Signaling for myocardial depression in hemorrhagic shock: roles of Toll-like receptor 4 and p55 TNF-α receptor

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Toll-like receptor 4 (TLR4) regulates cellular expression of Tumor Necrosis Factor-α (TNF-α), the role of TNF-α as a myocardial depressant factor in hemorrhagic shock remains to be determined. Moreover, it is unclear which TNF-α receptor mediates the myocardial depressive effects of TNF-α. Toll-like receptor 4 (TLR4) regulates cellular expression of proinflammatory mediators following lipopolysaccharide stimulation and may be involved in the tissue inflammatory response to injury. The contribution of TLR4 signaling to tissue TNF-α response to hemorrhagic shock and TLR4’s role in myocardial depression during hemorrhagic shock are presently unknown. We examined the relationship of TNF-α production to myocardial depression in a mouse model of nonresuscitated hemorrhagic shock, assessed the influence of TLR4 mutation, resulting in defective signaling, on TNF-α production and myocardial depression, and determined the roles of TNF-α and TNF-α receptors in myocardial depression using a gene knockout (KO) approach. Hemorrhagic shock resulted in increased plasma and myocardial TNF-α (4.9- and 4.5-fold, respectively) at 30 min and induced myocardial contractile depression at 4 h. TLR4 mutation abolished the TNF-α response and attenuated myocardial depression in left ventricular developed pressure of 43.0 ± 6.2 mmHg in TLR4 mutant vs. 30.0 ± 3.6 mmHg in wild type, \( P < 0.05 \). TNF-α KO also attenuated myocardial depression in hemorrhagic shock, and the p55 receptor KO, but not the p75 receptor KO, mimicked the effect of TNF-α KO. The results suggest that TLR4 plays a novel role in signaling to the TNF-α response during hemorrhagic shock and that TNF-α through the p55 receptor activates a pathway leading to myocardial depression. Thus TLR4 and the p55 TNF-α receptor represent therapeutic targets for preservation of cardiac mechanical function during hemorrhagic shock.

tumor necrosis factor-α receptors; mouse; cardiac contractile function; knockout

TUMOR NECROSIS FACTOR-ALPHA (TNF-α) is recognized as a cardiac depressant factor in septic shock and has been linked to heart failure associated with several different conditions (13, 37). Previous investigations have shown that transgenic overexpression of TNF-α results in congestive heart failure (8, 16). We have observed that TNF-α contributes to myocardial contractile dysfunction induced by either bacterial lipopolysaccharide (LPS) or myocardial ischemia-reperfusion (20–22). Although TNF-α is involved in endotoxic myocardial depression, there is a delay of at least 2 h between peak TNF-α production and myocardial depression (22). This observation suggests that TNF-α depresses myocardial contractility through the induction of downstream factors. Hemorrhagic shock also causes cardiac contractile dysfunction (15, 19), and this cardiodepressive effect is associated with the induction of TNF-α (2, 31, 35, 40). Although TNF-α antibody has been shown to improve survival in hemorrhagic shock (5, 41), the mechanistic role of TNF-α in myocardial depression during hemorrhagic shock remains to be determined.

Innate immunity plays a primary role in acute host defense. Toll-like receptor 4 (TLR4) regulates cellular expression of proinflammatory mediators following LPS stimulation (27). LPS activates the TLR4 signaling cascade through the TLR4 Toll/IL-1 receptor (TIR) domain, which uses a conserved signal transduction pathway requiring MyD88, IL-1-receptor-associated kinases (IRAKs), IKK, and NF-κB to induce a cytokine response (23). Indeed, TLR4 mediates cytokine production and myocardial depression in endotoxemia (6, 24). There are also data that suggest a broader role for TLR4 in the tissue inflammatory response. The contents of injured cells and the inducible heat shock protein 70 have been shown to provoke the inflammatory response through TLR4-mediated mechanisms in cultured mononuclear cells (4, 17, 38). Furthermore, TLR4 gene expression is upregulated in the liver in a model of nonresuscitated, fixed-volume hemorrhagic shock (30). These findings suggest that TLR4 signaling may contribute to tissue inflammatory response to injury. However, the contribution of TLR4 signaling to the tissue TNF-α response to hemorrhagic shock has not been determined, and the role of TLR4 in myocardial depression following hemorrhagic shock is presently unknown.

TNF-α signaling is mediated by two types of cell surface receptors (1, 11). The p55 TNF-α receptor is the dominant effector in TNF-α biology (26). Although the p75 TNF-α receptor is known to play an important role in ligand passing to the p55 receptor (1), several studies have found that the p75 TNF-α receptor suppresses p55 TNF-α receptor function (25, 26). We have found that mice with deficient p55 TNF-α receptor have attenuated lung neutrophil accumulation after hemorrhagic shock (34). Although the pulmonary inflammatory response to hemorrhagic shock involves the p55 TNF-α receptor, it remains unclear which TNF-α receptor is involved in myocardial depression following hemorrhagic shock.

The purposes of this study were 1) to examine whether TNF-α production temporally correlates with myocardial depression in hemorrhagic shock, 2) to assess the effect of
defective TLR4 signaling on the TNF-α response and myocardial depression during hemorrhagic shock, and 3) to test whether TNF-α deficiency or TNF-α receptor deficiency attenuates myocardial depression.

MATERIALS AND METHODS

Animals and experimental protocols. Male mice, 20–25 g body wt, were used in this study. Wild-type (BALB/cJ, B6/129 and C57 BL/6) and mutant [C3H-Tlr4<sup>−/−</sup>, TNF-α knockout (KO), p55 receptor KO, and p75 receptor KO] mice were obtained at Jackson Laboratory (Bar Harbor, ME). The C3H-Tlr4<sup>−/−</sup> mouse strain is a constructed mutant that has a mutation on the TLR4 TIR domain identical to that in C3HHeJ (39). Because this mutant is constructed on a BALB/cJ background, BALB/cJ mice were used as wild-type controls. The background strain for TNF-α KO is B6/129, and the background strain for both p55 TNF-α receptor KO and p75 TNF-α receptor KO is C57BL/6. The animals were quarantined and maintained on a standard pellet diet for 1 wk before initiation of the experiments. All animal experiments were approved by the Animal Care and Research Committee, University of Colorado Health Sciences Center. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892).

The mouse hemorrhagic shock model has been described previously (33, 34). Mice were anesthetized with methofane. Hemorrhagic shock was induced by removing 30% of the calculated total blood volume (0.27 ml per 10 g of body weight) over 60 s through a cardiac puncture. With this method, mean arterial pressure decreased to ∼40 mmHg and remained at this low level for ∼120 min (34). Overall mortality was <10%, and there was no evidence of bleeding into the pericardial space and hemotherax or signs of lung and cardiac confusion. The sham procedure involved cardiac puncture under methofane anesthesia, without removal of blood. Animals were killed at 0.5, 1, 2, and 4 h after treatment. After anesthetization and heparinization (60 mg/kg of pentobarbital sodium and 2,000 U/kg of heparin ip), the chest was opened. For measurement of TNF-α, blood was collected from the right ventricle. The heart was removed, and the coronary vessels were flushed with 2.0 ml of cold normal saline through the aortic root. Plasma and myocardial samples were stored at −70°C before TNF-α assay. Hearts were also isolated at 1, 2, and 4 h for the assessment of myocardial contractile function by Langendorff perfusion.

Isolated heart perfusion. Isolated heart perfusion was performed by a modified isovolumetric Langendorff technique as described elsewhere (28, 29). Hearts were excised into cold modified Krebs-Henseleit solution (in mM: 11 glucose, 119 NaCl, 1.2 CaCl<sub>2</sub>, 4.7 KCl, 25 NaHCO<sub>3</sub>, 1.18 KH<sub>2</sub>PO<sub>4</sub>, and 1.17 MgSO<sub>4</sub>). The aorta was cannulated with a 20-gauge needle, and the heart was perfused within 2 min after isolation. Hearts were perfused in an isovolumetric mode (70 mmHg) with the modified Krebs-Henseleit solution, which was saturated with 92.5% O<sub>2</sub>-7.5% CO<sub>2</sub> to achieve a P<sub>02</sub> of 420–460 Torr and a P<sub>CO2</sub> of 36–40 Torr. The myocardial temperature was maintained by placing the heart in a jacketed tissue chamber that was kept at 37°C by circulating warm water. A thin latex balloon was inserted into the left atrium from the right atrium, and the balloon was filled with water to achieve a left ventricular end-diastolic pressure of 8–15 mmHg. Pacing wires were fixed to the right atrium, and the heart was paced at 5.0 Hz (300 beats/min) during perfusion. After 15 min of equilibration, left ventricular developed pressure (LVDP) and its first derivatives (+dP/dt and −dP/dt) were continuously recorded for 15 min using a computerized pressure amplifier/digitizer (MacLab 8, ADInstruments, Cupertino, CA).

TNF-α assay. Myocardial homogenate was prepared in four parts of homogenate buffer [0.1 M sodium phosphate (pH 7.5) containing 0.1% Triton X-100, 2 mM EGTA, 1.0 mM benzamidine, and 1.0 mM PMSF]. Centrifugation was carried out at 4°C, 1,000 g for 15 min with an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY). The supernatant was collected for the measurement of TNF-α. TNF-α levels in plasma and in myocardial homogenate were measured by a mouse ELISA kit as described previously (29, 34).

RT-PCR. RT-PCR was performed to assess TNF-α gene transcription in the myocardium, using previously described primers (3). Total RNA was extracted from myocardial homogenate by phenol-chloroform extraction (10). Two micrograms of isolated RNA were subjected to RT-PCR with avian myeloblastosis virus reverse transcriptase and random hexanucleotides as previously described (32). RT-PCR was carried out at 42°C for 30 min, followed by enzyme inactivation at 99°C for 5 min. PCR SuperMix containing Taq DNA polymerase ( Gibco BRL, Gaithersburg, MD) was used to amplify cDNA obtained from the reverse transcription. GAPDH mRNA expression was assessed for control. The amplified products were separated in a 1.5% agarose gel containing 0.5× Tris-borate-EDTA buffer, pH 8.3, and 1% ethidium bromide.

Immunoblotting. Myocardial tissue was homogenized in cold homogenate buffer (50 mM PBS, pH 7.4, 1.0 mM benzamidine, 1.0 mM PMSF, and 1% Triton X-100). The homogenate was centrifuged at 800 g for 10 min at 4°C, and the supernatant was collected. After protein concentration was determined, the supernatant was mixed with an equal volume of SDS sample buffer. Size fraction and transfer were performed as described previously (33). The membrane was incubated with a goat polyclonal antibody against TLR4 (from Santa Cruz Biotechnology, 1:200 dilution with PBS containing 0.05% Tween 20 and 5% dry milk) for 60 min and then with peroxidase-labeled rabbit anti-goat IgG (1:2,000 dilution) for 45 min. After thorough wash with PBS, we detected bands using the enhanced chemiluminescence technique.

Statistical analysis. Data are presented as means ± SE. ANOVA with a post hoc Bonferroni-Dunn test was performed to analyze differences between experimental groups. Statistical significance was accepted within 95% confidence limits.

RESULTS

Myocardial depression following hemorrhage. Cardiac contractile function at 1, 2, and 4 h was similar following sham treatment. Mean LVDP in sham groups was comparable to that in untreated mice (54.3 ± 3.7 mmHg). Although myocardial contractility at 1 and 2 h after hemorrhage was not different from that of sham control, it was depressed at 4 h (Fig. 1). LVDP was 34.9 ± 2.8 mmHg at 4 h after hemorrhage (P < 0.05 vs. sham 4 h, Fig. 1A). Similarly, +dP/dt was attenuated at 4 h after hemorrhage (Fig. 1B).

TNF-α production following hemorrhage. The temporal changes in TNF-α levels are shown in Fig. 2, A and B. Data obtained at 0.5–4 h after sham treatment were combined because TNF-α levels did not change over time after sham treatment.

Low levels of TNF-α were detected in plasma and the myocardium of sham-treated mice (Fig. 2, A and B). A moderate increase in TNF-α levels was found at 0.5 and 1 h after hemorrhage (Fig. 2, A and B), whereas TNF-α was undetectable at 0.5 and 1 h after hemorrhage in either plasma or myocardium of TNF-α KO mice. TNF-α levels in plasma and the myocardium at 2 and 4 h after hemorrhage were not different from those of sham control. The increase in myocardial TNF-α levels was accompanied by an elevation of myocardial TNF-α mRNA (Fig. 2C).
and the myocardium following sham treatment (Fig. 3). However, TNF-α levels did not change at either 30 min or 1 h after hemorrhage, and TNF-α levels at both time points were lower than those of wild-type mice (Fig. 3, A and B).

Effect of TLR4 mutation on myocardial depression. Myocardial contractile function after sham treatment in TLR4 mutant mice was not different from that in sham-treated wild-type mice (Fig. 4). However, myocardial depression caused by hemorrhagic shock was markedly attenuated in TLR4 mutant mice (Fig. 4). LVDP was 43.0 ± 6.2 mmHg in TLR4 mutant mice at 4 h after hemorrhage (P < 0.05 vs. wild-type hemorrhagic shock, Fig. 4A), and it was slightly lower than that in sham-treated TLR4 mutant mice. Moreover, +dP/dt was improved in TLR4 mutant mice following hemorrhage (Fig. 4B).

Effect of TNF-α KO on myocardial depression. Myocardial contractile function following sham treatment in TNF-α KO mice was not different from that in sham-treated wild-type mice (Fig. 5). However, myocardial depression caused by hemorrhagic shock was attenuated in TNF-α KO mice (Fig. 5). LVDP was 47.0 ± 0.9 mmHg in TNF-α KO mice at 4 h after hemorrhage (P < 0.05 vs. wild-type hemorrhagic shock, Fig. 5A). In addition, +dP/dt was improved in TNF-α KO mice following hemorrhage (Fig. 5B).

Effect of TNF-α receptor KO on myocardial depression. Myocardial contractile function following sham treatment in the p55 TNF-α receptor (TNFR-I) KO mice and the p75 TNF-α receptor (TNFR-II) KO mice was not different from that in sham-treated wild-type mice (Fig. 6, A and B). Similar to the effect of TNF-α KO, TNFR-I KO attenuated myocardial depression at 4 h after hemorrhage. LVDP was 44.4 ± 2.2 mmHg in TNFR-I KO mice following hemorrhage (P < 0.05 vs. wild type hemorrhagic shock, Fig. 6A). TNFR-I KO also
improved +dP/dt following hemorrhage (Fig. 6B). In contrast, TNFR-II KO had a minimal influence on myocardial contractile function following hemorrhage (Fig. 6, A and B). There was no change in myocardial TLR4 protein level in TNFR-I KO mice (Fig. 6C).

DISCUSSION

This study demonstrates that TLR4 plays a novel role in the TNF-α response and myocardial depression following hemorrhagic shock and that TNF-α contributes to myocardial depression through the p55 receptor.

TNF-α has been demonstrated to be a cardiodepressant factor in a variety of animal models (20–22) and has been linked to heart failure in humans (13). In this study, we observed in a murine nonresuscitated hemorrhagic shock model that hemorrhage induces a rapid and transient increase in TNF-α levels in plasma and the myocardium. Both plasma and myocardial TNF-α levels peaked at 30 min and returned to baseline at 2 h after hemorrhage. The increase in myocardial TNF-α at 30 min is accompanied by an elevation of TNF-α mRNA, suggesting that myocardium is a source of increased TNF-α in the heart. However, myocardial depression did not occur until 4 h. The time lag between the TNF-α response and myocardial depression suggests that TNF-α is an indirect cardiac depressant factor in this model of hemorrhagic shock. This delayed depression temporally correlates with that observed in an endotoxemia model (22). Thus myocardial depression induced by either hemorrhagic shock or LPS is a delayed disorder, and these two forms of stress may share a signaling mechanism.

Toll-like receptors play important roles in the innate immune response. They were first identified as putative homologues of the Drosophila Toll protein; subsequently, several members of the Toll-like receptor family have now been identified in mammals (7). Toll-like receptors transmit signals from the ectodomain, consisting of multiple leucine-rich repeats, to the cytoplasm via the TIR domain. This process has been best described between LPS and TLR4 (27). In LPS-induced TLR4 signaling, the TIR domain uses a conserved signal transduction pathway requiring MyD88 (14). MyD88 interacts with TLR4 through its own carboxy-terminal TIR domain. Through its amino-terminal death domain, MyD88 recruits IRAK4, which phosphorylates IRAK1 to propagate the proinflammatory signal, leading to the phosphorylation of the IKK complex and MAPKs, including p38 and JNK (14). This phosphorylation event liberates bound NF-κB from the cytoplasm into the

Fig. 3. Influence of Toll-like receptor 4 (TLR4) mutation on TNF-α production following HS. HS was induced in wild-type (BALB/cJ) and TLR4-defective mutant (C3H-Tlr4d mice by removal of 30% of total blood volume. TNF-α levels in the plasma (A) and in the myocardium (B) were measured at 0.5 and 1 h after hemorrhage. TLR4 mutation abolished the TNF-α response. Values are means ± SE; n = 6 in each group. *P < 0.05 vs. BALB/cJ Sham; #P < 0.05 vs. BALB/cJ HS.

Fig. 4. Influence of TLR4 mutation on cardiac contractile dysfunction following HS. HS was induced in wild-type mice (BALB/cJ) and TLR4-defective mutant mice (C3H-Tlr4d mice by removal of 30% of total blood volume. Hearts were isolated and perfused by the Langendorff technique 4 h after hemorrhage. Myocardial depression induced by hemorrhagic shock was attenuated in TLR4 mutants. LVDP and +dP/dt are presented as means ± SE; n = 5 in each group. *P < 0.05 vs. BALB/cJ Sham. #P < 0.05 vs. BALB/cJ HS.
nucleus, resulting in the production of proinflammatory mediators. TLR4 seems to be involved in the cellular response to injury (17). Interestingly, TLR4 gene expression is upregulated in the liver in a similar model of nonresuscitated, fixed-volume hemorrhagic shock (30), and TLR4 signaling contributes to myocardial depression after burn injury (36). These findings, therefore, indicate that TLR4 ligands may include endogenous agents. It remains to be determined whether the TLR4 signaling cascade is activated during hemorrhagic shock and whether endogenous proteins mediate the inflammatory response to hemorrhagic shock.

In the present study, TNF-α KO markedly attenuates myocardial depression induced by hemorrhagic shock, thus con-
failing that TNF-α plays an important role in this disorder. However, TNF-α seems not to play a sole role in myocardial depression because TNF-α KO attenuates myocardial depression rather than abrogating it. TNF-α signaling is mediated by two types of cell surface receptors, the p55 TNF-α receptor and the p75 TNF-α receptor (11). We have found that mice lacking the p55 TNF-α receptor exhibit reduced lung neutrophil accumulation and attenuated lung injury following hemorrhagic shock (34). It is likely that TNF-α serves as a proximal mediator to initiate a pulmonary inflammatory cascade during hemorrhagic shock through the p55 receptor. In the present study, myocardial depression induced by hemorrhagic shock is attenuated by the p55 TNF-α receptor KO, suggesting that TNF-α signals through this receptor for the expression of depressant factors. Blockade of the p55 TNF-α receptor may protect cardiac function during stress that induces a TNF-α response.

It is unclear how TNF-α depresses myocardial contractility. TNF-α can directly depress myocardial contractility in vitro (9). Moreover, TNF-α has been shown to synergize with multiple cytokines to depress myocardial function (9, 18). However, it seems unlikely for TNF-α to be a direct depressant factor in myocardial depression in this model of hemorrhagic shock because plasma and myocardial TNF-α levels are normalized before myocardial depression occurs. Moreover, low concentrations of TNF-α alone are not sufficient to depress myocardial contractility (9). A spike in the myocardial TNF-α level appears to serve as a signal, in the setting of hemorrhagic shock, to induce myocardial depressant factors. Determination of the role of TNF-α-induced factors in myocardial depression would suggest therapeutic approaches for myocardial protection during hemorrhagic shock.

Hemorrhage is an important factor in the etiology of end-organ failure associated with trauma (30). The results of the present study demonstrate in a murine model of hemorrhagic shock that hemorrhage alone triggers a proinflammatory signaling cascade and that an early proinflammatory response is involved in the induction of cardiac dysfunction. These findings suggest that suppression of the early proinflammatory response to hemorrhage is critical for preservation of cardiac function in trauma with severe hemorrhage. However, extrapolation of these observations to clinical situations should be made with caution since complex factors associated with trauma and resuscitation are absent in this nonresuscitated hemorrhagic shock model.

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REFERENCES


