVA followed by Bonferroni corrections for post hoc t-tests. Western blot densities were analyzed with the Kruskal-Wallis test followed by Dunn’s post hoc test. All P values > 0.05 were considered statistically significant.

**RESULTS**

**Etanercept ameliorated MI/R injury.** To determine whether a single injection of etanercept during MI/R exerts cardioprotective effects, we evaluated cardiac function by echocardiography and invasive hemodynamic evaluation methods, determined myocardial infarct size by Evans blue/TTC staining, and observed myocardial apoptosis by TUNEL staining and caspase-3 activity assay. The echocardiography results showed that mice had significantly impaired cardiac function 1, 7, and 14 days after reperfusion (Fig. 1, A and B, P < 0.01). Administration of the TNF-α antagonist etanercept (a fusion protein of the soluble TNF-α receptor 2 and the Fc end of IgG1) significantly increased LVEF% at all the three time points tested (1 day: 41.9 ± 4.6% vs. 49.1 ± 2.3%; 7 days: 41.0 ± 3.8% vs. 48.2 ± 3.0%; and 14 days: 44.6 ± 4.7% vs. 54.1 ± 3.9%; P < 0.05, respectively). The results of invasive hemodynamic evaluation also showed that etanercept administration did not alter the heart rate but significantly attenuated the MI/R-induced elevated LVEDP and ±dp/dtmax depression (Fig. 1, C–F).

Compared with the vehicle, etanercept significantly reduced myocardial infarct size and cardiomyocyte apoptosis, demonstrated by Evans blue/TTC staining (Fig. 2A) as well as by TUNEL staining and caspase-3 activity assay (Fig. 2, B–D), respectively. In line with our previous studies, administrating gAD also significantly augmented the LVEF% (Fig. 1, A and B), reduced the infarct size, and attenuated cardiomyocyte apoptosis (Fig. 2, A–D). The effects of etanercept and gAD in
attenuating cardiac dysfunction and reducing infarct size and apoptosis were not significantly different from each other [except in LVEDP, MI/R + etanercept (E) vs. MI/R + gAD, P < 0.05]. However, the effects of gAD always occurred to a lesser extent compared with etanercept. Taken together, these data indicate that etanercept ameliorates MI/R injury in mice with a single injection 10 min before reperfusion.

**Etanercept increased plasma APN levels during MI/R.** Evidence exists that TNF-α inhibits APN expression in cultured adipocytes. We hypothesized that TNF-α may cause APN attenuation during MI/R and conducted several experiments. First, we observed the effect of etanercept on neutralizing TNF-α. Myocardial TNF-α was demonstrated by IHC staining 1 h after MI/R. Compared with the vehicle group, the mice in the MI/R group demonstrated obvious TNF-α immunostaining. However, TNF-α staining was significantly less in the MI/R + E group (Fig. 3A). Plasma TNF-α was tested by ELISA kits, using blood samples obtained 1, 3, and 8 h, and 1, 3, 5, 7, and 14 days after reperfusion, respectively. The results showed that plasma TNF-α concentration increased from 1 h to 3 days after MI/R. After etanercept injection, the TNF-α concentration significantly decreasing beginning at 1 h after MI/R, and was undetectable at 3 h and thereafter. These results indicate that TNF-α was neutralized after injecting etanercept (Fig. 3B).

ELISA was then performed on blood samples that were obtained at the time points listed above to assess dynamic changes in plasma APN concentrations. The results showed that plasma APN levels had started to decrease at 1 h post-MI/R, reached its nadir at 8 h post-MI/R, and returned to baseline 5 days after MI/R (Fig. 3C). Etanercept administration significantly ameliorated the decrease in APN at 3 h, 8 h, 1 day, and 3 days after reperfusion, respectively, compared with vehicle. Interestingly, APN concentrations were even higher in etanercept-treated mice compared with sham mice (P < 0.05) at 8 h after reperfusion. Because the phosphorylation of AMPK is critical in APN signaling for its cardioprotective effect, we also evaluated the phosphorylation of AMPK by immunoblotting, and found that the ratio of pAMPK/AMPK was higher in the etanercept treatment group compared with the vehicle group (Fig. 3, D and E).

To obtain more evidence to support a relationship between evaluated TNF-α and decreased APN in vivo, two additional experiments were performed. In the first series of experiments, mice from sham group were injected with 0.1 μg TNF-α protein. Using ELISA kits, we determined significantly decreased APN levels in the blood samples collected 8 h after injection (Fig. 3F). In the second series of experiments, etanercept was administered after MI/R surgery in mice. One day later, they were injected with 10 μg of TNF-α protein. APN level was also significantly reduced 8 h after treatment with TNF-α (Fig. 3G).

Taken together, these data indicate a causative relationship between TNF-α enhancement and APN attenuation in vivo and suggest that TNF-α neutralization ameliorates the reduction in plasma APN concentrations that occur during MI/R.

**The cardioprotective effects of etanercept were partially afforded by increasing APN.** Etanercept administration exhibited cardioprotective effects along with increasing APN levels during MI/R. Therefore, we sought to delineate the potential causative relationship between the TNF-α neutralization-induced APN upregulation and the resultant cardioprotective effects. We found that at 1, 7, and 14 days after MI/R, APN KO mice showed no significant improvement in LVEF% when treated with etanercept, compared with the vehicle (Fig. 4, A and B). Furthermore, the LVEF% increased by 7.26 ± 2.33% at 1 day after etanercept treatment in WT mice. However, LVEF% only increased by 2.74 ± 0.99% in APN KO mice (Figs. 1B and 4B, 1 day, P < 0.05; 7 day, WT 7.19 ± 0.79 vs. **
Fig. 3. Etanercept increased plasma adiponectin (APN) level during MI/R. A: myocardial TNF-α was demonstrated by IHC 1 h after MI/R. Plasma TNF-α (B) and APN concentrations (C) were evaluated by ELISA assay at indicated time points after reperfusion. D: phosphorylation of AMPK in the heart tissues was tested by immunoblotting. E: quantification of immunoblotting was indicated by the pAMPK/AMPK ratio. F: plasma APN concentration was determined after injecting 0.1 g TNF-α protein per mouse in the sham group. G: APN concentration in mice that were treated with etanercept during MI/R, then injected with 10 g TNF-α protein 1 day after. n = 6–8 animals/group. *P < 0.05, **P < 0.01 vs. WT MI/R; #P < 0.05, ##P < 0.01 vs. WT Sham; $$$P < 0.01 vs. WT Sham; &&P < 0.01 vs. WT MI/R + E.
KO 0.33 ± 0.11, P < 0.05; 14 day, WT 9.49 ± 0.80 vs. KO 3.98 ± 1.18, P < 0.05). Additionally, while myocardial infarct size was significantly reduced in WT mice treated with etanercept or gAD, the effect of etanercept in reducing myocardial infarct size was also significantly weakened in APN KO mice (Figs. 2A and 4C, reduced infarct size in WT 24.55 ± 3.36% vs. KO 8.56 ± 2.01%, P < 0.05). We found similar diluted cardioprotective effects of etanercept in reducing apoptosis in cardiac tissue, as observed by TUNEL staining (Figs. 2C and 4E, reduced TUNEL staining positive cells in WT 10.3 ± 3.46% vs. KO 4.82 ± 0.94%, P < 0.05) and caspase-3 activity assay (Figs. 2D and 4F, reduced in WT 4.23 ± 0.55 vs. KO 2.92 ± 0.46, P < 0.05). Not surprisingly, gAD could still significantly improve cardiac function and reduce infarct size and cardiomyocytes apoptosis compared with the vehicle. No significant differences were found between gAD and the etanercept treatment group (Fig. 4, A–F). These data showed that the cardioprotective effects of etanercept are weakened in APN KO mice, indicating its effects were partially mediated by APN.

The cardioprotective effects of etanercept were attenuated in AdipoR1 and AdipoR2 KD mice. To further investigate the relationship between APN signaling activation and the cardioprotective effects of etanercept, we used siRNA delivery to silence AdipoR1 and AdipoR2 in myocardium in vivo. Intramyocardial siRNA delivery successfully suppressed 70% AdipoR1 and -R2 expression in KD animals compared with WT animals (Fig. 5, A–C), with decreased AMPK phosphorylation when treated with etanercept or gAD during MI/R (Fig. 5, A and E). KD AdipoR1 and -R2 did not affect the APN levels (Fig. 3D). Administrating gAD in AdiopRs KD mice showed no significant cardioprotective effects (Fig. 5, F–I). Although etanercept could still significantly increase LVEF% and reduce infarct size in KD mice (P < 0.05, respectively), these effects were attenuated compared with the WT animals (Fig. 5F, increased LVEF% 1 day after MI/R: WT 7.26 ± 2.33% vs. 4.35 ± 0.42%, P < 0.05; Fig. 5G, reduced infarct size: WT 24.55 ± 3.36% vs. 8.78 ± 3.11%, P < 0.05). Etanercept also had less potential for reducing apoptosis, as observed by TUNEL staining (Fig. 5H, reduced in WT 10.3 ± 3.46% vs. KD 6.7 ± 0.99%, P = 0.053) and caspase-3 activity (Fig. 5I, reduced in WT 4.23 ± 0.55 vs. KD 2.1 ± 0.62, P < 0.05). These data suggest that the cardioprotective effects of etanercept are weakened by the loss of APN signaling activation.
DISCUSSION

In the current study, we have obtained evidence to support two major points. First, we provided evidence supporting the efficacy of acute neutralization of TNF-α by etanercept against MI/R injury. Second, we provided in vivo evidence demonstrating that elevated TNF-α is responsible for the hypoadiponectinemia that is observed during MI/R, such that neutralizing TNF-α can upregulate plasma APN levels, thereby conferring cardioprotective effects against MI/R injury. To our knowledge, we have demonstrated for the first time the causative relationship between TNF-α neutralization-induced APN upregulation and consequent cardioprotective effects.

Inflammation is considered as the initial response to myocardial ischemia-reperfusion (MI/R). MI/R increases the production of proinflammatory cytokines, including TNF-α, IL-1β, IL-2, IL-6, and IFN-γ. TNF-α is considered as a cytokine with pleiotropic effects; it can increase other inflammatory cytokines such as IL-1 and IL-6. TNF-α displays both beneficial and detrimental roles in the myocardium during MI/R injury, depending on its receptor subtype, concentration, and duration of exposure (21). Basal TNF-α improves contractile function via TNFR1 activation (23), and low concentrations of TNF-α are cardioprotective due to myocardial ischemic preconditioning (5, 26). During acute MI/R, TNF-α is excessively released within minutes. Studies have shown that treatment with TNF-α antibodies reduces infarct size (1, 10). Inhibiting circulating and cardiac TNF-α can also attenuate the MI/R-induced contractile dysfunction (3, 31). In contrast, there were studies demonstrating the mechanisms by
which MI/R can promote myocardial inflammation in a TNF-α-independent manner (22) and the ways by which endogenous TNF-α can protect the myocardium during acute myocardial infarction (14). In our studies, we demonstrated that neutralizing TNF-α preserves cardiac function and reduces infarct size and apoptosis. These results support the acute neutralization of TNF-α against MI/R injury.

Etanercept is a fusion protein of the soluble TNF-α receptor 2 and the Fc end of IgG1 which has been used clinically to neutralize circulating TNF-α. In previous clinical studies, the most frequent adverse events reported during etanercept therapy include injection-site reactions, infections, and allergic reactions. Etanercept is contraindicated in patients with sepsis or who are at risk of sepsis, and in those with other active infections. Currently, etanercept has been used for the treatment of patients with rheumatoid arthritis, psoriasis, and other diseases.

Etanercept is currently not indicated for use in the treatment of cardiovascular diseases. Most studies and clinical trials focused on investigating the effects of etanercept in the treatment of patients with congestive heart failure (CHF). In phase I and II studies, etanercept improved the LV ejection fraction, walking distance, and quality of life. Surprisingly, the results of these larger clinical trials showed no benefit in death and hospitalization with long-term etanercept treatment. The negative results were explained by the possibility that etanercept treatments are unable to sufficiently disrupt the complex network of inflammatory mediators such as APN, which are abnormally expressed or inhibited during CHF progression. Additionally, constant neutralization by TNF-α may cause TNF-α imbalances in the treatment of CHF. From these clinical trials, we have learned that the timing selection and the duration of TNF-α inhibition are important. However, these negative results do not argue against the hypothesis that TNF-α antagonists treatment is beneficial in the treatment of MI/R injury. Thus, rather than constant neutralization, in the present study, we performed a single injection of etanercept 10 min before reperfusion to restore the balance disrupted by the TNF-α increase that occurred shortly after MI/R. Compared with other TNF-α antagonists, the half-life of etanercept is 3 days and its formation with TNF-α is not irreversible. Therefore, it is likely that a single injection of etanercept only inhibited the “burst” of TNF-α production without affecting its subsequent effects after 3 days.

Furthermore, we determined that excessive TNF-α production is responsible for the attenuated APN levels during MI/R. Neutralizing TNF-α can increase APN levels, thereby exerting cardioprotective effects against MI/R injury. Mostly secreted by adipocytes, APN possesses anti-inflammatory and anti-ischemic properties (15) that are significantly downregulated after MI/R (17, 30, 32, 33). APN knockout mice manifested significantly enlarged infarct sizes and increased apoptosis during MI/R, which can be reversed by exogenous APN administration (25). APN exerts cardioprotective effects during MI/R that are linked to cyclooxygenase-2-mediated suppression of TNF-α signaling, AMPK-mediated inhibition of apoptosis, as well as the inhibition of excess peroxynitrite-induced oxidative-nitrate stress (9, 25, 30). APN administration has been shown conclusively to protect the myocardium from MI/R injury (30). Although promising as an ischemic injury therapeutic agent, APN comprises about 0.01% of all plasma proteins and already circulates in abundance. It would be clinically difficult to increase the concentration of APN by injecting purified protein. Thus far, no studies have reported on the effective restoration of the APN imbalance in animals during MI/R injury without directly administering APN. In our study, we found excessive TNF-α production, which is at a mechanistic point upstream of the pathology responsible for APN attenuation during MI/R. This may be a more effective strategy than direct intravenous or intraperitoneal APN supplement.

Previous studies have reported decreased plasma APN concentrations during MI/R injury (30, 33). However, the dynamic changes in plasma APN concentration during MI/R were still unclear. We found that APN levels started to decrease at 1 h post-MI/R, are maintained at a low level from 3 h to 1 day, and returned to baseline 5 days after MI/R. Our data suggest that APN responds to MI/R injury quickly, and might contribute less to the cardiac repairing process that occurs 5 days after MI/R. Another interesting finding is that APN concentration was significantly higher in mice treated with etanercept 8 h post-MI/R compared with the sham mice. These data indicate that there might be some unknown endogenous factors increasing APN levels in animals post-MI/R to counteract the effects of TNF-α or other cytokines that attenuate APN.

To prove that the neutralizing TNF-α upregulated the APN levels which subsequently conferred cardioprotective effects against MI/R injury, we used APN KO mice and AdipoR1 and AdipoR2 KO mice. AdipoR1 is abundantly expressed in skeletal muscle and cardiomyocytes, which has high affinity for globular APN. AdipoR2 is predominantly expressed in the liver and has high affinity for full-length APN. Both receptors are expressed in cardiomyocytes. In addition to these two receptors, T-cadherin is also a putative cell-surface binding protein for full-length and oligomeric APN. Because AdipoR1 and AdipoR2 knockout mice were not commercially available, we utilized siRNA gene silencing technique to KD AdipoR1 and AdipoR2 expression in the mouse heart. We tested the efficiency of siRNA silencing 24, 48, and 72 h after the delivery, which were found to be consistent with the results previously reported by Wang et al. (33) AdipoRs expression reaches its nadir 48 h after siRNA injection. However, there exists an important limitation of using siRNA gene silencing technique in our study. The siRNA delivery only suppressed 70% of AdipoR1 and -R2 expression in KD animals, causing an incomplete blockade of APN signaling activation.

In summary, our study provides evidence that 1) neutralizing TNF-α induced by a single injection of etanercept exerts cardioprotective effects; 2) TNF-α inhibits APN levels during MI/R; and 3) upregulated APN is responsible for the cardioprotection afforded by TNF neutralization. Our results provided additional insight on the signaling effects of TNF-α during MI/R and support the need for further studies in large animals or in clinical trials to validate the cardioprotective effects of etanercept treatment during MI/R.

GRANTS
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