Functional significance of inflammatory mediators in a murine model of resuscitated hemorrhagic shock

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Vallejo, Jesus G., Shintaro Nemoto, Masakuni Ishiyama, Bi Yu, Pascal Knuefermann, Abinav Diwan, J. Scott Baker, Gilberto Defreitas, David J. Tweardy, and Douglas L. Mann. Functional significance of inflammatory mediators in a murine model of resuscitated hemorrhagic shock. Am J Physiol Heart Circ Physiol 288: H1272–H1277, 2005; doi:10.1152/ajpheart.01003.2003.—The mechanisms that underlie the development of myocardial dysfunction after resuscitated hemorrhagic shock (HS) are not known. Recent studies suggest that systemic activation of inflammatory mediators may contribute to cellular dysfunction and/or cell death in various organs, including the heart. However, the precise role that inflammatory mediators play in the heart in the setting of resuscitated HS is not known. Accordingly, the purpose of the present study was to use a well-defined murine model of resuscitated HS to characterize the functional significance of inflammatory mediators in the heart in vivo. Mice were subjected to sham operation or resuscitated HS. Left ventricular (LV) function was assessed by two-dimensional echocardiography 6 h after resuscitation. Myocardial TNF, IL-1β, and IL-6 proteins were measured 1 and 6 h after resuscitation. To determine the role of TNF in HS-induced LV dysfunction, mice were treated with a soluble TNF receptor antagonist (etanercept) before HS or at the time of resuscitation. LV fractional shortening was significantly depressed (P < 0.05) in resuscitated HS mice (28 ± 1.5%) compared with sham controls (35.8 ± 1.0%). TNF and IL-1β levels were significantly increased (P < 0.05) in resuscitated HS mice. Pretreatment with etanercept abrogated resuscitated HS-induced LV dysfunction, whereas treatment at the time of resuscitation significantly attenuated, but did not abrogate, LV dysfunction. Together, these data suggest that TNF plays a critical role in HS-induced LV dysfunction; however, once the deleterious consequences of reperfusion injury are initiated, TNF contributes to, but is not necessary for, the development of LV dysfunction.

MATERIALS AND METHODS

Animal care. Eight-week-old male and female C57BL/6 mice were used for these studies. Mice were maintained in standard conditions under a 12:12-h light-dark cycle and were provided standard food and water at libitum. The animal protocols were approved by the Baylor College of Medicine Institutional Review Board for animal experimentation and were performed in accordance with the guidelines outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Murine model of resuscitated HS. C57BL/6 mice were randomly subjected to resuscitated HS or a sham operation as previously described (7). Briefly, mice were anesthetized with isoflurane. Both superficial femoral arteries were surgically prepared and cannulated. The left femoral artery was used for continuous blood pressure monitoring. The right femoral artery was used for blood withdrawal and blood and fluid administration. Animals were subjected to HS by continuous withdrawal of blood over 10 min to achieve a mean arterial pressure (MAP) of 30 mmHg. MAP was maintained at 30 mmHg for 180 min with continuous monitoring of blood pressure and withdrawal and return of blood as needed. The animals were resuscitated to a MAP of 80 mmHg by administration of the remaining shed blood plus an intra-arterial injection of lactated Ringer solution equal to two times the total shed volume administered over 30 min. The incisions were closed, and the animals were allowed to recover for 6 h.

Characterization of LV function and structure. Six hours after resuscitated HS or sham operation, the mice were anesthetized intravenously with pentobarbital and transverse abdominal incisions were made. The peritoneal cavity was opened, and the superficial femoral arteries were cannulated and infused with a heparinized Ringer’s solution. The heart was excised, and Langendorff perfusion was established. The cLVM was isolated, and Langendorff perfusion was continued for 1 h. Then, the heart was perfused with a 15% solution of paraformaldehyde and osmium tetroxide and immersed in 2% osmium tetroxide overnight. Tissue was processed for subsequent en face sectioning and immunohistochemical staining.
peritoneally with a mixture of ketamine (100 mg/kg), xylazine (2.5 mg/kg), and heparin (5,000 U/kg); additional doses were given as needed. Animals were placed in the supine position under a heat lamp to maintain body temperature at 37°C. Mice were allowed to breathe spontaneously using 1 l/min of oxygen given via a nasal cone. We performed transthoracic echocardiographic examinations using an Acuson Sequoia cardiac system equipped with a 15-MHz linear transducer (C256 and 15L8, Acuson, Mountain View, CA) as described (15).

After a parasternal short-axis view was obtained, two-dimensional targeted M-mode tracings were recorded through the anterior and posterior LV walls at a sweep speed of 200 mm/s. All images were digitally acquired and stored for off-line analysis. Echocardiographic measurements of LV dimensions were recorded at end diastole (EDD) and end systole (ESD) from three consecutive cardiac cycles using the leading edge method (5). LV fractional shortening (%FS) was calculated as:

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\text{%FS} = \frac{\text{ESD} - \text{ESD}}{\text{EDD}} \times 100.
\]

**Myocardial histology.** Hearts from sham-operated and resuscitated HS mice were harvested at 6 h, perfusion fixed in buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for routine histological examination, as described (15).

**Myeloperoxidase activity.** Samples from sham-operated and resuscitated HS mice were homogenized [1-20 (wt/vol)] in ice-cold 20 mM KPO4 buffer (pH 7.4). After the supernatants were removed (12,000 g, 4°C, 30 min), pellets were again resuspended in ice-cold 20 mM KPO4 buffer (pH 7.4), followed by two additional centrifugations. Next, 0.5% (wt/vol) hexacyltrimethylammonium bromide-10 mM EDTA (Sigma, St. Louis, MO) in 50 mM KPO4 (pH 6.0) was added to the remaining pellet (6:1 buffer-to-pellet ratio). Suspensions were sonicated for 5 × 1 s on ice, freeze thawed three times, and incubated for 20 min at 4°C. After final centrifugation (12,000 g, 15 min, 4°C), supernatants were used to measure myeloperoxidase (MPO) activity. In duplicate, assay buffer (0.2 mg/ml o-dianisidine and 158 µM H2O2 in 50 mM KPO4, pH 6.0) was added to the supernatant at a ratio of 4:1. Purified MPO (Sigma) was used as standard. Changes in absorbance were recorded at 460 nm over 3.5 min. The linear part of the resulting curve was used for calculating MPO activity. Results were expressed as units of MPO per millgram of protein of supernatant as determined by bicinchoninic acid assay (Pierce Chemical, Rockford, IL) with BSA as a standard.

**TNF and IL-1β ribonuclease protection assay.** Hearts were harvested 1 h after resuscitation and homogenized, and total RNA was extracted by the guanidinium thiocyanate method. TNF and IL-1β gene expressions were determined with a custom-designed multiprobe RNase protection assay system according to the manufacturer’s protocol (RiboQuant, Pharmingen, San Diego, CA).

**Myocardial TNF, IL-1β, and IL-6 protein levels.** Hearts were harvested at 1 and 6 h after resuscitation and were homogenized in 1 ml of ice-cold extraction buffer containing 20 mM HEPES (pH 7.4), 20 mM glycerophosphate, 20 mM sodium pyrophosphate, 0.2 mM Na3VO4, 2 mM EDTA, 20 mM sodium fluoride, 10 mM benzamidine, 1 mM DTT, 20 ng/ml leupeptin, 0.4 mM Pefabloc SC, and 0.01% Triton X-100. The homogenate was centrifuged at 14,000 g for 15 min at 4°C. The supernatant was collected, and the protein concentration was determined with the bicinchoninic acid assay. The resulting supernatant was used for determination of TNF, IL-1β, and IL-6 by ELISA (R&D Systems), according to the manufacturer’s suggestions. Absorbance of standards and samples was determined spectrophotometrically at 450 nm with a microplate reader (Dynex Technologies, Chantilly, VA). Results were plotted against the linear portion of the standard curve. Final results were expressed as picograms cytokine protein per millgram of protein.

**TNF immunohistochemistry.** To localize the cellular source of TNF expression, we performed immunohistochemistry studies using a rabbit anti-TNF antibody (MBL International, Woburn, MA). Paraffin-embedded sections were used for immunostaining, which was performed with an immunoenzymatic staining kit (DAKO EnVision+ Systems, peroxidase, Dako, Carpentera, CA) as recommended by the manufacturer. Counterstaining was performed with hematoxylin, and each immunostained slide was evaluated by light microscopy.

**TNF neutralization.** For these experiments, mice were randomized into two different groups. The first group received normal saline (100 µl) or 150 µg of etanercept, a soluble TNF antagonist, in 100 µl of normal saline. The second group received a single dose of normal saline (100 µl) or 150 µg of etanercept in 100 µl of normal saline administered at the time of resuscitation. After completion of the experimental protocol, LV function was assessed as described in Characterization of LV function and structure. The investigators performing the resuscitated HS studies, as well as the investigators performing the evaluation of LV function, were blinded to the randomization protocol.

**Statistical analysis.** All values are expressed as means ± SE. A nonpaired t-test was used to assess differences in cardiac function between sham-operated and resuscitated HS mice. ANOVA followed by Fisher’s paired least-significant difference was used to determine significant differences in cytokine protein expression at different time points in the sham-operated and resuscitated HS animals. Statistical significance was determined at the P < 0.05 level.

**RESULTS**

**Characterization of LV structure and function in resuscitated HS mice.** LV structure and function were assessed by two-dimensional echocardiography 6 h after sham operation or resuscitated HS. Figure 1A shows representative M-mode echocardiograms from sham-operated and resuscitated HS mice. As shown in Fig. 1B, the extent of %FS was significantly (P < 0.05) depressed in resuscitated HS mice compared with sham-operated mice. These differences in LV function persisted for up to 12 h after resuscitation (data not shown). As
shown in Table 1, the ESD was greater in resuscitated HS mice than in sham-operated mice. Analyses of group data (n = 6/group) showed that there was a significant (P = 0.04) increase in ESD in resuscitated HS mice (0.30 ± 0.001 cm) compared with sham-operated mice (0.25 ± 0.02). Given that HS mice were resuscitated to maintain a stable blood pressure of 80 mmHg, the increase in ESD likely reflects a true decrease in cardiac contractility. Importantly, there was no significant difference in heart rate between sham-operated mice and resuscitated HS mice (Table 1).

Representative examples of the histology of the hearts from sham-operated mice and resuscitated HS mice are depicted in Fig. 2. There were no obvious qualitative differences in the morphological appearance of the myocardium between the two groups. Importantly, we did not observe evidence of increased neutrophil sequestration in the hearts of resuscitated HS mice at 6 h, when we observed significant LV dysfunction. To confirm the qualitative histological findings, we also performed MPO assays on hearts from the resuscitated HS and sham-operated control mice. As shown in Fig. 2C, there was no difference in MPO activity between these two groups of mice.

Myocardial TNF and IL-1β. Insofar as proinflammatory cytokines have been linked to the development of LV dysfunction, we next examined the expression of myocardial TNF and IL-1β in sham-operated and resuscitated HS animals. Figure 3A shows that resuscitated HS induced the expression of TNF and IL-1β mRNA within the cardiac compartment. To determine the cellular source for TNF expression, immunostaining was performed in hearts that had been subjected to resuscitated HS or sham operation. Figure 3, B–E, summarizes the results of the immunostaining studies that used a mouse specific anti-TNF antibody. Consistent with the mRNA findings, a small amount of TNF protein was detected in the hearts of sham-operated mice (Fig. 3B). However, there was clear increase in cytoplasmic labeling of endothelial cells in blood vessels and cytoplasmic labeling of the cardiac myocytes with the TNF antibody in the hearts of resuscitated HS mice (Fig. 3, C–E).

Both TNF (Fig. 3F) and IL-1β (Fig. 3G) protein levels were significantly greater at 1 h in the resuscitated HS mice compared with sham-operated animals. Six hours after resuscitated HS, the myocardial levels of TNF and IL-1β were not significantly different between the two groups. Myocardial IL-6 production did not differ between sham-operated animals and resuscitated HS mice at 1 h (3.2 ± 0.66 vs. 3.6 ± 0.87 pg/mg protein). Serum TNF levels were also significantly increased in the resuscitated HS mice compared with sham-operated animals (Fig. 3H); however, this increase was not detectable until 6 h after resuscitated HS and occurred after the increase in myocardial TNF expression.

Effect of etanercept on LV function in resuscitated HS. Previous studies with an isolated Langendorff preparation have shown that administration of TNF antibodies before the onset of the HS attenuates LV dysfunction in rats (18, 19). To determine whether TNF played a role in the onset of LV dysfunction in vivo, mice were treated with the soluble TNF receptor antagonist etanercept or diluent (normal saline) before the onset of HS as well as at the time of resuscitation. Figure 3A shows that pretreatment with etanercept completely abrogated LV dysfunction when administered before the onset of resuscitated HS. That is, there was no difference in the extent of LV fractional shortening between sham-operated mice (33 ± 5.3%) and resuscitated HS mice (37 ± 1.3%). In contrast, resuscitated HS mice that were pretreated with diluent developed significant LV dysfunction compared with sham-operated controls. We then asked whether administration of etanercept at the time of resuscitation would modulate the LV dysfunction associated with resuscitated HS. Figure 3B shows that etanercept administered at the time of resuscitation significantly attenuated LV dysfunction. Resuscitated HS mice treated with diluent had a 21 ± 2.2% decrease in fractional shortening compared with sham-operated mice. In contrast, mice treated with etanercept at the time of resuscitation had a 11 ± 3.5% decrease in the extent of LV fractional shortening compared with the appropriate controls. However, the extent of fractional shortening in the etanercept-treated resuscitated HS mice was still significantly depressed (P < 0.01) compared with sham-operated mice. Thus administration of a soluble TNF antagonist at the time of reperfusion significantly attenuated, but did not abrogate, resuscitated HS-induced LV dysfunction.

**DISCUSSION**

The mechanisms for the worsening LV function after restoration of circulating blood volume have been the subject of intense scrutiny for nearly 60 yr and have been attributed to the ill-defined “rude unhinging of the machinery of life” proposed by Gross (6) and to more specific mechanisms, including...
decreased coronary perfusion pressure, metabolic acidosis, and progressive loss of adrenergic support, as well as metabolic factors, including abnormalities in myocardial glycolysis, oxygen free radicals, and/or increased elaboration of circulating myocardial depressant factors (9, 17, 20). Most recently, it has been suggested that HS and/or resuscitation leads to increased expression of a portfolio of inflammatory mediators related to the so-called innate immune system, including TNF, IL-1β, IL-6, and nitric oxide (1, 22). Here, we show for the first time that the induction of myocardial inflammation is necessary for the onset of LV dysfunction in vivo in a murine model of resuscitated HS. Two major sets of experimental observations support this statement. First, there was a significant increase (1 h) in myocardial levels of TNF and IL-1β in the resuscitated HS animals compared with sham-operated controls (Fig. 3).

Although this study was not designed to determine the source of myocardial cytokine production, the observation that there was no evidence of inflammatory cells in the hearts of the resuscitated HS animals either by histological analysis or by MPO staining indicates that the increased synthesis of proinflammatory cytokines following resuscitated HS was not secondary to neutrophils influxing into the myocardium. Second, administration of the soluble TNF antagonist before the onset of hemorrhage and resuscitation completely abrogated the LV dysfunction that was observed following resuscitated HS. That is, there was a significant 21% decrease in fractional shortening in the resuscitated HS mice compared with sham-operated controls (Fig. 3).

Fig. 3. Myocardial TNF and IL-1β mRNA expression and protein production in R-HS. A: TNF and IL-1β mRNA expression in the heart. B–E: results of immunostaining with an anti-TNF antibody. F and G: myocardial levels of TNF and IL-1β protein at 1 and 6 h after resuscitation in sham-operated (open bars) and hemorrhagic shock (solid bars) mice (n = 5 hearts/group for each time point). H: serum TNF levels after R-HS or sham operation. *P < 0.05.
were pretreated with etanercept (Fig. 4A). Perhaps more importantly from a therapeutic standpoint, we show for the first time that administration of a soluble TNF antagonist at the time of resuscitation significantly attenuated \( P < 0.035 \) the extent of LV dysfunction (Fig. 4B) induced by resuscitated HS. Mice that were administered dinitrofluorobenzene at the time of resuscitation developed a 21% decrease in fractional shortening, whereas mice that were treated with etanercept at the time of resuscitation had an 11% decrease in fractional shortening compared with the sham-operated controls. However, it is important to note that the extent of fractional shortening in the mice treated with etanercept at the time of resuscitation was still significantly depressed (\( P = 0.01 \)) compared with etanercept-treated sham-operated controls. Taken together, these data suggest that inflammatory mediators such as TNF play a critical upstream role in the development of resuscitated HS-induced LV dysfunction; however, once the deleterious consequences of reperfusion injury are initiated, TNF contributes to, but is not necessary for, the development of LV dysfunction.

The results of the present in vivo study both confirm and expand on previous in vitro studies, wherein pretreatment of rats with anti-TNF antibodies before resuscitation attenuated LV dysfunction observed in Langendorff-perfused hearts that were harvested from experimental models of lower torso ischemia and resuscitated HS (18, 19). Moreover, the present study is consistent with work from a number of laboratories that has implicated TNF in the LV dysfunction that supervenes in a variety of different shock models, including splanchic artery occlusion (21), intestinal ischemia-reperfusion (24), and hypovolemic HS (25). However, the present study differs somewhat from a previous in vivo study in rats in which treatment with an anti-TNF antibody administered 15 min after resuscitation had no effect on LV stroke volume but did lead to an overall increase in cardiac output because of an increase in heart rate in the anti-TNF-treated rats. Whether the greater degree of attenuation of LV dysfunction observed in the present study represents a difference in timing or the type of anti-TNF strategy that was employed, a difference in species, or a difference in resuscitated HS protocols cannot be determined from the present study.

**Limitation of the present study.** Treatment with etanercept at the time of resuscitation only partially prevented the development of LV dysfunction after HS. As noted above, we consider that the most likely explanation for this finding is that, once the deleterious consequences of reperfusion injury are initiated, TNF contributes to, but is not necessary for, the development of LV dysfunction. Nonetheless, because we did not perform TNF bioassays (for technical reasons), we cannot exclude the possibility that the dose of etanercept used in the present study was insufficient to completely neutralize TNF.

In conclusion, the results of the present study suggest that inflammatory mediators play a critical upstream role in the subsequent development of the LV dysfunction that supervenes following resuscitated HS. Accordingly, approaches to neutralize TNF in the early stages of HS may be beneficial from a therapeutic standpoint. This statement notwithstanding, the results of the present study suggest that anti-inflammatory therapies need to be administered at the time of resuscitation or shortly thereafter to maximize their potential for myocardial salvage. Accordingly, it will be important in future studies to more precisely define the timing of the “therapeutic window” for anti-inflammatory strategies as well as to determine the molecules that are immediately downstream from the inflammatory cascade, with the intent of extending the window of opportunity for treating the LV dysfunction that supervenes after resuscitated HS.

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