

## Role of TNF- $\alpha$ in myocardial dysfunction after hemorrhagic shock and lower-torso ischemia

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**Shahani, Rohan, John G. Marshall, Barry B. Rubin, Ren-Ke Li, Paul M. Walker, and Thomas F. Lindsay.** Role of TNF- $\alpha$  in myocardial dysfunction after hemorrhagic shock and lower-torso ischemia. *Am. J. Physiol. Heart Circ. Physiol.* 278: H942–H950, 2000.—Ruptured abdominal aortic aneurysm (RAAA) repair, a combination of hemorrhagic shock and lower-torso ischemia, is associated with a 50–70% mortality. Myocardial dysfunction may contribute to the high rate of mortality after aneurysm repair. We attempted to determine whether RAAA repair results in cardiac dysfunction mediated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). We modeled aortic rupture and repair in the rat by inducing hemorrhagic shock to a mean blood pressure of 50 mmHg for 1 h, followed by supramesenteric clamping of the aorta for 45 min. After 90 min of reperfusion, cardiac contractile function was assessed with a Langendorff preparation. Myocardial TNF- $\alpha$ , ATP and creatine phosphate (CP) levels, and markers of oxidant stress ( $F_2$ -isoprostanes) were measured. Cardiac function in the combined shock and clamp rats was significantly depressed compared with sham-operated control rats but was similar to that noted in animals subjected to shock alone. Myocardial TNF- $\alpha$  concentrations increased 10-fold in the combined shock and clamp rats compared with sham rats, although there was no difference in myocardial ATP, CP, or  $F_2$ -isoprostanes. TNF- $\alpha$  neutralization improved cardiac function by 50% in the combined shock and clamp rats. Hemorrhagic shock is the primary insult inducing cardiac dysfunction in this model of RAAA repair. An improvement in cardiac contractile function after immunoneutralization of TNF- $\alpha$  indicates that TNF- $\alpha$  mediates a significant portion of the myocardial dysfunction in this model.

ruptured abdominal aortic aneurysm; left ventricular function; cytokines; neutrophils; oxidant stress

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RUPTURE OF an abdominal aortic aneurysm (RAAA) remains a major cause of mortality in males over the age of 65 (8). The survival rate after RAAA repair has not improved over the last decade, despite a drop in the mortality of elective abdominal aortic aneurysm repair (22). RAAA induces hypotension secondary to bleeding into the retroperitoneum or abdomen. When repair is undertaken the aorta must be clamped, inducing lower-torso and possibly mesenteric ischemia, depending on the location of clamp application. Thus the rupture and

repair of an abdominal aortic aneurysm is a combination of two sequential ischemia-reperfusion events (hemorrhagic shock and lower-torso ischemia). The causes of mortality after RAAA repair include irreversible shock, bleeding, myocardial infarction, and cardiac failure. It has been suggested that a significant portion of the postoperative mortality and morbidity is secondary to myocardial dysfunction (38). Optimal myocardial function is required to withstand the sequential insults of hypotension, aortic clamping, and reperfusion to enable postoperative recovery. Hence, we have developed a model to test interventions that may enhance cardiac function after the combination of hemorrhagic shock and lower-torso ischemia.

In this model, hemorrhagic shock represents the event of aortic rupture in a patient with RAAA. Hemorrhagic shock followed by resuscitation represents a whole body ischemia-reperfusion event and has been found to result in multiple organ injury (37). Horton (16) previously demonstrated that hemorrhage to a mean arterial pressure (MAP) of 30 mmHg for 2 h, followed by resuscitation for 20 min, results in a 45% reduction in cardiac contractile function. The resultant depression in myocardial function can further exacerbate the hypoperfusion of various organ systems, which may potentiate organ injury after shock. Other inflammatory and ischemic injuries remote from the heart, including burn shock and intestinal ischemia-reperfusion, have also been shown to result in cardiac dysfunction (17, 18). Hemorrhagic shock also may act as a priming stimulus for neutrophils (9, 27), and the addition of a subsequent injury after hemorrhagic shock may result in significant increases in neutrophil-mediated organ injury. Addition of a soft tissue injury in a swine model that includes hemorrhagic shock resulted in severe depression of cardiac function compared with hemorrhagic shock alone (33). The combination of hemorrhagic shock and lower-torso ischemia has been shown to act synergistically to induce increased lung and intestinal protein leak, neutrophil sequestration, and oxidative injury (4, 27). Hence, we examined whether hemorrhagic shock may prime the heart to respond in an exaggerated fashion to a second stimulus, such as aortic clamping, to result in depressed myocardial function.

Recent evidence has linked several cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$ , and interleukin-6 to cardiac dysfunction in various models

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of injury (12, 23, 24). Administration of TNF- $\alpha$  to both isolated cardiomyocytes as well as isolated whole heart preparations has been found to cause depressed contractile function (7, 21, 28, 39). In addition, there is considerable evidence indicating that TNF- $\alpha$  plays a significant role in the cardiac dysfunction associated with endotoxemia, chronic heart failure, and myocardial infarction (5, 20, 29, 36). Rat myocardial TNF- $\alpha$  synthesis has been found to increase 10-fold after hemorrhagic shock and resuscitation and is associated with the translocation of the transcription factor, nuclear factor- $\kappa$ B (NF- $\kappa$ B), to the nucleus (30). Neutralization of TNF- $\alpha$  by a soluble dimeric TNF- $\alpha$  receptor (TNFR:Fc) was able to significantly diminish the cardiac dysfunction seen 24 h after a surface burn injury (11). These studies demonstrate that TNF- $\alpha$  may play a role in the cardiac dysfunction seen in a variety of pathological proinflammatory states.

The primary purpose of this study was to determine whether the combination of hemorrhagic shock and lower-torso ischemia, which individually induce cardiac dysfunction, act synergistically to induce depressed cardiac contractile function. We also sought to determine whether TNF- $\alpha$  is a mediator of the myocardial dysfunction observed after simulated RAAA repair.

## MATERIALS AND METHODS

### *Surgical Procedure*

In a model of RAAA repair designed in our laboratory, adult male Sprague-Dawley rats weighing 350–400 g (Charles River, Wilmington, MA) were allowed to acclimatize for 5 days with water and rat chow ad libitum. All experiments were carried out in accordance with the requirements of the Animals for Research Act of the province of Ontario and with the regulations of the Toronto Hospital Animal Care Committee. Rats were pretreated intramuscularly with atropine sulfate (25  $\mu$ g/kg) and anesthetized intraperitoneally with pentobarbital sodium (50 mg/kg). Catheters (22 gauge) were placed in the tail vein and carotid artery. Supplemental anesthetic, return of withdrawn blood, and fluid resuscitation (lactated Ringer solution) were administered through the intravenous line; the carotid artery was utilized for measurement of mean arterial blood pressure (Hewlett-Packard model 78304A, Palo Alto, CA) and removal of blood for the induction of hemorrhagic shock. A tracheostomy (14-gauge catheter) and laparotomy were performed. The abdominal aorta was isolated between the celiac axis and the superior mesenteric artery and immediately proximal to the aortic bifurcation. The abdomen was then closed and the animal allowed to stabilize for 30 min.

### *Experimental Groups*

Rats were divided into four groups. The first group consisted of sham-operated control rats. The second group underwent shock alone. After 30 min of stabilization and 30 min of baseline measurements, blood was withdrawn from the carotid artery to maintain a MAP of 50 mmHg for a period of 60 min. After this period, half the shed blood volume was returned and the second half returned 45 min later. The animals then underwent 90 min of reperfusion. In the third group (clamp alone), after stabilization and a baseline and 60-min time-matched monitoring period, animals underwent 45 min of aortic occlusion. An atraumatic microvascular clip

was applied to the abdominal aorta proximal to the superior mesenteric artery; a second clip was applied proximal to the aortic bifurcation. After clamp removal, animals were allowed to reperfuse for 90 min. The final group (S + C) underwent a sequential period of hemorrhagic shock (MAP = 50 mmHg for 60 min) and supramesenteric aortic clamping (45 min), followed by 90 min of reperfusion. All animals were supplemented with lactated Ringer solution to maintain a MAP of 100 mmHg during the 90-min reperfusion period (see Fig. 1).

### *Assessment of Left Ventricular Function*

After the 90-min reperfusion period, heparin (200 IU) was given to prevent coagulation, and the hearts were rapidly excised and placed in 4°C Krebs-Henseleit bicarbonate (KHB) buffer. The KHB buffer used in this study was similar to that previously reported with isolated heart muscle preparations (1). The solution contained the following (in mM): 118 NaCl, 4.7 KCl, 21 NaHCO<sub>3</sub>, 1.25 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, and 11 glucose. All solutions were prepared daily with deionized water and bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub>. The pH of the solution was 7.4 and the temperature was maintained at 37°C. The ascending aorta was cannulated with an 18-gauge cannula that was subsequently connected through glass tubing to a KHB buffer reservoir for perfusion of the coronary circulation at a constant pressure of 120 cmH<sub>2</sub>O. Intraventricular pressure was measured with a saline-filled latex balloon attached to a polyethylene tube and threaded into the left ventricular chamber through the left auricle. Left ventricular pressure was measured with a mini pressure transducer (Gould Electronics, Valley View, OH) attached to the balloon cannula. Left ventricular maximal pressure increase and decrease over time (+ and - dP/dt<sub>max</sub>) values were obtained with the use of an electronic differentiator (model 13-4615-17, Gould Electronics) and recorded with a Windo-Graph chart recording system (Gould Electronics). After 20 min of stabilization, coronary effluent was collected over a 2-min period to determine coronary flow rates (26).

A Starling relationship for the different groups was determined by plotting left ventricular peak systolic pressure (PSP), +dP/dt<sub>max</sub> (a measure of contractility), and -dP/dt<sub>max</sub> (a measure of relaxation) against the physical parameters of increasing left ventricular volume and increasing end-diastolic pressure. As a second method of determining cardiac contractile function independent of alterations in ventricular volume, we chemically stimulated the isolated heart with the  $\beta$ -adrenergic stimulant isoproterenol as previously described (19). Left ventricular pressure was maintained at 5 mmHg on isoproterenol stimulation. Increasing concentrations of isoproterenol were utilized, and it was noted that maximal cardiac stimulation occurred at a concentration of 50 ng/ml.

The relationship between left ventricular capacity and balloon volume was determined by plotting the pressure-volume relationship of the isolated balloon. All experiments were performed on the flat portion of the balloon pressure-volume curve.

### *Biochemical Analysis of Myocardium*

Heart biopsies were collected for analyses of TNF- $\alpha$  and energy metabolites, markers of lipid peroxidation, and neutrophil sequestration.

*Heart collection.* Rats underwent sham operation, shock alone, aortic clamping alone, or the combination of shock and aortic clamping (6 per group). The hearts were collected for biochemical analyses after the 90-min reperfusion period by in situ freeze-clamping (3), flash frozen in liquid N<sub>2</sub>, and stored at -80°C until analysis.

**Myocardial TNF- $\alpha$  quantification.** Frozen biopsies were homogenized according to the method of Torre-Amione et al. (35). Briefly, samples were suspended in phosphate-buffered saline (PBS) containing phenylmethylsulfonyl fluoride (PMSF, 1.49 mM), leupeptin (475.6  $\mu$ M), and aprotinin (0.31  $\mu$ M). The homogenates were centrifuged for 20 min at 20,000 *g*. The pellet was solubilized according to the method of Stauber et al. (34) by resuspension in an equal volume of PBS containing PMSF (1 mM), aprotinin (50  $\mu$ l), and 1% Triton X-100. After 1 h of incubation at 4°C, the solubilized protein was centrifuged for 20 min at 20,000 *g*. The supernatant was analyzed in duplicate through the use of the Cytoscreen rat TNF- $\alpha$  ELISA kit (Biosource International, Camarillo, CA). This assay is linear between 0 and 1,000 pg/ml. TNF- $\alpha$  levels were standardized to total soluble protein content, determined using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL).

**Myocardial energy stores.** Determination of myocardial ATP and creatine phosphate (CP) was adapted from Harris et al. (14). Each sample was freeze-dried for 24 h (Lyph-Lock 6 Lyophilizer, Labconco, Kansas City, MO) and stored at -80°C. On analysis, dried muscle tissue was separated from any remaining blood or connective tissue and homogenized in 0.5 M perchloric acid (PCA) with 1 mM EDTA. Samples were centrifuged at 3,000 *g*, and the supernatant was collected and neutralized with 2 M KOH and 0.5 M PCA. Twenty microliters of neutralized substrate were added to 2 ml of sample buffer [including 1 M Tris·HCl, pH 8.1, 0.1 M MgCl<sub>2</sub>, 50 mM dithiothreitol, 50 mM NADP, 10 mM glucose, and 10  $\mu$ l glucose-6-phosphate in a final volume of 50 ml]. Enzymatic reactions utilizing hexokinase and creatine phosphokinase were performed, and NADPH production was measured by fluorometric analysis (COBAS FARA, Roche Diagnostic Systems, Nutley, NJ) at an excitation wavelength of 340 nm and an emission wavelength of 470 nm. Each unit of NADPH produced represented 1 unit of ATP or CP.

**F<sub>2</sub>-isoprostane quantification.** The extent of membrane lipid peroxidation was estimated using the production of F<sub>2</sub>-isoprostanes, which have previously been shown to provide a reliable index of lipid peroxidation (4, 31). Frozen biopsies were thawed and homogenized with a blade homogenizer in PBS. Tritiated prostaglandin F<sub>2 $\alpha$</sub>  (PGF<sub>2 $\alpha$</sub> ) was added to each sample to determine recoveries after extraction. Protein was extracted by ethanol precipitation, centrifuged, and discarded. Fatty acid side chains, including F<sub>2</sub>-isoprostanes, were cleaved from their glycerol backbone by alkaline hydrolysis. The samples were then acidified with HCl to a pH <4 and loaded onto an activated C<sub>18</sub> reverse-phase SPE cartridge (Sep-Pak Column, Waters, Mississauga, Ontario, Canada). The column was washed with ultrapure water and hexane, the F<sub>2</sub>-isoprostanes eluted with ethyl acetate containing 1% methanol, and the eluent evaporated to dryness under nitrogen and stored at -80°C. Samples were reconstituted and analyzed in duplicate utilizing a commercially available 8-epi F<sub>2</sub>-isoprostane enzyme immunoassay (Cayman, Ann Arbor, MI). Extraction efficiency from the purification step was analyzed by scintillation counting of each sample and averaged 70%. F<sub>2</sub>-isoprostane values were standardized to tissue protein content.

**Myocardial myeloperoxidase assay.** Neutrophil sequestration in the heart was quantified by the presence of myeloperoxidase by the method of Mullane et al. (32). Tissue samples were homogenized with a blade homogenizer (Polytron, Brinkman, Westbury, NY) in ice-cold phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA. The homogenate was then sonicated (Vibra Cell Sonicator, Sonics and Materials, Danbury, CT) and centri-

fuged at 12,000 rpm. One hundred microliters of supernatant were added to 2.9 ml of assay buffer containing Na<sub>2</sub>PO<sub>4</sub>, 0.3% H<sub>2</sub>O<sub>2</sub>, and 0.1% o-dianisidine hydrochloride in a final volume of 50 ml. The H<sub>2</sub>O<sub>2</sub>-dependent oxidation of o-dianisidine hydrochloride was used as an index of myeloperoxidase activity. One unit of myeloperoxidase was defined as the amount of myeloperoxidase required to degrade 1  $\mu$ mol H<sub>2</sub>O<sub>2</sub>/min at 25°C. Myeloperoxidase values were standardized to tissue protein content.

**TNF- $\alpha$  neutralization.** A third set of 16 S + C animals were randomized into two groups. The first group received a polyclonal rabbit anti-mouse TNF- $\alpha$  neutralizing antibody (600  $\mu$ l/kg of IP-400; Genzyme Diagnostics, Cambridge, MA) 5 min before the onset of hemorrhagic shock. The second group received a control rabbit IgG molecule (500  $\mu$ l/kg, Zymed Laboratories, San Francisco, CA). After completion of the experimental protocol, hearts were excised and left ventricular function was assessed as described above.

**Statistical analysis.** All values are expressed as means  $\pm$  SE. Statistical comparisons include ordinary *t*-test and one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test for multiple pairwise comparisons performed by SPSS for Windows statistical software (SPSS, Chicago, IL). *P* < 0.05 was considered significant.

## RESULTS

### *In Vivo Response of Experimental Groups*

We measured *in vivo* responses to hemorrhagic shock and aortic clamping, including fluid resuscitation requirements and blood gases, to validate the severity of the model before assessment of cardiac contractile function. The volume of blood removed throughout the hemorrhagic shock period to maintain a MAP at 50 mmHg was 20.9 ml/kg in the shock-alone group and 19.1 ml/kg in the S + C group. To maintain a MAP of 100 mmHg during the reperfusion period (Fig. 1), the S + C animals required significantly higher volumes of supplemental fluid (219.4 ml/kg) compared with sham-operated (35.6 ml/kg), shock-alone (62.8 ml/kg), and clamp-alone animals (142.6 ml/kg; *P* < 0.001). Arterial blood gases were performed immediately before the completion of the experimental protocol, and no significant differences were noted in PO<sub>2</sub>, PCO<sub>2</sub>, O<sub>2</sub> saturation, and pH between groups (data not shown). The significant increase in fluid requirements suggested that a substantial injury resulted from the induction of hemorrhagic shock followed by lower-torso ischemia. This led us to investigate the impact of this model on cardiac contractile function.

### *Assessment of Left Ventricular Function*

Myocardial function was subsequently assessed by recording peak systolic pressure (PSP), +dP/dt<sub>max</sub>, and -dP/dt<sub>max</sub> in a total of 36 animals (12 shams, 8 shock controls, 8 clamp controls, and 8 S + C) on a Langendorff perfusion apparatus. No difference in end-diastolic pressure was noted between groups as ventricular volume (preload) was increased (data not shown). Significant myocardial systolic dysfunction was noted after hemorrhagic shock alone (shock alone), lower-torso ischemia alone (clamp alone), and in the combined shock and clamp (S + C) group, as indicated by

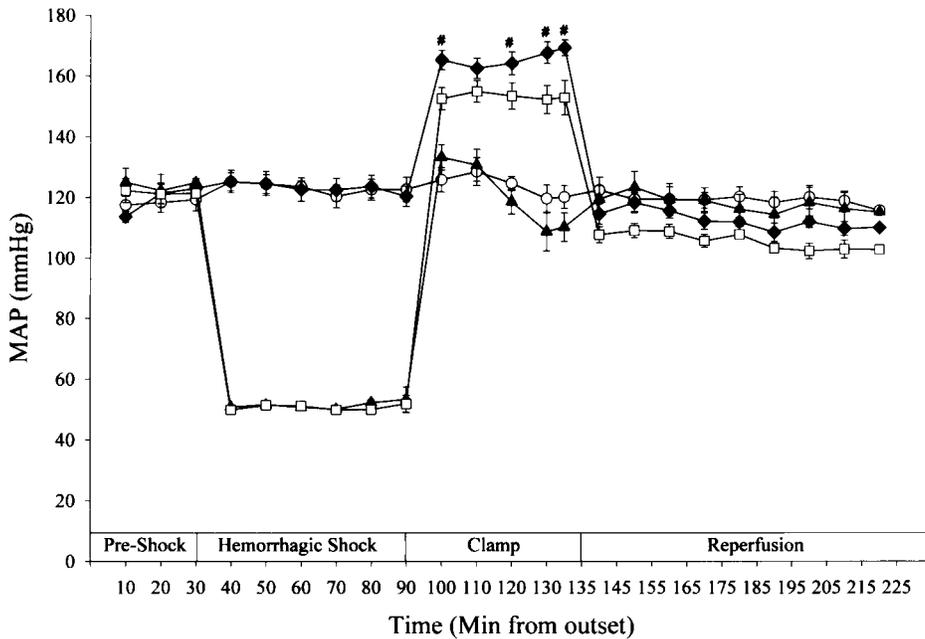


Fig. 1. In vivo response of experimental groups [mean arterial pressure (MAP)].  $\circ$ , Sham-operated control group;  $\blacklozenge$ , clamp-alone group;  $\blacktriangle$ , hemorrhagic shock-alone group;  $\square$ , shock and clamp (S + C) group. All values are expressed as means  $\pm$  SE. # $P < 0.05$  vs. S + C group during clamp period.

the downward shift of the function curves (Fig. 2). An intermediate dysfunction was noted after lower-torso ischemia (clamp alone) compared with sham-operated controls. Hearts from the shock-alone and combined S + C animals demonstrated a significant cardiac dysfunction compared with sham-operated controls. In the clamp-alone, shock-alone, and combined S + C groups, the initial PSP (Fig. 2A),  $+dP/dt_{\max}$  (Fig. 2B), and  $-dP/dt_{\max}$  (Fig. 2C) were significantly lower than in sham-operated animals ( $P < 0.001$ ; Fig. 2). The PSP and both  $+$  and  $-dP/dt_{\max}$  in the shock-alone and the S + C groups remained depressed as preload was increased. However, the clamp-alone group responded differently. Increasing preload resulted in improved

PSP and  $+dP/dt_{\max}$ , which approached sham-operated control levels. When left ventricular end-diastolic pressure was increased, peak systolic pressure,  $+$  and  $-dP/dt_{\max}$  displayed similar results as those in Fig. 2 (data not shown).

We stimulated the isolated heart with isoproterenol (an  $\beta$ -adrenergic agonist) as a preload-independent control. Initially, the PSP of the clamp-alone, shock-alone, and S + C groups were significantly depressed compared with sham-operated controls ( $P < 0.05$  vs. sham-operated control at 0 ng/ml; Fig. 3). On isoproterenol stimulation, PSP in the clamp-alone group rose to 100% of sham-stimulated levels (sham-operated control at 50 ng/ml of isoproterenol). However, cardiac

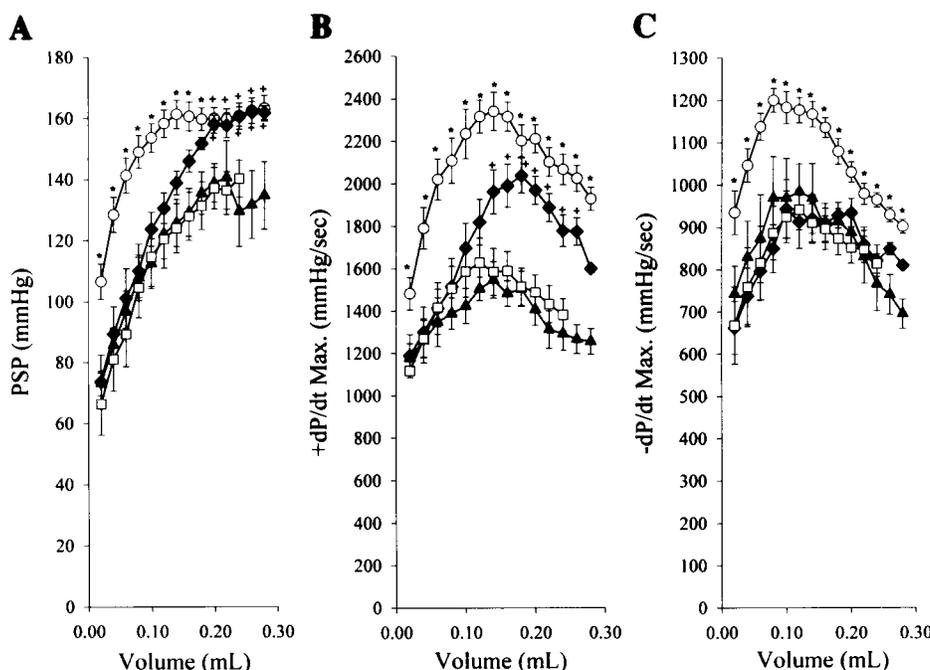
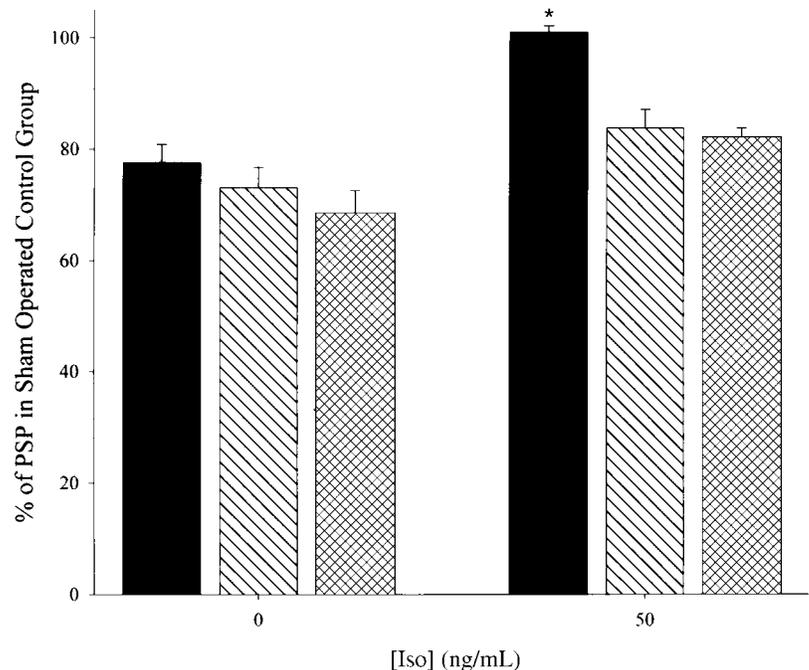


Fig. 2. Assessment of left ventricular function at increasing left ventricular volume. A: peak systolic pressure (PSP). B: maximal pressure increase over time ( $+dP/dt_{\max}$ ). C: maximal pressure decrease over time ( $-dP/dt_{\max}$ ).  $\circ$ , Sham-operated control group;  $\blacklozenge$ , clamp-alone group;  $\blacktriangle$ , hemorrhagic shock-alone group;  $\square$ , S + C group. All values are expressed as means  $\pm$  SE. \* $P < 0.05$  vs. clamp-alone, shock-alone, and S + C groups; + $P < 0.05$  vs. shock-alone and S + C groups.

Fig. 3. Assessment of left ventricular function in response to isoproterenol (Iso) stimulation. PSP as a percentage of sham-operated control group at either 0 or 50 ng/ml is shown. Solid bars, clamp-alone group; hatched bars, hemorrhagic shock-alone group; cross-hatched bars, S + C group. All values are expressed as means  $\pm$  SE. \* $P < 0.05$  vs. clamp-alone, shock-alone, and S + C groups at 0 ng/ml; + $P < 0.05$  vs. shock-alone and S + C groups at 50 ng/ml.



function in the shock-alone and S + C groups returned to only 80% of sham-stimulated levels. Thus, whereas isoproterenol stimulation significantly improved PSP in the clamp-alone group, cardiac contractile function remained significantly depressed in both the shock-alone and S + C groups compared with sham-operated control and clamp-alone hearts ( $P < 0.001$ ). Increases in  $+dP/dt_{\max}$  were similar to those noted in the PSP for each of the treatments (data not shown).

We attempted to determine whether altered heart rate or increased tissue fluid content were responsible for inducing the observed cardiac dysfunction. Coronary flow rates increased from 14.0 ml/min in the sham-operated control group to 15.1 ml/min in the clamp-alone group, 15.9 ml/min in the shock-alone group, and 18.8 ml/min in the combined S + C group. Heart rate and myocardial edema did not differ among the four groups in this model ( $P = 0.5$ , ANOVA; data not shown).

#### Myocardial TNF- $\alpha$ Concentration

We sought to correlate myocardial TNF- $\alpha$  levels with the degree of cardiac dysfunction noted in this model. Myocardial TNF- $\alpha$  levels increased threefold from the sham-operated controls (22.2 pg/mg of soluble protein) to the clamp-alone hearts (71.7 pg/mg). Hemorrhagic shock resulted in a twofold increase of myocardial TNF- $\alpha$  levels (145.2 pg/mg) compared with hearts from clamp-alone animals and a sixfold increase compared with sham-operated controls ( $P < 0.05$  vs. sham and clamp-alone groups). The combination of hemorrhagic shock and aortic clamping (S + C) resulted in a pronounced increase in myocardial TNF- $\alpha$  levels to 222.2 pg/mg, which was significantly greater than the myocardial TNF- $\alpha$  levels seen in the shock-alone and clamp-alone animals ( $P < 0.001$  vs. sham, clamp-alone, and shock-alone groups; Fig. 4). Therefore, an interme-

diante level of myocardial TNF- $\alpha$  seen in the clamp-alone animals was associated with an intermediate degree of cardiac contractile dysfunction. The larger increase in myocardial TNF- $\alpha$  seen in both the shock-alone and the combined S + C groups was associated with an extensive depression in cardiac function.

#### TNF- $\alpha$ Neutralization

To determine whether TNF- $\alpha$  is a mediator of the cardiac dysfunction seen in the S + C animals, we neutralized TNF- $\alpha$  before the onset of hemorrhagic shock. Administration of a neutralizing anti-TNF- $\alpha$  antibody resulted in a significant improvement in MAP during the clamp period from 146.4 mmHg in the S + C group given a control antibody to 158.2 mmHg in the anti-TNF- $\alpha$  antibody group ( $P < 0.005$  vs. S + C with control antibody). The volume of supplemental fluid required during the reperfusion period was significantly reduced to 131.1 ml/kg from 230.4 ml/kg in the control antibody-treated group ( $P < 0.001$  vs. S + C with control antibody).

Immunoneutralization of TNF- $\alpha$  in the S + C animals resulted in a significant improvement in cardiac contractile function as indicated by the increased PSP (Fig. 5A), contractility (Fig. 5B), and relaxation (Fig. 5C) toward sham-operated levels ( $P < 0.025$  vs. S + C with control antibody) on increased preload. Cardiac function remained depressed in the S + C group given the control antibody ( $P < 0.001$  vs. sham-operated control group) and was not significantly different compared with untreated S + C animals (Fig. 2). When cardiac contractile function was evaluated by increasing left ventricular end-diastolic pressure, improvements in cardiac function similar to those noted with increasing left ventricular volume were observed (data not shown).

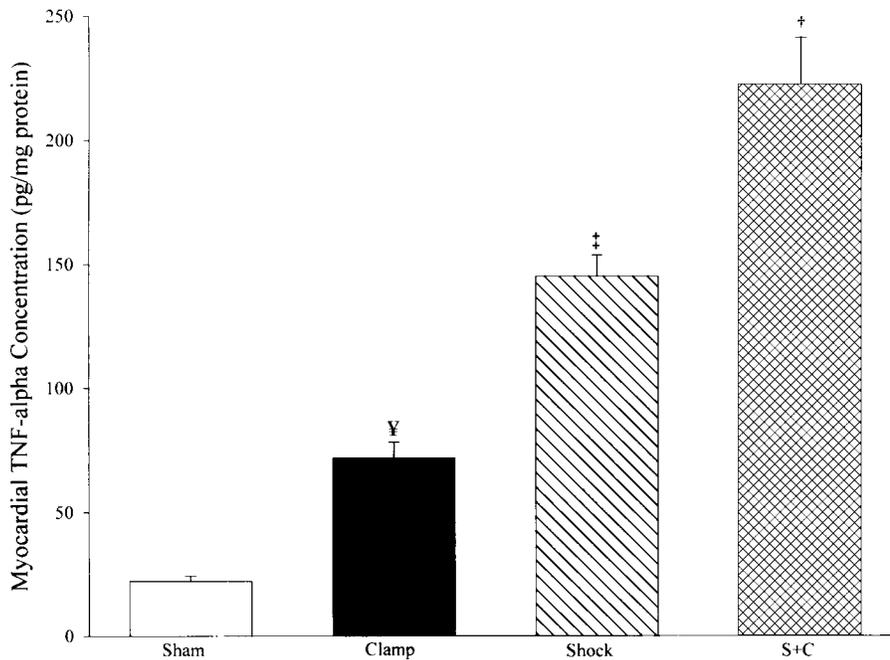


Fig. 4. Concentration of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in myocardium. Open bar, sham-operated control group; solid bar, clamp-alone group; hatched bar, hemorrhagic shock-alone; crosshatched bar, S + C group. All values are expressed as means  $\pm$  SE.  $\dagger P < 0.05$  vs. sham, superior mesenteric artery (SMA) clamp controls, and hemorrhagic shock controls;  $\ddagger P < 0.05$  vs. sham and SMA clamp controls;  $\S P < 0.05$  vs. sham.

Before inotropic stimulation, animals treated with the anti-TNF- $\alpha$  antibody displayed a significant improvement in PSP compared with control antibody-treated rats ( $P < 0.05$ ; Fig. 6). The  $\beta$ -adrenergic response in the anti-TNF- $\alpha$ -treated group was significantly greater than in the control antibody-treated group. PSP in the hearts of animals receiving the anti-TNF- $\alpha$  antibody increased to 95% of that seen in the stimulated sham-operated control group ( $P < 0.05$  vs. S + C with anti-TNF- $\alpha$  at 0 ng/ml). However, myocardial function in the control antibody-treated group remained significantly depressed compared with the anti-TNF- $\alpha$ -treated group ( $P < 0.001$ ), and PSP

rose to only 80% of sham-stimulated levels after isoproterenol stimulation. Cardiac contractile function in the control antibody-treated group increased to a similar degree noted after isoproterenol stimulation in the untreated S + C group (Fig. 3).

#### Biochemical Analysis of Myocardium

Neutralization of TNF- $\alpha$  did not return cardiac function to sham-operated levels. Therefore, we wanted to determine other potential mediators for the cardiac dysfunction seen in the S + C group. Myocardial ATP and CP remained unchanged in the sham-operated,

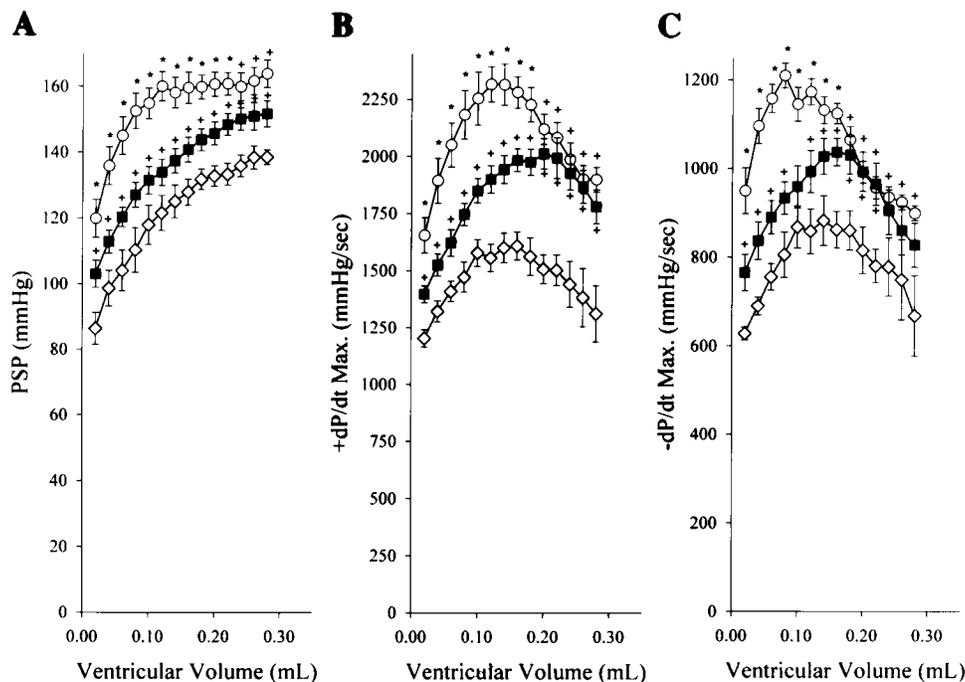


Fig. 5. Cardiac function at increasing left ventricular volume with TNF- $\alpha$  neutralization. A: PSP. B:  $+dP/dt_{max}$ . C:  $-dP/dt_{max}$ .  $\circ$ , Sham-operated control group;  $\blacksquare$ , S + C + anti-TNF- $\alpha$  antibody-treated group;  $\diamond$ , S + C + control antibody-treated group. All values are expressed as means  $\pm$  SE.  $* P < 0.05$  vs. S + C + anti-TNF antibody and S + C + control antibody groups;  $+ P < 0.05$  vs. S + C + control antibody group.

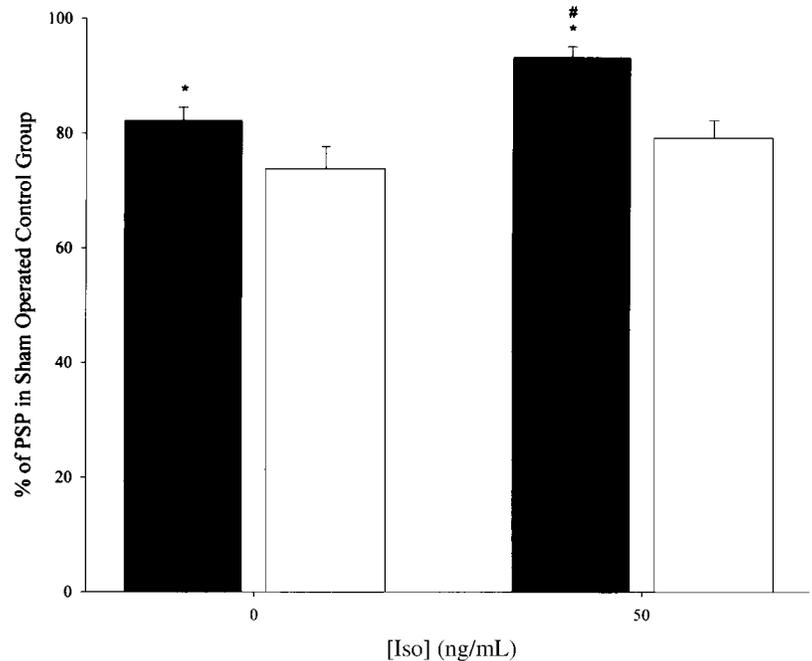


Fig. 6. Cardiac function in response to Iso stimulation with TNF- $\alpha$  neutralization. PSP as a percentage of sham-operated control group at either 0 or 50 ng/ml is shown. Solid bars, S + C + anti-TNF- $\alpha$  antibody-treated group; open bars, S + C + control antibody-treated group. All values are expressed as means  $\pm$  SE. \*  $P < 0.001$  vs. S + C + control antibody group; #  $P < 0.05$  vs. S + C + anti-TNF- $\alpha$  antibody group at 0 ng/ml.

shock-alone, clamp-alone, and S + C groups (Table 1). Myocardial oxidant injury was not noted because F<sub>2</sub>-isoprostane levels were not significantly elevated in any of the experimental groups. Myocardial neutrophil sequestration, as assessed by myocardial myeloperoxidase content, was significantly increased in the clamp-alone group (1.85 units/mg protein) compared with the sham-operated controls (1.09 units/mg protein) and the shock-alone group (1.43 units/mg protein). Myeloperoxidase content doubled (2.16 units/mg protein) after the combination of hemorrhagic shock and lower-torso ischemia ( $P < 0.001$  vs. sham controls). Additional studies indicated that no myocyte necrosis developed 24 h after hemorrhagic shock (data not shown).

## DISCUSSION

Cardiac contractile function was impaired in our model of ruptured abdominal aortic aneurysms. Myocardial dysfunction was observed in the clamp-alone, shock-alone, and combined hemorrhagic shock and lower-torso ischemia (S + C) groups. Clamping of the aorta above the superior mesenteric artery (SMA) resulted in a decrease in the initial peak systolic pressure, + and  $-dP/dt_{max}$ . As ventricular volume or pressure (preload) was increased, the cardiac function of hearts from the clamp-alone group returned to sham levels. However, cardiac function in the hearts from the

shock-alone and the combined S + C groups remained depressed, despite increases in preload. The cardiac dysfunction noted in the shock-alone and in the S + C groups were similar. The injury in the S + C group did not demonstrate any synergy between the hemorrhagic shock simulating aortic rupture and the supramesenteric clamping of aortic repair. Previous investigations have noted the influence of hemorrhagic shock alone (16) and gut ischemia alone (18) on cardiac contractile function; however, the combination of the two injuries has not been previously addressed. We conclude that the majority of the myocardial dysfunction in the S + C group resulted from hemorrhagic shock.

Investigation of the potential mechanisms mediating this injury revealed that the cardiac contractile dysfunction seen in this model differs from that seen after acute myocardial infarction. In contrast with other models of myocardial ischemia-reperfusion, we saw no depletion in myocardial energy stores (ATP and CP), no increase in oxidant stress (by F<sub>2</sub>-isoprostanes), and no myocyte necrosis 24 h after hemorrhagic shock, which are all characteristic of ischemic myocardial injury (15). Neutrophils did not appear to be a significant mediator of this injury because both the shock-alone and combined S + C groups displayed similar cardiac dysfunction but differing levels of neutrophil sequestration.

Table 1. Biochemical analysis of myocardium

	Sham	Clamp	Shock	S + C
<i>n</i>	12	8	8	8
ATP, $\mu\text{mol/g}$ dry wt	33.63 $\pm$ 3.71	33.38 $\pm$ 2.57	33.76 $\pm$ 3.33	33.35 $\pm$ 0.82
Creatine phosphate, $\mu\text{mol/g}$ dry wt	46.39 $\pm$ 4.16	47.96 $\pm$ 3.99	48.93 $\pm$ 4.54	44.47 $\pm$ 4.13
F <sub>2</sub> -isoprostanes, pg/mg protein	68.77 $\pm$ 9.39	76.78 $\pm$ 23.10	87.36 $\pm$ 13.96	64.40 $\pm$ 7.85
Myeloperoxidase, units/mg protein	1.09 $\pm$ 0.13	1.85 $\pm$ 0.18†	1.43 $\pm$ 0.30	2.16 $\pm$ 0.17*

All values are means  $\pm$  SE; *n* = number of rats. S + C, shock + clamp. \*  $P < 0.05$  vs. sham, clamp, and shock; †  $P < 0.05$  vs. sham and shock.

We observed a progressive increase in the levels of myocardial TNF- $\alpha$  in the clamp-alone group and both groups experiencing hemorrhagic shock coupled with a concomitant decrease in cardiac contractile function. Furthermore, neutralization of TNF- $\alpha$  activity by administration of an anti-TNF- $\alpha$  antibody significantly improved cardiac function toward sham control levels. Thus we conclude that TNF- $\alpha$  is responsible for a significant component of the myocardial dysfunction noted in our model of RAAA repair. This is the first study to demonstrate improved cardiac contractile function by TNF- $\alpha$  immunoneutralization after hemorrhagic shock.

Hemorrhagic shock has been shown to induce increases in TNF- $\alpha$  synthesis (30), and inhibition of TNF- $\alpha$  improves cardiac function in a model of burn shock (11). Previous studies using isolated cardiomyocytes and whole heart preparations have shown that incubation or perfusion with TNF- $\alpha$  results in depressed contractile function (28, 39). The heart is known to be a TNF- $\alpha$ -generating organ, and as much as 50% of the total TNF- $\alpha$  found within the heart can be produced by cardiomyocytes (20). After synthesis, TNF- $\alpha$  is secreted and can act extracellularly on membrane-bound receptors to activate intracellular signaling cascades (2). Consequently, sphingosine and nitric oxide (NO) may be produced. Sphingosine has been shown to mediate the early depression due to TNF- $\alpha$  administration (25), whereas NO production results in late cardiac depression (10, 12). The mechanisms by which TNF- $\alpha$  induces cardiac dysfunction in this model are currently under investigation. Because the neutralizing antibody utilized is unable to enter cardiomyocytes, our results indicate that TNF- $\alpha$  may remain in the interstitial space after secretion and continue to induce cardiac dysfunction.

The antibody used in this model is highly specific to neutralize TNF- $\alpha$  (6) and may diffuse from the circulation into the interstitial space of the heart. Cardiac dysfunction seen after TNF- $\alpha$  administration to isolated cardiomyocytes was reversed after a 30-min washout period (12, 39). After administration of the antibody to the S + C animals, improvements in cardiac function were noted by the increase in MAP during the clamp phase, and by the improvements in PSP and both + and  $-dP/dt_{max}$  measured on the Langendorff apparatus. The efficacy of the anti-TNF- $\alpha$  antibody (through the experimental protocol and cardiac functional measurements) suggests that it diffuses to the site of TNF- $\alpha$  activity, thereby influencing the chain of signaling events to improve cardiac function. Thus we observed both in vivo and cardiac functional benefits of this therapy.

The hearts of clamp animals initially showed cardiac contractile impairment, but function returned to sham levels on stimulation (by increasing preload or inotropic stimulation). It has previously been shown that TNF- $\alpha$  induces cardiac dysfunction in a dose-dependent manner (12). Only a fourfold increase in myocar-

dial TNF- $\alpha$  concentrations from sham hearts to clamp hearts was seen. In contrast, a similar level of cardiac depression was observed in the shock-alone and S + C groups despite significantly higher TNF- $\alpha$  levels in the S + C group compared with shock alone. An intermediate dysfunction was noted in hearts expressing the lowest increase in TNF- $\alpha$  concentrations (clamp-alone group) and a more pronounced depression in cardiac function was seen in hearts associated with a significantly greater amount of TNF- $\alpha$  (shock-alone and S + C groups). This supports the concept of a dose-dependent relationship between TNF- $\alpha$  and cardiac contractile dysfunction.

A differential response to  $\beta$ -adrenergic stimulation was noted between hearts from animals undergoing hemorrhagic shock or lower-torso ischemia. Inotropic stimulation returned PSP in the clamp-alone group to 100% of sham-stimulated levels; however, function remained significantly depressed in the shock-alone and the combined S + C groups. Thus there appears to be a differing biochemical basis of the depressed cardiac contractile function between the two insults. Studies have shown that TNF- $\alpha$  reduces  $\beta$ -adrenergic stimulation without altering the density of  $\beta$ -adrenergic receptors (13). After immunoneutralization of TNF- $\alpha$  in the S + C group, the response to  $\beta$ -adrenergic stimulation was significantly improved. Thus the depressed cardiac contractile response in this model of RAAA repair may be due to a reduction in  $\beta$ -adrenergic responsiveness, secondary to TNF- $\alpha$ .

In summary, hemorrhagic shock and supramesenteric aortic clamping resulted in a 65% reduction in myocardial contractile function. This dysfunction is associated with a 10-fold increase in myocardial TNF- $\alpha$ , and immunoneutralization of TNF- $\alpha$  significantly improves contractile dysfunction after S + C. These results imply that therapies directed at attenuating the physiological actions of TNF- $\alpha$  may improve contractile dysfunction after RAAA repair.

We thank Sherwin Nicholson for technical assistance.

This research was partially supported by the Canadian Society for Vascular Surgery.

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Received 19 May 1999; accepted in final form 1 October 1999.

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