Role of tumor necrosis factor-α in myocardial dysfunction and apoptosis during hindlimb ischemia and reperfusion

Xiangru Lu, MD; Joel A. Hamilton, MSc; Ji Shen, MD, MSc; Theresa Pang, BSc; Douglas L. Jones, PhD; Richard F. Potter, PhD; J. Malcolm O. Arnold, MD; Qingping Feng, MD, PhD

Objective: Peripheral vascular surgery involving limb ischemia/reperfusion is associated with tumor necrosis factor-α production and an increased risk of cardiac complications. The objective of this study was to investigate the role of tumor necrosis factor-α in myocardial apoptosis and dysfunction following hindlimb ischemia/reperfusion.

Design: Randomized perspective animal study.

Setting: Research laboratory.

Subjects: Adults male tumor necrosis factor-α−/− and littermate wild-type mice.

Interventions: Bilateral hindlimb ischemia/reperfusion was induced in wild-type and tumor necrosis factor-α−/− mice using tourniquet occlusion. After 2 hrs of hindlimb ischemia, the tourniquets were released, allowing reperfusion for 0.5–24 hrs.

Measurements and Main Results: In wild-type mice, hindlimb ischemia/reperfusion resulted in myocardial depression early during the reperfusion period (p < .05). These effects were temporally correlated with enhanced levels of myocardial and plasma tumor necrosis factor-α. All variables were restored to baseline levels by 24 hrs of reperfusion. Myocardial apoptosis, assessed by cell death enzyme-linked immunosorbent assay, terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling staining, and caspase-3 activity, was also significantly higher at 6 hrs of reperfusion (p < .05) but returned to baseline levels by 24 hrs. Interestingly, cardiac dysfunction and myocardial apoptosis were abolished in tumor necrosis factor-α−/− mice subjected to the same degree of hindlimb ischemia/reperfusion as the wild-type mice. Treatment of etanercept restored cardiac function in wild-type mice.

Conclusions: Tumor necrosis factor-α contributes significantly to myocardial dysfunction and apoptosis in hindlimb ischemia/reperfusion. Although a causal link between myocardial apoptosis and cardiac dysfunction is not established, our study does suggest that tumor necrosis factor-α may be a potential therapeutic target for cardiac injury in clinical situations involving prolonged remote ischemia/reperfusion. (Crit Care Med 2006; 34:484–491)

Key Words: hindlimb ischemia and reperfusion; cardiac dysfunction; tumor necrosis factor-α; myocardial apoptosis; systemic inflammatory response syndrome

Prolonged limb ischemia followed by reperfusion (I/R) induces systemic inflammatory response syndrome (SIRS) (1, 2). Clinical studies suggest that the heart is at risk in the setting of SIRS. Patients undergoing complex vascular surgery that involves prolonged clamping of the aorta have been shown to develop SIRS (3–6) and exhibit a four-fold increased risk of cardiac events including heart failure following surgery (7, 8). Given that the major clinical consequences of SIRS include multiple organ dysfunction and/or death, it is important to understand the underlying mechanisms responsible for these phenomena. Tumor necrosis factor (TNF)-α is considered one of the primary mediators of SIRS (9–12). Although the main sources of TNF-α are resident macrophages found within organs, recent evidence indicates that cardiomyocytes themselves are a rich source of TNF-α production in the heart (13–15). The cardiovascular effects of TNF-α include myocardial depression, hypotension, and a decrease in systemic vascular resistance (16–18). In addition, TNF-α also induces cardiomyocyte apoptosis (19–22).

The goal of the present study was to investigate the role of TNF-α in myocardial dysfunction and apoptosis resulting from noncardiac injury. We used a murine model of SIRS induced by bilateral hindlimb I/R. Previous studies have shown that hindlimb I/R increases circulating TNF-α levels and causes remote organ injuries including lungs and liver (11, 23). We hypothesized that TNF-α production contributes to cardiac dysfunction and myocardial apoptosis following hindlimb I/R. We showed that hindlimb I/R injury caused myocardial dysfunction only in the presence of TNF-α and that animals lacking TNF-α or treated with a TNF-α blocker were protected. Furthermore, the myocardial dysfunction seen following hindlimb I/R was temporally correlated with increased myocardial apoptosis, indicating that ap-
optosis may in part contribute to the development of cardiac dysfunction post hindlimb I/R.

METHODS

Animals. Breeding pairs of TNF-α−/− and C57BL/6 mice were purchased from the Jackson Laboratory. TNF-α−/− mice were backcrossed to C57BL/6 background for at least six generations. Male adult TNF-α−/− and wild-type littermates (age 2–5 months) were used for the experiments. All mice were genotyped by polymerase chain reaction using genomic DNA extracted from the tail. All animals were maintained on normal mouse chow and given water ad libitum in a 12/12-hr light-dark cycle. Animals used in this study were handled in accordance with the guidelines of the Canadian Council on Animal Care, and study protocols were approved by the Animal Use Subcommittee at the University of Western Ontario, London, Canada.

Hindlimb Ischemia and Reperfusion. TNF-α−/− and wild-type mice were anesthetized by sodium pentobarbital injection (50 mg/kg intraperitoneally) and were placed on a heating pad to maintain body temperature at 37°C throughout the procedure. Ischemia was achieved using a tourniquet (#2 silk suture) above the greater trochanter of each hindlimb. After 2 hrs of ischemia, the tourniquets were released to allow hindlimb reperfusion. Analgesic (buprenorphine, 0.03 mg/kg subcutaneously) was administered along with 1.5 mL of saline for fluid resuscitation. Reperfusion of the hindlimbs occurred for various periods of time (0.5, 3, 6, 12, 18, and 24 hrs). Sham ligations were used as controls, where animals received similar anesthetic treatment to I/R animals but no hindlimb I/R was induced. Some wild-type mice were treated with etanercept (2 mg/kg intraperitoneally), a TNF-α blocking protein containing the extracellular binding portion of the p75 TNF-α receptor (recombinant human p75 receptor Fc fusion protein, TNFR/Fc) immediately after induction of hindlimb ischemia. The dose of etanercept was chosen from a previous study that showed improvements in cardiac function in endotoxemic mice (24).

In Vivo Measurements of Cardiac Function. Following reperfusion of various times, mice were reanesthetized, intubated, and mechanically ventilated (SAR830, CWE, Ardmore, PA) with room air for catheter placements. A Millar tip pressure transducer (1.4 Fr, Millar Instruments) was inserted into the right carotid artery to obtain mean arterial blood pressure and heart rate. The catheter was further advanced to the left ventricle for measurement of left ventricular systolic pressure, left ventricular end-diastolic pressure, and maximal rate of pressure development (+dP/dt max) and maximal rate of relaxation of left ventricle (−dP/dt min). All animals were kept on a proportional heating pad throughout the experiment to maintain the body temperature at 37°C. All measurements were recorded using a Gould recorder (2400S). After the measurement of hemodynamic variables was complete, whole blood samples, left ventricle (LV) tissue, and skeletal muscle tissue were taken and stored at −70°C before further studies.

Isolated Heart Preparation. Mouse hearts were isolated and perfused in a Langendorff system with Krebs-Henseleit buffer at 2 mL/min constant flow. The perfusion buffer was maintained at 37°C and bubbled continuously with a mixture of 95% oxygen and 5% CO₂. Myocardial function was assessed according to our recent report (25). Briefly, a 6–0 silk suture was placed through the apex of the left ventricle and threaded through a lightweight rigid coupling rod, which was connected to a force-displacement transducer (FT03) to record tension and heart rate. The force rate was calculated by multiplying the force (g) by the heart rate (beats/min). Maximal and minimal first derivative of pressure (+dP/dt max and −dP/dt min) as the rate of contraction and relaxation were analyzed by PowerLab Chart program (ADInstruments, Mountain View, CA).

Enzyme Immunoassay for Cytoplasmic Histone-Associated DNA Fragments. Apoptosis was quantified by measuring cytoplastic histone-associated DNA fragments (mono- and oligonucleotides) using a photometric enzyme immunoassay (Cell Death Detection ELISA, Roche Molecular Biochemicals, Indianapolis, IN) according to our previous report (26).

In Situ Detection of Apoptotic Cells. To determine the cell type undergoing apoptosis, apoptotic cells in the myocardium were identified using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL), which incorporates fluorescein into the DNA strand breaks (Roche Molecular Biochemicals) (26). The signal of TUNEL was then detected by an antifluorescein antibody conjugated with peroxidase using diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin. The number of apoptotic nuclei was quantified by two blinded observers as we previously described (27).

Caspase-3 Activity. Caspase-3 activity was measured using a caspase-3 fluorescent assay kit according to the manufacturer’s protocol (BIOMOL Research Laboratories, PA). This assay is based on fluorescent intensity of the chromophore 7-amino-4-methylcoumarin after cleavage from the C-terminus of the peptide substrate (26). Caspase-3 activity was quantified using a fluorescent spectrophotometer (excitation at 355 nm, emission at 460 nm) and normalized using inhibitor-treated samples as background. Caspase-3 activity was expressed as nmol 7-amino-4-methylcoumarin cleaved per mg sample protein per hour.

Measurement of TNF-α. TNF-α levels in the left ventricle and present in the blood were measured using a solid-phase sandwich enzyme-linked immunosorbent assay (Biosource International, Camarillo, CA) as we previously described (24). For the measurement of myocardial TNF-α, excised LV tissue was mechanically homogenized for 1 min in ice-cold phosphate-buffered saline. For measurement of circulating TNF-α, 50 μL of plasma was added to the microtiter plates. The amount of TNF-α in each sample was determined from a linearized standard curve and was expressed as ng/mg protein and pg/mL for LV tissues and plasma, respectively.

Immunohistochemistry. Sections of formalin-fixed and paraffin-embedded myocardial tissue were analyzed by an indirect immunoperoxidase technique (27). Briefly, 5-μm sections were incubated with goat anti-TNF-α antibody (Santa Cruz Technology, Santa Cruz, CA) overnight at 4°C and then with peroxidase-conjugated donkey anti-goat immunoglobulin G antibody. The reaction was developed with diaminobenzidine and H₂O₂. Sections were counterstained with hematoxylin, dehydrated, and mounted by routine methods.

Lactate Dehydrogenase (LDH) Assay. LDH is a constitutively expressed enzyme present in muscle tissue, which on cell membrane rupture is released into the circulation. For determination of skeletal leg muscle tissue damage, we measured residual LDH enzymatic activity (units/μg protein) in leg muscle samples using a photometric enzyme assay (Sigma Chemical, St. Louis, MO) as previously described (28). Briefly, leg muscle samples were homogenized. After protein concentrations were determined using the Bradford assay (29), samples (2 μg protein) were reacted with excess LDH substrates (i.e., pyruvate, reduced nicotinamide adenine dinucleotide, and H⁺) and changes in absorbance were measured at 340 nm using a spectrophotometer.

Western Blot Analysis. Cardiac tissue samples (30 μg protein) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 15% gels, followed by electrotransfer to nitrocellulose membranes. Both intact and cleaved troponin-I bands were determined similarly to a recent report (30) by probing the blots using goat anti-troponin-I antibody (sc-8118, Santa Cruz Technology, 1:1000), followed by enhanced chemiluminescence detection.

Data Analysis. Data are expressed as mean ± SEM. All graphs were generated using the GraphPad Prism computer program (San Diego, CA). Data were analyzed using analysis of variance followed by Student-Newman-Keuls’ test. We considered p < .05 as statistically significant.

RESULTS

Assessment of Cardiac Function. Cardiac function was assessed in vivo in a total of 92 animals (53 wild-type [WT] and 39 TNF-α−/− mice). LV +dP/dt max was significantly lower in WT mice after 2 hrs of hindlimb ischemia followed by
0.5–6 hrs of reperfusion (Table 1, p < .05). However, no significant changes of LV + dP/dt max were seen at any time points of reperfusion in TNF-α−/− animals (p = nonsignificant). As a result, LV + dP/dt max was significantly increased at 3 and 6 hrs of reperfusion in TNF-α−/− compared with WT mice (p < .01). Mean arterial blood pressure was significantly lower in WT animals during the first 6 hrs of hindlimb reperfusion (Table 1, p < .05). By 12 hrs of hindlimb reperfusion, all hemodynamic variables were recovered to baseline levels. By contrast, no changes in mean arterial blood pressure were seen in TNF-α−/− animals during the course of reperfusion.

To eliminate reflex influences and loading conditions during *in vivo* measurements, cardiac function was also measured in isolated hearts (Table 2). Mice underwent 2 hrs of hindlimb ischemia followed by 3 hrs of reperfusion. Consistent with *in vivo* measurements, the maximal rate of force development (+dP/dt) and heart work were significantly decreased at baseline in WT but not in TNF-α−/− mice. Response to a β1-adrenergic agonist dobutamine (2 µg added to the perfusate) was significantly decreased following hindlimb I/R in WT but not in TNF-α−/− mice. Treatment with a recombinant soluble TNF-α receptor, etanercept (2 mg/kg intraperitoneally), immediately after induction of hindlimb ischemia significantly improved basal and dobutamine-stimulated cardiac function in WT (p < .05). Heart rate was not significantly altered in TNF-α−/−, WT, or etanercept treatment group. In summary, both *in vivo* and isolated heart measurements showed that cardiac function was higher in TNF-α−/− compared with WT mice, and inhibition of TNF-α restored cardiac function following hindlimb I/R in WT mice.

**Assessment of Myocardial Apoptosis.** The presence of apoptotic cell death in the LV myocardium following hindlimb I/R was demonstrated by DNA fragmentation and caspase-3 activation, hall-

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**Table 1. In vivo assessment of cardiovascular function after 2 hrs of hindlimb ischemia followed by 0.5–24 hrs of reperfusion in wild-type (WT) and tumor necrosis factor (TNF)-α−/− mice**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Genotype</th>
<th>Sham</th>
<th>0.5 Hrs</th>
<th>3 Hrs</th>
<th>6 Hrs</th>
<th>12 Hrs</th>
<th>18 Hrs</th>
<th>24 Hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>WT</td>
<td>438 ± 27</td>
<td>413 ± 20</td>
<td>468 ± 24</td>
<td>443 ± 19</td>
<td>465 ± 24</td>
<td>436 ± 9</td>
<td>426 ± 10</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>421 ± 8</td>
<td>410 ± 19</td>
<td>452 ± 8</td>
<td>444 ± 12</td>
<td>432 ± 10</td>
<td>416 ± 10</td>
<td>430 ± 13</td>
<td></td>
</tr>
<tr>
<td>MAP</td>
<td>WT</td>
<td>77 ± 3</td>
<td>66 ± 2</td>
<td>62 ± 4a</td>
<td>67 ± 3</td>
<td>70 ± 3</td>
<td>67 ± 2</td>
<td>66 ± 3</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>79 ± 4</td>
<td>69 ± 3</td>
<td>76 ± 6b</td>
<td>72 ± 3</td>
<td>76 ± 4</td>
<td>76 ± 4</td>
<td>71 ± 4</td>
<td></td>
</tr>
<tr>
<td>LV + dP/dt max</td>
<td>WT</td>
<td>5.1 ± 0.8</td>
<td>6.1 ± 0.8</td>
<td>5.1 ± 0.6</td>
<td>5.7 ± 0.9</td>
<td>5.6 ± 1.8</td>
<td>6.5 ± 1.5</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>LV − dP/dt min</td>
<td>WT</td>
<td>97 ± 3</td>
<td>87 ± 3</td>
<td>89 ± 4</td>
<td>90 ± 3</td>
<td>94 ± 2</td>
<td>94 ± 3</td>
<td>90 ± 2</td>
</tr>
<tr>
<td>LVSP</td>
<td>WT</td>
<td>104 ± 5</td>
<td>94 ± 4</td>
<td>104 ± 6a</td>
<td>98 ± 4</td>
<td>97 ± 1</td>
<td>110 ± 9a</td>
<td>113 ± 3b</td>
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<tr>
<td>LVSP</td>
<td>TNF-α−/−</td>
<td>4.8 ± 0.6</td>
<td>5.8 ± 0.7</td>
<td>5.2 ± 0.5</td>
<td>5.6 ± 0.5</td>
<td>4.3 ± 0.6</td>
<td>6.4 ± 0.5</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>LVSP</td>
<td>TNF-α−/−</td>
<td>7035 ± 269</td>
<td>4968 ± 145a</td>
<td>5050 ± 392a</td>
<td>5027 ± 199a</td>
<td>6531 ± 265</td>
<td>6211 ± 263</td>
<td>5683 ± 358</td>
</tr>
<tr>
<td>LVSP</td>
<td>TNF-α−/−</td>
<td>6621 ± 482</td>
<td>6605 ± 254</td>
<td>6600 ± 485b</td>
<td>6650 ± 278b</td>
<td>6060 ± 413</td>
<td>6300 ± 713</td>
<td>6750 ± 712</td>
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<tr>
<td>LVSP</td>
<td>TNF-α−/−</td>
<td>6785 ± 258</td>
<td>5031 ± 99c</td>
<td>5100 ± 405c</td>
<td>4944 ± 355c</td>
<td>6250 ± 183</td>
<td>5846 ± 237</td>
<td>5733 ± 325</td>
</tr>
<tr>
<td>LVSP</td>
<td>TNF-α−/−</td>
<td>6786 ± 594</td>
<td>6050 ± 320</td>
<td>6900 ± 367b</td>
<td>6350 ± 145c</td>
<td>6300 ± 496</td>
<td>6650 ± 840</td>
<td>6750 ± 523</td>
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</tbody>
</table>

HR, heart rate (beats/min); MAP, mean arterial pressure (mm Hg); LVEDP, left ventricular end diastolic pressure (mm Hg); LVSP, left ventricular systolic pressure (mm Hg); LV + dP/dt max, maximum rate of left ventricular pressure development (mm Hg/sec); -dP/dt min, maximum rate of left ventricular relaxation (mm Hg/sec).

*p < .01 vs. corresponding sham; **p < .05 vs. wild-type; ***p < .05 vs. corresponding sham. Results are expressed as mean ± SEM. n = 5–7 and 7–8 for each time point in TNF-α−/− and wild-type, respectively.

**Table 2. Ex vivo assessment of cardiac function using an isolated heart preparation after 2 hrs of hindlimb ischemia followed by 3 hrs of reperfusion (I/R) in wild-type mice (WT, n = 7), tumor necrosis factor (TNF)-α−/− mice (n = 6), and WT mice treated with etanercept (n = 6)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Genotype</th>
<th>Sham</th>
<th>I/R</th>
<th>Sham 2 µg Dobutamine</th>
<th>I/R 2 µg Dobutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, beats/min</td>
<td>WT</td>
<td>354 ± 25</td>
<td>329 ± 26</td>
<td>460 ± 20</td>
<td>424 ± 34</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>375 ± 23</td>
<td>337 ± 24</td>
<td>458 ± 15</td>
<td>476 ± 36</td>
<td></td>
</tr>
<tr>
<td>WT + etanercept</td>
<td>361 ± 14</td>
<td>361 ± 14</td>
<td>480 ± 42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Force, g</td>
<td>WT</td>
<td>3.8 ± 0.35</td>
<td>2.9 ± 0.28</td>
<td>5.3 ± 0.41</td>
<td>3.7 ± 0.47</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>3.5 ± 0.33</td>
<td>4.0 ± 0.27a</td>
<td>4.4 ± 0.29</td>
<td>5.2 ± 0.25a</td>
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</tr>
<tr>
<td>WT + etanercept</td>
<td>3.63 ± 0.28</td>
<td>3.63 ± 0.28</td>
<td>5.4 ± 0.2c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+dP/dt max, g/sec</td>
<td>WT</td>
<td>99 ± 9</td>
<td>58 ± 9</td>
<td>205 ± 24</td>
<td>109 ± 16</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>99 ± 11</td>
<td>94 ± 14a</td>
<td>161 ± 18</td>
<td>191 ± 20b</td>
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<tr>
<td>WT + etanercept</td>
<td>82 ± 6b</td>
<td>82 ± 6b</td>
<td>166 ± 12c</td>
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<tr>
<td>-dP/dt min, g/sec</td>
<td>WT</td>
<td>107 ± 9</td>
<td>77 ± 4</td>
<td>186 ± 16</td>
<td>126 ± 16c</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>106 ± 12</td>
<td>105 ± 13</td>
<td>156 ± 13</td>
<td>191 ± 18a</td>
<td></td>
</tr>
<tr>
<td>WT + etanercept</td>
<td>93 ± 5b</td>
<td>93 ± 5b</td>
<td>160 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart work, g · beats/min</td>
<td>WT</td>
<td>1356 ± 184</td>
<td>916 ± 74</td>
<td>2443 ± 227</td>
<td>1535 ± 213c</td>
</tr>
<tr>
<td>TNF-α−/−</td>
<td>1322 ± 185</td>
<td>1352 ± 151a</td>
<td>2634 ± 154</td>
<td>2492 ± 256b</td>
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<tr>
<td>WT + etanercept</td>
<td>1300 ± 94a</td>
<td>1300 ± 94a</td>
<td>2609 ± 293c</td>
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</table>

HR, heart rate; +dP/dt max, maximum rate of force development; -dP/dt min, maximum rate of relaxation.

*p < .05 vs. WT; **p < .01 vs. corresponding sham; ***p < .05 vs. corresponding sham; ****p < .01 vs. WT. Dobutamine (2 µg) was added to the perfusate to measure contractile response to dobutamine stimulation. Etanercept (2 mg/kg intraperitoneally) was administered immediately after hindlimb ischemia. Results are expressed as mean ± SEM.
marks of apoptosis. First, the cytosolic DNA fragments in the myocardium were determined using cell death detection enzyme-linked immunosorbent assay. Cytoplasmic DNA fragments in the LV myocardium were determined by cell death detection enzyme-linked immunosorbent assay, and percent change of control is shown following various times of reperfusion. Cytoplasmic DNA fragments in the LV myocardium were significantly increased after 3 and 6 hrs of reperfusion in WT mice (\( p < .05 \), \( ** p < .01 \) vs. sham, respectively). Myocardial DNA fragments were significantly decreased in TNF-\( \alpha \)-/− mice compared with WT mice (\( p < .05 \)). n = 5–7 and 7–8 for each time point in TNF-\( \alpha \)-/− and WT mice, respectively.

To determine the cell type (myocyte vs. nonmyocyte) in the myocardium undergoing apoptosis, TUNEL staining was used. Myocytes were identified by their distinct morphology under bright field. Since the TUNEL signal was dianinobenzoidine, which was visualized under bright field, troponin-I double staining was not needed. Representative images are shown in Figure 2A. Very few TUNEL-positive nuclei were observed in sham-operated animals. After 2 hrs of hindlimb ischemia followed by 6 hrs of reperfusion, WT mice showed a significant increase in TUNEL-positive nuclei compared with sham controls (\( p < .05 \)). The number of TUNEL-positive nuclei was significantly decreased in TNF-\( \alpha \)-/− compared with WT mice (\( p < .05 \), Fig. 2B). Almost all of the TUNEL-positive nuclei observed were within cardiac myocytes, suggesting that myocytes are the primary cell type undergoing apoptosis in the LV myocardium during hindlimb I/R.

Further confirmation of the presence of apoptosis was provided through the determination of caspase-3 activity. Caspases represent a family of aspartate-associated proteins that are involved in nuclear DNA cleavage. Caspase-3 activity was significantly increased in the LV myocardium of WT animals after 6 hrs of hindlimb reperfusion vs. sham controls (\( p < .05 \)). Furthermore, myocardial caspase-3 activity after 6 hrs of hindlimb reperfusion was significantly decreased in TNF-\( \alpha \)-/− compared with WT mice (\( p < .05 \), Fig. 3).

Myocardial and Plasma TNF-\( \alpha \) Levels. The levels of TNF-\( \alpha \) in the LV myocardium and plasma were investigated in an attempt to correlate levels of TNF-\( \alpha \) to the degree of myocardial dysfunction following hindlimb I/R. Figure 4A shows that plasma TNF-\( \alpha \) levels peaked at 30 mins of hindlimb reperfusion in WT mice...
cytes (Fig. 5, edly increased in the myocardium of WT throughout the time course of reperfu-
and residual LDH activity, was similar 
significant). Thus, the extent of skeletal muscle 
staining was present in TNF-
Immunohistochemical staining con-
reperfusion relative to sham levels (p < .05). Therefore, myocardial TNF-
D, F, and H
two-fold compared with sham controls 
skeletal leg muscle injury was investi-
gated to determine whether there were 
differences between TNF-α−/− and WT mice. Wet/dry ratio, which measures tis-
several days later. In fact, the fact that the 
Figure 3. Myocardial caspase-3 activity after 2 hrs of hindlimb ischemia followed by 6 hrs of reperfu-
sion (I/R) in wild-type (WT) and tumor necrosis factor (TNF-α−/−) mice. Hindlimb I/R significantly 
creased myocardial caspase-3 activity compared with sham operation in WT mice (**p < .05). However, myocardial caspase-3 activity was 
significantly decreased in TNF-α−/− compared with WT mice (p < .05). n = 5–7 per group.

(p < .05) and returned to control levels by 3 hrs of reperfusion. Interestingly, myocardial TNF-α levels also signifi-
cantly increased at 30 mins of hindlimb 
pressure relative to sham levels (p < .01). However, these levels persisted over 12 hrs following reperfusion (Fig. 4B).

DISCUSSION

In the present study, we demonstrated that hindlimb I/R induced myocardial 
TNF-α expression, cardiomyocyte apoptosis, and cardiac dysfunction in WT mice. Interestingly, these changes were 
absent or significantly decreased in TNF-α−/− mice with the same degree of hindlimb skeletal muscle injury as the WT mice. Our study suggests that TNF-α plays an important role in myocardial dysfunction and apoptosis during hindlimb I/R.

TNF-α has been implicated as a primary mediator of various cardiac pathol-
gies, including heart failure, myocardial I/R, and sepsis. In the present study, we 
showed that hindlimb I/R induced a re-
versible reduction in cardiac function that coincided with a time-dependent in-
crease in plasma and myocardial TNF-α levels. The sharp peak of TNF-α levels in the plasma occurring 30 mins after hindlimb reperfusion has been observed in 
previous studies (32, 33). These studies indicate that the plasma TNF-α spike comes from the TNF-α made during hindlimb ischemia and flushed out after 
reperfusion (32, 33). The fact that the elevated systemic TNF-α levels were not 
maintained beyond 3 hrs of hindlimb reperfusion suggests that TNF-α is 
cleared rapidly from the circulation. In-

Skeletal Muscle Injury. The level of skeletal leg muscle injury was investi-
gated to determine whether there were 
differences between TNF-α−/− and WT mice. Wet/dry ratio, which measures tis-

Apoptosis is a highly regulated physi-
ologic process that is crucial to tissue 
development and/or remodeling. How-
ever, excessive apoptosis has been shown to 
contribute to various pathologies in-
cluding sepsis and heart failure (35–37).

In the present study, we provided strong 
evidence for an increased myocardial ap-
optosis following hindlimb I/R in WT 
mice. Myocardial levels of cytoplasmic his-
tone-associated DNA fragments and 
caspase-3 activity were significantly in-
creased in WT mice subjected to hind-
limb I/R. We also observed TUNEL-

Figure 4. Effects of hindlimb ischemia and reper-
fusion (I/R) on plasma (A) and myocardial (B) tumor necrosis factor (TNF-α) levels in wild-type 
(WT) mice. Animals were subjected to 2 hrs of ischemia followed by 0.5–24 hrs of reperfusion or sham operation (time 0). Plasma and myocardial 
TNF-α levels were determined by enzyme-linked 
imunosorbent assay. *p < .05 vs. sham, **p < 
.01 vs. sham, n = 4–7 for each time point per 
group.

Figure 5. Immunohistochemical staining for 
TNF-α in WT and TNF-α−/− hearts 
(Fig. 5, D, F, and H).

Skeletal Muscle Injury. The level of skeletal leg muscle injury was investi-
gated to determine whether there were 
differences between TNF-α−/− and WT mice. Wet/dry ratio, which measures tis-

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ologic process that is crucial to tissue 
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cluding sepsis and heart failure (35–37).

In the present study, we provided strong 
evidence for an increased myocardial ap-
optosis following hindlimb I/R in WT 
mice. Myocardial levels of cytoplasmic his-
tone-associated DNA fragments and 
caspase-3 activity were significantly in-
creased in WT mice subjected to hind-
limb I/R. We also observed TUNEL-
positive staining mainly present in cardiomyocytes. Taken together, all three 
measures of apoptosis indicated that 
hindlimb I/R induced a significant in-
crease in myocardial apoptosis in WT 
mice. Interestingly, myocardial apoptosis 
was almost abrogated in TNF-α−/− mice, 
suggesting that TNF-α plays a critical 
role in cardiomyocyte apoptosis following 
hindlimb I/R.

It is well known that TNF-α impairs 
myocardial function and has been impli-
cated in clinical pathologies including heart failure and sepsis (16, 17). In the present study, we demonstrated that myocardial TNF-α expression was markedly increased and was mainly produced in cardiomyocytes following hindlimb I/R. Increased myocardial TNF-α expression was associated with decreased cardiac function in WT mice. Decreased cardiac function was demonstrated in both in vivo whole animal models and isolated hearts. To study the specific role of TNF-α in cardiac dysfunction during hindlimb I/R, we employed TNF-α−/− mice. We showed that cardiac function in both in vivo and isolated heart preparations was well preserved in TNF-α−/− mice. Furthermore, inhibition of TNF-α by etanercept restored cardiac function in WT mice. These results suggest an important role of TNF-α in cardiac dysfunction post hindlimb I/R.

In the present study, skeletal muscle injury following hindlimb I/R was similar between TNF-α−/− and WT mice as determined by wet/dry ratio and residual LDH activity. However, cleavage of cardiac troponin-I was barely detectable in TNF-α−/− mice. The fact that TNF-α−/− mice had much less cardiac troponin-I cleavage but similar skeletal muscle injuries with the WT further supports the notion that TNF-α is pivotal in the development of myocardial apoptosis and dysfunction post hindlimb I/R.

CONCLUSIONS

Hindlimb I/R induced myocardial TNF-α expression, cardiomyocyte apoptosis, and cardiac dysfunction in WT mice. Deficiency in TNF-α protected the myocardium from apoptosis and improved cardiac function during the same degree of hindlimb injury. We conclude that TNF-α contributes significantly to myocardial dysfunction and apoptosis in hindlimb I/R. Although a causal link between myocardial apoptosis and cardiac dysfunction is not established, our study does suggest that TNF-α may be a potential therapeutic tar-

Figures 5 and 6.
get for cardiac injury in clinical situations involving prolonged remote I/R.

REFERENCES

22. Carlson DL, Willis MS, White DJ, et al: Tu-


