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

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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-$\alpha^{-/-}$ and littermate wild-type mice.**

Interventions: **Bilateral hindlimb ischemia/reperfusion was induced in wild-type and tumor necrosis factor-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-

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optosis may in part contribute to the development of cardiac dysfunction post hindlimb I/R.

METHODS

Animals. Breeding pairs of TNF-$\alpha^{-/-}$ and C57BL/6 mice were purchased from the Jackson Laboratory. TNF-$\alpha^{-/-}$ mice were backcrossed to C57BL/6 background for at least six generations. Male adult TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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, maximal rate of pressure development ($+dP/dt_{max}$), and maximal rate of relaxation of left ventricle ($-dP/dt_{max}$). 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% CO_2. 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 heart work was calculated by multiplying the force (g) by the heart rate (beats/min). Maximal and minimal first derivative of force ($+dF/dt_{max}$ and $-dF/dt_{min}$) 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 cytoplasmic 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 H_2O_2. 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ animals (p = nonsignificant). As a result, LV $+dP/dt_{max}$ was significantly increased at 3 and 6 hrs of reperfusion in TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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 (+dF/dt) and heart work were significantly decreased at baseline in WT but not in TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ 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-$\alpha^{-/-}$, WT, or etanercept treatment group. In summary, both *in vivo* and isolated heart measurements showed that cardiac function was higher in TNF-$\alpha^{-/-}$ 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-

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)-$\alpha^{-/-}$ mice

Variables	Genotype	Sham	0.5 Hrs	3 Hrs	6 Hrs	12 Hrs	18 Hrs	24 Hrs
HR	Wild-type	438 ± 27	433 ± 24	468 ± 24	434 ± 19	465 ± 24	436 ± 9	426 ± 10
	TNF-$\alpha^{-/-}$	421 ± 8	410 ± 19	452 ± 8	444 ± 12	432 ± 10	416 ± 10	430 ± 13
MAP	Wild-type	77 ± 3	66 ± 2	62 ± 4[a]	67 ± 3	70 ± 3	67 ± 2	66 ± 3
	TNF-$\alpha^{-/-}$	79 ± 4	69 ± 3	76 ± 6[b]	72 ± 3	76 ± 4	76 ± 4	71 ± 4
LVEDP	Wild-type	4.8 ± 0.6	5.8 ± 0.7	5.2 ± 0.5	5.6 ± 0.5	4.3 ± 0.6	6.4 ± 0.5	4.8 ± 0.6
	TNF-$\alpha^{-/-}$	5.1 ± 0.8	6.1 ± 0.8	5.1 ± 0.6	5.7 ± 0.9	5.6 ± 1.8	6.5 ± 1.5	3.1 ± 1.2
LVSP	Wild-type	97 ± 3	87 ± 2	89 ± 4	90 ± 3	94 ± 2	94 ± 3	90 ± 2
	TNF-$\alpha^{-/-}$	104 ± 5	94 ± 4	104 ± 6[b]	98 ± 4	97 ± 1	110 ± 9[c]	113 ± 3[b]
LV $+dP/dt_{max}$	Wild-type	7035 ± 269	4968 ± 145[a]	5050 ± 392[a]	5027 ± 199[a]	6531 ± 265	6211 ± 263	5683 ± 358
	TNF-$\alpha^{-/-}$	6621 ± 482	6050 ± 254	6600 ± 485[b]	6550 ± 278[c]	6050 ± 413	6300 ± 713	6750 ± 712
LV $-dP/dt_{min}$	Wild-type	6785 ± 258	5031 ± 99[d]	5100 ± 405[a]	4944 ± 355[d]	6250 ± 183	5846 ± 237	5733 ± 325
	TNF-$\alpha^{-/-}$	6786 ± 594	6050 ± 320	6900 ± 367[b]	6350 ± 145[c]	6300 ± 496	6650 ± 840	6750 ± 523

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).

[a] $p < .01$ vs. corresponding sham; [b] $p < .01$ vs. wild-type; [c] $p < .05$ vs. wild-type; [d] $p < .05$ vs. corresponding sham. Results are expressed as mean ± SEM. n = 5–7 and 7–8 for each time point in TNF-$\alpha^{-/-}$ 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)-$\alpha^{-/-}$ mice (n = 6), and WT mice treated with etanercept (n = 6)

Parameters	Genotype	Sham	I/R	Sham 2 μg Dobutamine	I/R 2 μg Dobutamine
HR, beats/min	WT	354 ± 25	329 ± 26	460 ± 20	424 ± 34
	TNF-$\alpha^{-/-}$	375 ± 23	337 ± 24	458 ± 15	476 ± 36
	WT + etanercept		361 ± 14		480 ± 42
Force, g	WT	3.8 ± 0.35	2.9 ± 0.28	5.3 ± 0.41	3.7 ± 0.47
	TNF-$\alpha^{-/-}$	3.5 ± 0.33	4.0 ± 0.27[a]	4.4 ± 0.29	5.2 ± 0.25[a]
	WT + etanercept		3.63 ± 0.28		5.4 ± 0.2[a]
$+dF/dt_{max}$, g/sec	WT	99 ± 9	58 ± 5[b]	205 ± 24	109 ± 16[c]
	TNF-$\alpha^{-/-}$	99 ± 11	94 ± 14[a]	161 ± 18	191 ± 20[d]
	WT + etanercept		82 ± 6[a]		166 ± 12[a]
$-dF/dt_{min}$, g/sec	WT	107 ± 9	77 ± 4[c]	186 ± 16	126 ± 16[c]
	TNF-$\alpha^{-/-}$	106 ± 12	105 ± 13	156 ± 13	191 ± 18[a]
	WT + etanercept		93 ± 5[a]		160 ± 9
Heart work, g · beats/min	WT	1356 ± 184	916 ± 74	2443 ± 227	1535 ± 213[c]
	TNF-$\alpha^{-/-}$	1322 ± 185	1352 ± 151[a]	2034 ± 154	2492 ± 256[d]
	WT + etanercept		1300 ± 94[a]		2609 ± 293[a]

HR, heart rate; $+dF/dt_{max}$, maximum rate of force development; $-dF/dt_{min}$, maximum rate of relaxation.

[a] $p < .05$ vs. WT; [b] $p < .01$ vs. corresponding sham; [c] $p < .05$ vs. corresponding sham; [d] $p < .01$ 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.

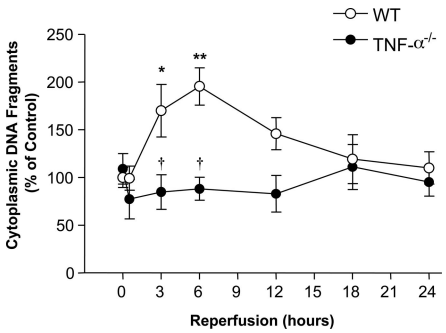

Figure 1. Accumulation of cytoplasmic DNA fragments in the left ventricular (LV) myocardium during hindlimb ischemia and reperfusion in wild-type (*WT*) and tumor necrosis factor (*TNF*)-$\alpha^{-/-}$ mice. Animals were subjected to 2 hrs of hindlimb ischemia followed by 0.5–24 hrs of reperfusion. Sham animals (time 0) underwent anesthesia but without hindlimb ischemia/reperfusion. Cytoplasmic DNA fragments from 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-$\alpha^{-/-}$ compared with WT mice (†$p < .05$). n = 5–7 and 7–8 for each time point in TNF-$\alpha^{-/-}$ and WT mice, respectively.

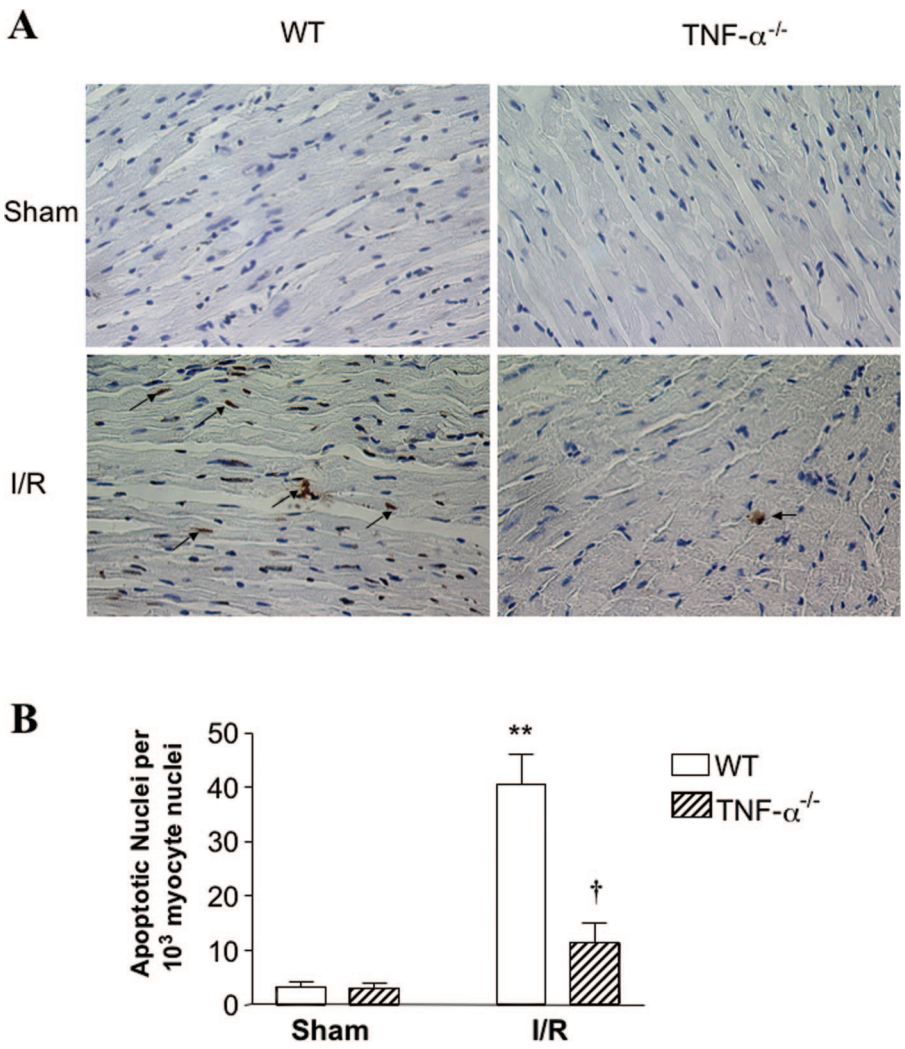

Figure 2. Myocardial apoptosis as measured by terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL) staining. Animals were subjected to sham operation or 2 hrs of hindlimb ischemia followed by 6 hrs of reperfusion (*I/R*) in wild-type (*WT*) and tumor necrosis factor (*TNF*)-$\alpha^{-/-}$ mice. TUNEL staining was performed on paraffin-embedded heart sections. *A*, representative images (original magnification ×400). Sham-operated mice showed very few TUNEL-positive nuclei in WT and TNF-$\alpha^{-/-}$ mice. A marked increase in the number of TUNEL-positive nuclei (*arrows*) was evident in the left ventricle (LV) myocardium of WT compared with that of TNF-$\alpha^{-/-}$ mice. *B*, quantitative analysis of myocyte apoptosis. Apoptotic nuclei in the LV myocardium were significantly increased after I/R compared with sham operation in WT mice (**$p < .01$). Apoptotic nuclei after I/R were significantly decreased in TNF-$\alpha^{-/-}$ compared with WT mice (†$p < .05$). n = 4 per group.

marks of apoptosis. First, the cytosolic DNA fragments in the myocardium were determined using cell death detection enzyme-linked immunosorbent assay. Cytosolic DNA fragments were significantly increased at 3 and 6 hrs of reperfusion in WT mice (Fig. 1, $p < .05$) and gradually returned to control levels after 12 hrs of reperfusion. By contrast, animals lacking TNF-α showed no significant increase in the level of myocardial DNA fragmentation at any time points following hindlimb reperfusion (p = nonsignificant).

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 diaminobenzidine, which was visualized under bright field, troponin-I double staining was not needed. Representative images are shown in Figure 2*A*. 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 ($p < .05$, Fig. 2*B*). The number of TUNEL-positive nuclei was significantly decreased in TNF-$\alpha^{-/-}$ compared with WT mice ($p < .05$, Fig. 2*B*). 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-α Levels. The levels of TNF-α in the LV myocardium and plasma were investigated in an attempt to correlate levels of TNF-α to the degree of myocardial dysfunction following hindlimb I/R. Figure 4*A* shows that plasma TNF-α levels peaked at 30 mins of hindlimb reperfusion in WT mice

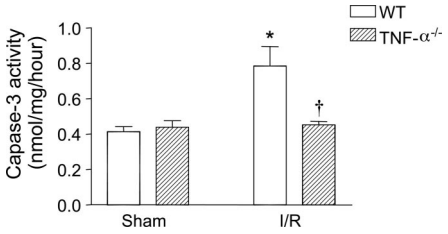

Figure 3. Myocardial caspase-3 activity after 2 hrs of hindlimb ischemia followed by 6 hrs of reperfusion (*I/R*) in wild-type (*WT*) and tumor necrosis factor (*TNF*)-$\alpha^{-/-}$ mice. Hindlimb I/R significantly increased myocardial caspase-3 activity compared with sham operation in WT mice (*$p <$ .05). However, myocardial caspase-3 activity was significantly decreased in TNF-$\alpha^{-/-}$ 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 significantly increased at 30 mins of hindlimb reperfusion relative to sham levels ($p <$ .01). However, these levels persisted over 12 hrs following reperfusion (Fig. 4*B*). Immunohistochemical staining confirmed myocardial TNF-α production. Following 0.5, 3, and 6 hrs of hindlimb reperfusion, TNF-α staining was markedly increased in the myocardium of WT mice and mainly present in cardiomyocytes (Fig. 5, *C, E, and G*). No TNF-α staining was present in TNF-$\alpha^{-/-}$ hearts (Fig. 5, *D, F, and H*).

Skeletal Muscle Injury. The level of skeletal leg muscle injury was investigated to determine whether there were differences between TNF-$\alpha^{-/-}$ and WT mice. Wet/dry ratio, which measures tissue edema, was significantly increased in the leg muscle following hindlimb I/R, peaking at the 6-hr time point by about two-fold compared with sham controls (Fig. 6). This response was similar between TNF-$\alpha^{-/-}$ and WT mice (p = nonsignificant). Skeletal leg muscle injury was further assessed by measuring the activity of LDH, a constitutively expressed enzyme present in muscle tissue (Fig. 7*A*). As expected, LDH activity levels in the tissue were high in sham animals and significantly decreased after 6 hrs of reperfusion ($p <$ .05). However, there was no significant difference between TNF-$\alpha^{-/-}$ and WT mice (p = nonsignificant). Thus, the extent of skeletal muscle injury, as measured by both wet/dry ratio and residual LDH activity, was similar throughout the time course of reperfusion for both TNF-$\alpha^{-/-}$ and WT animals.

Cardiac Injury. To verify cardiac injury, both TNF-$\alpha^{-/-}$ and WT mice were subjected to 2 hrs of hindlimb ischemia followed by 3 hrs of reperfusion. Troponin-I cleavage in the LV myocardium, an index of cardiac injury, was clearly seen in WT mice but barely detectable in TNF-$\alpha^{-/-}$ mice (Fig. 7*B*). There was no troponin-I cleavage in sham animals. An LV sample from a WT mouse 30 days after myocardial infarction served as a positive control (31). Our data showed that cardiac injury following hindlimb I/R was attenuated in TNF-$\alpha^{-/-}$ mice.

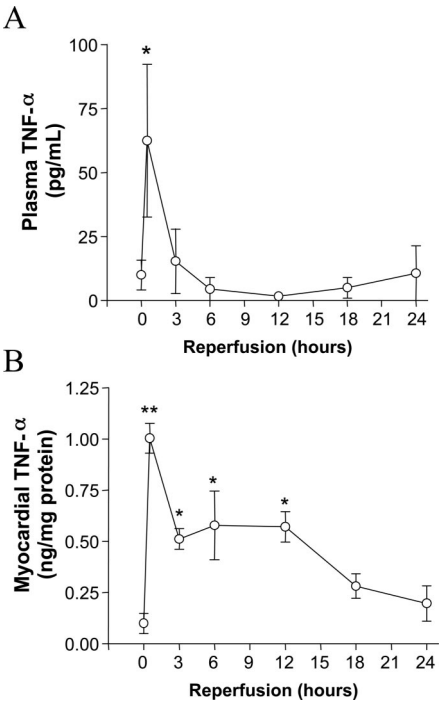

Figure 4. Effects of hindlimb ischemia and reperfusion (I/R) on plasma (*A*) and myocardial (*B*) tumor necrosis factor (*TNF*)-α levels in wild-type (WT) mice. Animals were subjected to 2 hrs of ischemia followed by 0.5–24 hrs of reperfusion or sham operation (time 0). Plasma and myocardial TNF-α levels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

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-$\alpha^{-/-}$ 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 pathologies, including heart failure, myocardial I/R, and sepsis. In the present study, we showed that hindlimb I/R induced a reversible reduction in cardiac function that coincided with a time-dependent increase 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. Interestingly, myocardial TNF-α levels also peaked at 30 mins but persisted over 12 hrs following hindlimb reperfusion. One potential explanation for the rapid reduction in systemic TNF-α levels is that TNF-α binds to its receptors in organs and tissues including the heart, thus removing it from circulation. The rapid increase in plasma levels of TNF-α may trigger a sustained local production of TNF-α in the heart, possibly via reactive oxygen species (34). In fact, cardiomyocytes are the major site of TNF-α production in the heart following hindlimb I/R as demonstrated by our immunohistochemical staining.

Apoptosis is a highly regulated physiologic process that is crucial to tissue development and/or remodeling. However, excessive apoptosis has been shown to contribute to various pathologies including sepsis and heart failure (35–37). In the present study, we provided strong evidence for an increased myocardial apoptosis following hindlimb I/R in WT mice. Myocardial levels of cytoplasmic histone-associated DNA fragments and caspase-3 activity were significantly increased in WT mice subjected to hindlimb 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 increase in myocardial apoptosis in WT mice. Interestingly, myocardial apoptosis was almost abrogated in TNF-$\alpha^{-/-}$ 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-

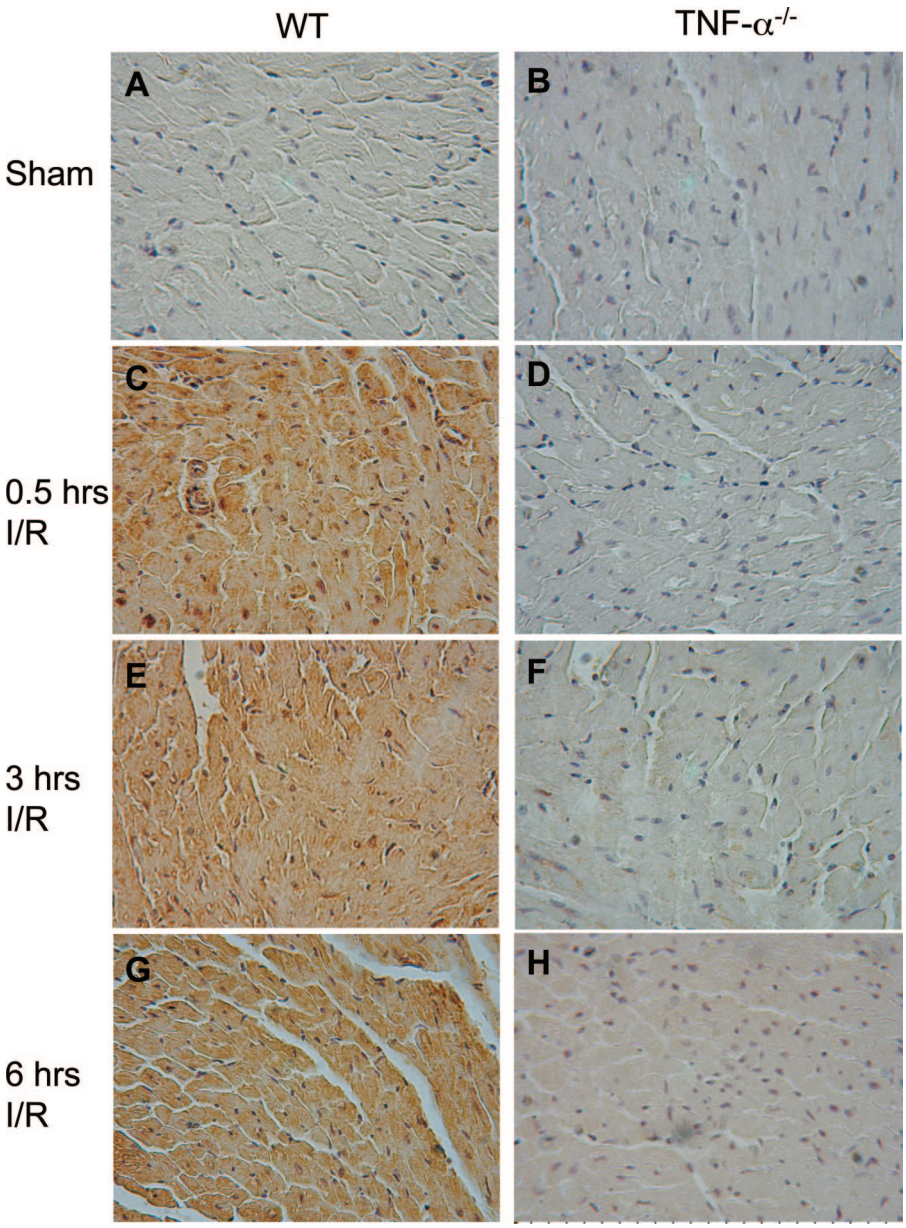

Figure 5. Immunohistochemical staining of tumor necrosis factor (*TNF*)-α in the left ventricle (LV) myocardium after 2 hrs of ischemia followed by 0.5, 3, and 6 hrs of reperfusion (*I/R*) in wild-type (*WT*) and TNF-$\alpha^{-/-}$ mice. Sham and TNF-$\alpha^{-/-}$ mice showed no TNF-α staining (*A, B, D, F, H*). Marked TNF-α staining was seen in the myocardium after 0.5, 3, and 6 hrs of reperfusion in WT mice (*C, E, G*). Shown are representatives of three independent experiments. (Original magnification ×500).

Tumor necrosis factor-α contributes significantly to myocardial dysfunction and apoptosis in hindlimb ischemia/reperfusion.

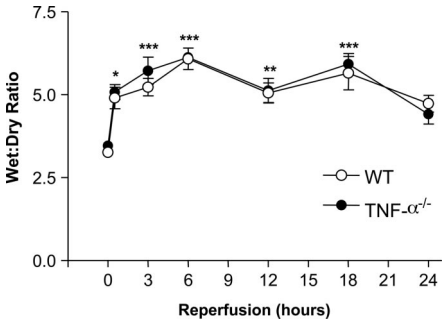

Figure 6. Changes of skeletal muscle wet/dry ratio after 2 hrs of hindlimb ischemia followed by 0.5–24 hrs of reperfusion in wild-type (*WT*) and tumor necrosis factor (*TNF*)-$\alpha^{-/-}$ mice. Following reperfusion, the wet/dry ratio was significantly increased in both WT and TNF-$\alpha^{-/-}$ mice. However, there was no statistical difference between the two groups. *$p < .05$, **$p < .01$, ***$p < .001$ vs. corresponding sham (time 0), respectively. n = 4–7 for each time point per group.

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-$\alpha^{-/-}$ mice. We showed that cardiac function in both *in vivo* and isolated heart preparations was well preserved in TNF-$\alpha^{-/-}$ 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-$\alpha^{-/-}$ and WT mice as determined by wet/dry ratio and residual LDH activity. However, cleavage of cardiac troponin-I was barely detectable in TNF-$\alpha^{-/-}$ mice. The fact that TNF-$\alpha^{-/-}$ 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-

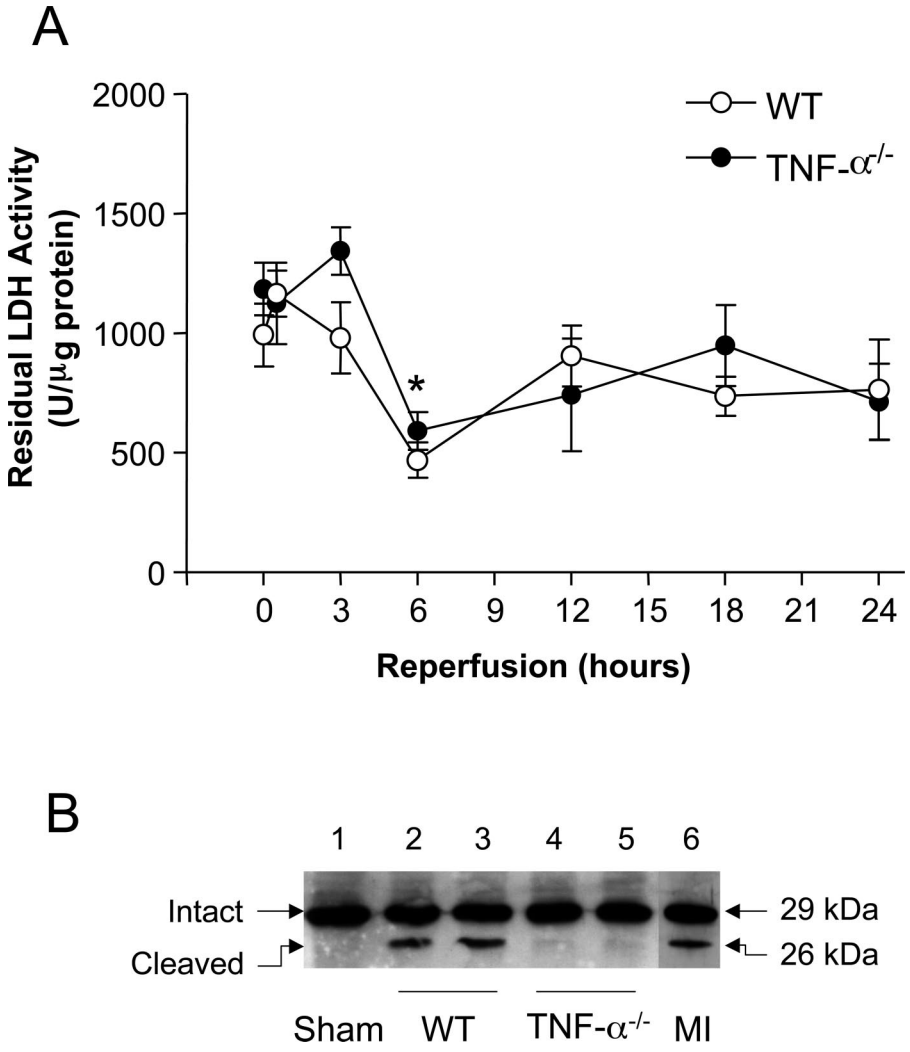

Figure 7. Skeletal muscle and cardiac injury. *A*, changes of residual skeletal muscle lactate dehydrogenase (*LDH*) activity after 2 hrs of hindlimb ischemia followed by 0.5–24 hrs of reperfusion or sham operation (time 0) in wild-type (*WT*) and tumor necrosis factor (*TNF*)-$\alpha^{-/-}$ mice. Following 6 hrs of reperfusion, LDH activity was significantly decreased in both WT and TNF-$\alpha^{-/-}$ mice. However, there was no statistical difference between the two groups. *$p < .05$ vs. corresponding sham. n = 4–7 for each time point per group. *B*, representative Western blot analysis of cardiac troponin-I. Marked cleavage of troponin-I from the left ventricle (LV) myocardium was detected in WT mice after 2 hrs of hindlimb ischemia followed by 3 hrs of reperfusion (I/R, *lanes 2* and *3*). The cleaved band was barely detectable in TNF-$\alpha^{-/-}$ mice subjected to the same hindlimb I/R protocol (*lanes 4* and *5*). The noninfarcted LV myocardium from mice subjected to coronary artery ligation (*MI*) for 30 days served as positive controls (*lane 6*). Each lane represents a sample from an individual mouse. n = 4 for each I/R group. n = 2 for each sham group.

get for cardiac injury in clinical situations involving prolonged remote I/R.

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