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A Cardiovascular Drug Rescues Mice from Lethal Sepsis by Selectively Attenuating a Late-Acting Proinflammatory Mediator, High Mobility Group Box 1

Wei Li,* Jianhua Li,† Mala Ashok,* Rongqian Wu,‡ Dazhi Chen,‡ Lihong Yang,‡ Huan Yang,‡ Kevin J. Tracey,* Ping Wang,** Andrew E. Sama,* and Haichao Wang‡*†

The pathogenesis of sepsis is mediated in part by bacterial endotoxin, which stimulates macrophages/monocytes to sequentially release early (e.g., TNF, IL-1, and IFN-γ) and late (e.g., high mobility group box 1 (HMGB1) protein) proinflammatory cytokines. The recent discovery of HMGB1 as a late mediator of lethal sepsis has prompted investigation for development of new experimental therapeutics. We found that many steroidal drugs (such as dexamethasone and cortisone) and nonsteroidal anti-inflammatory drugs (such as aspirin, ibuprofen, and indomethacin) failed to influence endotoxin-induced HMGB1 release even at superpharmacological concentrations (up to 10–25 μM). However, several steroid-like pigments (tanshinone I, tanshinone IIA, and cryptotanshinone) of a popular Chinese herb, Danshen (Salvia miltiorrhiza), dose dependently attenuated endotoxin-induced HMGB1 release in macrophage/monocyte cultures. A water-soluble tanshinone IIA sodium sulfonate derivative (TSNIIA-SS), which has been widely used as a Chinese medicine for patients with cardiovascular disorders, selectively abrogated endotoxin-induced HMGB1 cytoplasmic translocation and release in a glucocorticoid receptor-independent manner. Administration of TSNIIA-SS significantly protected mice against lethal endotoxemia and rescued mice from lethal sepsis even when the first dose was given 24 h after the onset of sepsis. The therapeutic effects were partly attributable to attenuation of systemic accumulation of HMGB1 (but not TNF and NO) and improvement of cardiovascular physiologic parameters (e.g., decrease in total peripheral vascular resistance and increase in cardiac stroke volume) in septic animals. Taken together, these data re-enforce the pathogenic role of HMGB1 in lethal sepsis, and support a therapeutic potential for TSNIIA-SS in the treatment of human sepsis. The Journal of Immunology, 2007, 178: 3856–3864.

Sepsis is defined as a systemic inflammatory response syndrome resulting from a microbial infection. As a continuum of increasing clinical severity, severe sepsis is defined as sepsis associated with one or more acute organ dysfunctions (1). Despite recent advances in antibiotic therapy and intensive care, sepsis is still the most common cause of death in intensive care units, claiming ~225,000 victims annually in the United States alone. The pathogenesis of sepsis is attributable, at least in part, to dysregulated systemic inflammatory responses characterized by excessive accumulation of various proinflammatory mediators such as TNF or IL-1 (2), IFN-γ (3), NO (4), and macrophage migration inhibitory factor (5–7).

We recently discovered that a ubiquitous protein, high mobility group box 1 (HMGB1), is released by activated macrophages/monocytes (8–11) and functions as a late mediator of lethal endotoxemia and sepsis (8, 12–14). Circulating HMGB1 levels are elevated in a delayed fashion (after 16–32 h) in endotoxemic and septic mice (8, 12) and in patients with sepsis (8). Administration of recombinant HMGB1 to mice recapitulates many clinical signs of sepsis, including fever (15), derangement of intestinal barrier function (16), tissue injury (17), and multiple organ failure (8). Administration of anti-HMGB1 Abs or inhibitors (e.g., ethyl pyruvate, nicotine, or stearoyl lysophosphatidylcholine) significantly protects mice against LPS-induced acute tissue injury (17, 18) and lethal endotoxemia (8, 13, 19, 20). Notably, these anti-HMGB1 reagents are capable of rescuing mice from lethal experimental sepsis even when the first doses are given 24 h after the onset of sepsis (12, 13, 21, 22), indicating a wider window for HMGB1-targeted therapeutic strategies. Therefore, agents proven clinically safe, and yet still capable of attenuating HMGB1 release, may hold potential in the prevention and treatment of inflammatory diseases.

Throughout human history, herbal medicine has formed the basis of folk remedies for various inflammatory ailments. The use of willow bark extract to reduce pain and fever was documented by a Greek physician (Hippocrates) in the 5th century B.C. The subsequent discovery of salicylic acid as a pain or fever relief active component gave rise to the first synthetic nonsteroidal anti-inflammatory drug (NSAID), aspirin, and the birth of the pharmaceutical industry. Among thousands of Chinese medicinal herbs, only a few have been entitled Shen (e.g., Ren Shen (ginseng), Dan Shen (Salvia miltiorrhiza)). Danshen refers to a medicinal herb (termed shen) containing substance of premier medicinal value (termed Dan, cinnabar), and has been widely used in China for patients with cardiovascular disorders (23). Its beneficial effects are attributable to several red pigments including tanshinones I, II,
IV, and cryptotanshinone (24, 25), which exhibit various anti-inflammatory properties (26–28). In this study, we evaluated their capacity in inhibiting endotoxin-induced HMGB1 release, and explored their therapeutic potential in animal models of lethal endotoxemia and sepsis.

Materials and Methods

Cell culture

Murine macrophage-like RAW 264.7 cells were obtained from the American Type Culture Collection, and primary peritoneal macrophages were isolated from BALB/c mice (male, 7–8 wk, 20–25 g) at 2–3 days after i.p. injection of 2 ml thioglycolate broth (4%) as previously described (9, 10). Murine macrophages were precultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FBS and 2 mM glutamine. Human blood was obtained from anonymous healthy donors recruited by the Long Island Blood Bank. The study was considered to be exempt by the North Shore-Long Island Jewish Health System, Office of the Institutional Review Board, in accordance with the guidelines for human subject protection. Human PBMC were isolated from the blood of healthy donors by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS; Amersham Biosciences), and cultured in RPMI 1640 supplemented with 10% heat-inactivated human serum/2 mM l-glutamine as previously described (9).

LPS stimulation

Adherent macrophages or monocytes were gently washed with, and cultured in, serum-free OptiMEM I medium 2 h before stimulation with bacterial endotoxin (LPS, Escherichia coli O111:B4; Sigma-Aldrich). At 16 h after LPS stimulation, levels of HMGB1 in the culture medium were determined as previously described (8–10).

Preparation of herbal extract

Various Chinese herbs were obtained from New York Tongrentang and extracted in water (85°C) for 4 h. The water-soluble fraction was cleared sequentially by centrifugation (3300 × g for 20 min, 4°C) and filtration (through a 0.2-μm filter), and the filtrate fraction was examined for HMGB1-suppressing activities.

Chemical sources and stock solutions

Dexamethasone, cortisone, and RU486 were obtained from Sigma-Aldrich, and 10 mM stock solutions were prepared in 100% ethanol. Aspirin (catalog no. A0819), ibuprofen (catalog no. I1048), indomethacin (catalog no. I5315) were obtained from the LKT Laboratories, and 10 mM stock solution were prepared in water (20 mM). Tanshinone I (catalog no. T0153, 98.6% HPLC purity), tanshinone IIA (catalog no. T0154, 93.0% HPLC purity), and cryptotanshinone (catalog no. C7007, 98.8% HPLC purity) were obtained from the LKT Laboratories, and 20 mM stock solutions were prepared in DMSO. Tanshinone IIA sodium sulfonate (TNSIIA-SS) was obtained from the Shanghai No. 1 Biochemical & Pharmaceutical, and its purity was determined by HPLC using a Nova-Pak C18 column (3.9 × 150 mm) and 0.065% trifluoroacetic acid (v/v, in water) as the mobile phase. The sample was eluted by a linear gradient of 0–59% acetoniitrile (v/v, in 0.065% trifluoroacetic acid) over 12 min at a flow rate of 1.0 ml/min, and monitored at a wavelength of 245.2 nm. Each HPLC peak was analyzed using a Liquid Chromatography-Mass Spectrometry (LCQ DECA XP PLUS; Thermo Electron Corporation). The major peak (at a retention time of 8.3 min) accounted for >80% of the total area under the HPLC peaks and contained a red pigment with a mass-to-charge ratio of m/z = 373.07 (100.0%), corresponding to an empirical formula of C13H10O6S2 (TNSIIA-SS).

Animal models of endotoxemia and sepsis

This study was approved and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at the Feinstein Institute for Medical Research (Manhasset, NY). Endotoxemia was induced in BALB/c mice (male, 7–8 wk) by i.p. injection of bacterial endotoxin (LPS, 15 mg/kg) as previously described (8, 19, 29). Sepsis was induced in male BALB/c mice (7–8 wk, 20–25 g) or Sprague-Dawley rats (200–310 g) by cecal ligation and puncture (CLP) as previously described (29, 30). Herbal components were administered i.p. into mice at indicated doses and time points, and mice were monitored for survival for up to 2 wk. In parallel experiments, mice were euthanized to collect blood at 28 h (following two doses of herbal components at −0.5 and +24 h) after endotoxemia and at 52 h (following two doses of herbal components at +24 and +48 h) after CLP.

TNF ELISA

The levels of TNF in the culture medium or serum were determined using commercial ELISA kits (catalog no. MTA00; R&D Systems) with reference to standard curves of purified recombinant TNF at various dilutions as previously described (9, 10, 29).

NO assay

The levels of NO in the culture medium or serum were determined indirectly by measuring the NO2 concentrations with reference to a standard curve generated with sodium nitrite at various dilutions.

HMGB1 Western blotting analysis

The levels of HMGB1 in the culture medium or serum were determined by Western blot analysis as previously described (8–10). The relative band intensity was quantified by using the NIH Image 1.59 software to determine HMGB1 levels with reference to standard curves generated with purified HMGB1.

Cytokine Ab array

Murine cytokine Ab array (catalog no. M0308003; RayBiotech), which detects 62 cytokines on one membrane, was used to determine the profile of cytokines in the culture medium following the manufacturer’s instructions. Briefly, the membranes were sequentially incubated with equal volume of cell-conditioned culture medium, primary biot-in-conjugated Ab, and HRP-conjugated streptavidin. After exposing to x-ray film, the relative signal intensity was determined using NIH Image 1.59 software with reference to the positive controls on the membrane.

Immunocytochemistry and cell fractionation or Western blot

At 16 h after LPS stimulation, cellular HMGB1 was immunostained with anti-HMGB1 polyclonal Abs, and images were acquired using fluorescent microscope (Carl Zeiss Microimaging) as previously described (9, 10). Alternatively, localization of HMGB1 was examined by a cell fractionation (Western blotting) technique as previously described (11). Cell fractionation is based on differential lysis of plasma and nuclear membranes by nonionic detergent (Nonidet P-40). Briefly, after selective lysis of the plasma membrane in low salt buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1% Nonidet P-40), the intact nuclei was collected by a quick centrifugation step (7000 × g for 1 min at 4°C), leaving the cytoplasmic fraction in the supernatant. The nuclei pellet was resuspended in Nonidet P-40 high salt buffer (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1% Nonidet P-40), and briefly sonicated to generate the nuclear fraction. After fractionation, the protein content of different fractions was determined by a Bradford method, and each fraction was assayed for levels of various protein by Western blot analysis using primary Abs specific for HMGB1, a cytoplasmic protein (β-actin; Santa Cruz Biotechnology), and a proliferating cell nuclear Ag protein (BD Biosciences).

Cell viability assays

Cell viability was assessed by trypan blue exclusion assays as previously described (9). Briefly, trypan blue was added to cell cultures at a final concentration of 0.08%. After incubation for 5 min at 25°C, cell viability was assessed by the percentage of dye-excluding cells in five × 40 microscope fields.

Measurement of cardiac output

We used a radiolabeled microsphere technique to measure cardiac output at 20 h after CLP or sham operation as previously described (30). Briefly, a bolus of 145Ce-labeled microspheres (40 μCi; DuPont NEN) was injected into the left ventricle, where they were mixed uniformly with the oxygenated blood at the root of the aorta and subsequently distributed via aortic blood flow to the capillary beds within each organ. The reference blood sample was withdrawn from the femoral arterial catheter (at a rate of 0.7 ml/min) with a pump (Harvard Apparatus), after which isotonic sodium chloride solution was infused manually to replace the volume of blood lost. At 20 h after CLP, i.p. animals were sacrificed to harvest various organs for measurement of radioactivity with an automatic gamma counter (1470 Wizard; Wallac). Cardiac output and organ blood flow were calculated according to the following equations, respectively: ((RBF × Ct)/Cr) ×
FIGURE 1. Danshen extract (A) and components (tanshinone I, tanshinone IIA, and cryptotanshinone) (B and C) attenuated endotoxin-induced HMGB1 release. Murine macrophage-like RAW 264.7 cells were stimulated with LPS in the absence or presence of herbal extract (A), and tanshinone I (TSN I), tanshinone IIA (TSN IIA), or cryptotanshinone (C-TSN) (B and C) for 16 h, and levels of HMGB1 in the culture medium were determined by Western blot analysis. A representative Western blot of three independent experiments with similar results is shown (bottom panel). *, p < 0.05 vs controls (+LPS alone).

1/100 and ((RBF × Cg)/Cr) × 1/100, where RBF is the reference blood sample withdrawal rate (0.7 ml/min), Cg is cpm of total injected dose, Cc is cpm per gram of tissue, and Cr is cpm in the reference blood sample. Stroke volume and total peripheral resistance were calculated as previously described (30).

Statistical analysis

Data are expressed as mean ± SD of two independent experiments in triplicate. One-way ANOVA was used for comparison among all different groups. When the ANOVA was significant, posthoc testing of differences between groups was performed using Tukey’s test. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A value of p < 0.05 was considered statistically significant.

Results

Danshen (Salvia miltiorrhiza) extract and components attenuated endotoxin-induced HMGB1 release

The successful identification of salicylic acid as the active principle of willow bark to reduce fever and pain gave rise to the first synthetic NSAID, aspirin. Many NSAIDs (such as aspirin, ibuprofen, and indomethacin) can inhibit cyclooxygenases, but are unable to protect animals against lethal sepsis (31–33). In this study, we found that none of these cyclooxygenase inhibitors (e.g., aspirin, ibuprofen, and indomethacin) significantly attenuated LPS-induced HMGB1 release, with levels of HMGB1 in the culture medium determined by Western blot analysis. A representative Western blot of three independent experiments with similar results is shown (bottom panel). *, p < 0.05 vs controls (+LPS alone).

Water-soluble TSNIIA-SS selectively attenuated endotoxin-induced HMGB1 release

Most tanshinones are barely water-soluble at physiological temperature, and this poor solubility may adversely affect their bioavailability in vivo. We thus examined a water-soluble derivative TSNIIA-SS (Fig. 1B) for HMGB1-inhibiting activities. TSNIIA-SS is a widely used Chinese medicine for patients with cardiovascular disorders (23), and was obtained from the Shanghai No. 1 Biochemical & Pharmaceutical. HPLC analysis revealed a major peak (>80%, at a retention time of 8.3 min) containing a red pigment with an m/z = 373.07, corresponding to an empirical formula of C19H15O6S (tanshinone IIA sulfonate). The minor HPLC peak (<20%, at a retention time of 6.8 min) contained a red pigment with an m/z = 389.11, corresponding to an empirical formula of C19H15O6S (tanshinone IIA sulfonate). The moderately purified TSNIIA-SS preparation (containing both TSNIIA-SS (C19H15O6S, >80%) and TSNIIA-SS analog (C19H15O6S, <20%)) dose dependently inhibited HMGB1 release, with an estimated IC50 = 5 µM (Fig. 2A). Similarly, it effectively inhibited LPS-induced HMGB1 release in cultures of primary human PBMC (Fig. 2B) and murine peritoneal macrophages (data not shown).

To identify the active principles of the moderately purified TSNIIA-SS preparation, we screened each HPLC fractions for potential activities to inhibit endotoxin-induced HMGB1 release. The HMGB1-inhibiting activities were coeluted specifically with two HPLC peaks (monitored at a wavelength of 452.2 nm, at a retention time of 6.8 and 8.3 min, respectively) corresponding to TSNIIA-SS (C19H15O6S) or TSNIIA-SS analog (C19H15O6S) (data not shown). In light of the observations that all tanshinones tested (including I, IIA, cryptotanshinone, TSNIIA-SS, and TSNIIA-SS analog) were capable of inhibiting endotoxin-induced HMGB1 release, we performed most studies using the moderately purified TSNIIA-SS preparation (containing >80% TSNIIA and <20% TSNIIA analog). To further evaluate its anti-inflammatory properties, we determined the potential effects of TSNIIA-SS on LPS-induced release of other proinflammatory mediators. At concentrations (100 µM) that completely abrogated LPS-induced HMGB1 release,
TSNIIA-SS did not completely block LPS-induced release of NO (Fig. 2A, top) or TNF (Fig. 2B, bottom). Furthermore, its inhibitory effects on TNF (but not HMGB1) release were often lost when macrophages were stimulated with LPS at higher concentrations (>200 ng/ml), indicating selectivity for HMGB1 suppression. To evaluate this specificity, we determined its effects on the release of 62 cytokines using a cytokine Ab array. At concentrations that completely abrogated LPS-induced HMGB1 release (100 μM, data not shown), TSNIIA-SS did not completely block LPS-induced release of NO (A), TNF (B), or IL-1α, platelet factor 4 (PF-4), IL-12p70, and MCP-5 (C). A representative cytokine array (C) of two independent experiments with similar results is shown.

FIGURE 2. TSNIIA-SS specifically abrogated endotoxin-induced HMGB1 release. Murine macrophage-like RAW 264.7 cells, primary murine peritoneal macrophages, or human PBMC were stimulated with LPS in the absence or presence of TSNIIA-SS at indicated concentrations. At 16 h after LPS stimulation, levels of HMGB1 (A and B), NO (A), or TNF (B and C) in the culture medium were determined by Western blot analysis (A and B), Griess reaction (A), ELISA (B), or cytokine array (C). Note that at concentrations that completely abrogated LPS-induced HMGB1 release, TSNIIA-SS did not completely block LPS-induced release of NO (A), TNF (B), or IL-1α, platelet factor 4 (PF-4), IL-12p70, and MCP-5 (C). A representative cytokine array (C) of two independent experiments with similar results is shown.

Together, these data indicate that TSNIIA-SS selectively attenuates LPS-induced release of HMGB1 as opposed to other proinflammatory cytokines.

Delayed administration of TSNIIA-SS still significantly attenuated endotoxin-induced HMGB1 release

As compared with early proinflammatory cytokines (such as TNF), HMGB1 is released in a delayed fashion following endotoxin stimulation. It is intriguing to consider whether TSNIIA-SS can inhibit HMGB1 release if added after LPS stimulation. Whereas concurrent administration of TSNIIA-SS with LPS was maximally effective in suppressing HMGB1 release, significant inhibition was retained when it was added 2–6 h after LPS stimulation. It may thus be feasible to pharmacologically attenuate late-acting proinflammatory mediators (such as HMGB1) by strategically administering TSNIIA-SS in a delayed fashion.
inflammatory drugs (Fig. 5A), we evaluated: 1) whether cortico-
steroids similarly attenuated LPS-induced HMGB1 release and 2) whether TSNIIA-SS abrogates HMGB1 release in a glucocor-
ticoid receptor-dependent mechanism. Even at concentrations
up to 10 μM, dexamethasone and cortisol failed to reduce
LPS-induced HMGB1 release (Fig. 5B), although they effec-
tively attenuated LPS-mediated TNF secretion (Fig. 5C). Steroi-
dal anti-inflammatory drugs inhibit cytokines through binding to
intracellular glucocorticoid receptor (35), which can be over-ridden
by specific glucocorticoid receptor antagonists (such as RU486). Indeed, RU486 almost completely abrogated dexametha-
sone-mediated suppression of TNF secretion (Fig. 5C), but did not
affect TSNIIA-SS-mediated inhibition of TNF (Fig. 5C), or
HMGB1 release (data not shown). Taken together, these data in-
dicate that TSNIIA-SS and dexamethasone use distinct mecha-
nisms to suppress endotoxin-induced cytokine release.

TSNIIA-SS protected against lethal endotoxemia

In light of the capacity of TSNIIA-SS in attenuating LPS-induced
HMGB1 release, we explored its efficacy in an animal model of
lethal endotoxemia. Administration with a single dose of
TSNIIA-SS 30 min after an LD75 dose of LPS did not significantly
improve animal survival rate (25% for control receiving saline
(n = 20 mice/group) vs 37.5% for experimental group receiving

FIGURE 3. Delayed administration of TSNIIA-SS still significantly at-
tenuated endotoxin-induced HMGB1 release. Murine macrophage-like
RAW 264.7 cells were stimulated with LPS, and TSNIIA-SS (25 μM (A)
or 50 μM (B)) was added at 0, 2, 6, and 12 h after LPS stimulation. Levels of
HMGB1 levels in the culture medium were determined at 16 h after LPS
stimulation and expressed (in arbitrary unit, AU) as the mean ± SD of two
independent experiments. A representative Western blot is shown at bottom
of both experiments. *, p < 0.05 vs controls (+LPS alone).

FIGURE 4. TSNIIA-SS blocked endotoxin-induced cytoplasmic
HMGB1 translocation. Macrophage cultures were stimulated with LPS in
the absence or presence of TSNIIA-SS, and assayed for HMGB1 cytoplas-
ic translocation by immunohistochemistry (A) or cell fractionation
(Western blot technique) (B) at 16 h after LPS stimulation. Note in A that
HMGB1 was predominantly localized in the nuclear region of unstimulated
macrophages (control) (left column), in both cytoplasmic and nuclear re-
gions of LPS-stimulated macrophages (middle column). TSNIIA-SS (100
μM) preserved HMGB1 in the nuclear regions (LPS + TSNIIA-SS) (right
column). Following LPS stimulation, cytoplasmic (C) and nuclear (N) frac-
tions were isolated and assayed for levels of HMGB1, a proliferating cell
nuclear Ag protein, or cytoplasmic (β-actin) protein using Western blot
analysis. Equal loading of samples was confirmed by Western blot analysis
of fractions with cytoplasmic (β-actin) or proliferating cell nuclear Ag
protein markers. Blots are representative of two independent experiments
with similar results. To further validate the above hypothesis, cytoplasmic and
nuclear fractions were isolated, and immunoblotted with Abs specific
for HMGB1, a proliferating cell nuclear Ag protein, or β-actin (a
cytoplasmic protein), respectively. Consistently, levels of HMGB1
in the cytoplasmic fractions were dramatically increased after LPS
stimulation (data not shown), but were dramatically reduced by
TSNIIA-SS treatment (Fig. 4B), confirming that TSNIIA-SS at-
tenuates HMGB1 release by interfering with its cytoplasmic
translocation.

TSNIIA-SS inhibited endotoxin-induced HMGB1 release in a
steroidal glucocorticoid receptor-independent mechanism

In light of the structural resemblance (i.e., the presence of a four-
fused ring structure) between tanshinones and steroidal anti-
inflammatory drugs (Fig. 5A), we evaluated: 1) whether cortico-

TSNIIA-SS inhibited endotoxin-induced HMGB1 release by
blocking its cytoplasmic translocation

To investigate a mechanism of tanshinone-mediated suppression
of HMGB1 release, we determined its effect on endotoxin-induced
HMGB1 cytoplasmic translocation, an essential step for HMGB1
release (9, 10, 34). Consistent with previous reports (10), quiescent
macrophages constitutively expressed HMGB1 and maintained an
intracellular “pool” of HMGB1 predominantly in the nucleus (Fig.
4A, left column). At 16 h after LPS stimulation, we observed sig-
nificant HMGB1 staining in cytoplasmic vesicles (Fig. 4A, middle
column), confirming that LPS stimulates macrophages to actively
translocate nuclear HMGB1 to the cytoplasm before releasing it
into the extracellular milieu. Although the TSNIIA-SS did not af-
flect the nuclear localization of HMGB1 in resting cells (data not
shown), it almost completely abrogated LPS-induced HMGB1 cytop-
lasmic translocation in most endotoxin-stimulated cells (Fig.
4A, right column), indicating that TSNIIA-SS attenuates HMGB1
release by interfering with its cytoplasmic translocation.

To further validate the above hypothesis, cytoplasmic and nu-
clear fractions were isolated, and immunoblotted with Abs specific
for HMGB1, a proliferating cell nuclear Ag protein, or β-actin (a
cytoplasmic protein), respectively. Consistently, levels of HMGB1
in the cytoplasmic fractions were dramatically increased after LPS
stimulation (data not shown), but were dramatically reduced by
TSNIIA-SS treatment (Fig. 4B), confirming that TSNIIA-SS at-
tenuates HMGB1 release by interfering with its cytoplasmic
translocation.
TSNIIA-SS at 15 mg/kg (n = 20 mice/group); p > 0.05). By treating animals with three additional doses of TSNIIA-SS (+24, +48, and +72 h), we observed a dose-dependent improvement in animal survival (from 20% to 80%) (Fig. 6A). Furthermore, administration of TSNIIA-SS dose dependently attenuated circulating HMGB1 levels (140 ± 20 ng/ml, LPS plus vehicle vs 40 ± 25 ng/ml (n = 10 mice); p < 0.01), suggesting that TSNIIA-SS protects animals against lethal endotoxemia partly through attenuating systemic HMGB1 accumulation.

**TSNIIA-SS rescues mice from lethal sepsis**

Although endotoxemia is useful to investigate the complex cytokine cascades, more clinically relevant animal models are necessary to explore therapeutic agents for the treatment of human sepsis. One well-characterized, standardized animal model of sepsis is induced by CLP. In light of the late and prolonged kinetics of HMGB1 accumulation in experimental sepsis (12), we reasoned that it might be possible to rescue mice from lethal sepsis even if TSNIIA-SS is administered after the onset of sepsis. The first dose of TSNIIA-SS was given 24 h after the onset of sepsis, a time point at which mice developed clear signs of sepsis (including lethargy, diarrhea, piloerection). An i.p. administration with a single dose of TSNIIA-SS 24 h after the onset of sepsis failed to improve survival rate (33% for control receiving saline (n = 24 mice/group) vs 50% for experimental group receiving TSNIIA-SS at 15 mg/kg (n = 24 mice/group); p > 0.05). However, repeated administration of TSNIIA-SS beginning 24 h after the onset of sepsis (followed by additional doses at 48, 72, and 96 h after sepsis) conferred a dose-dependent protection against lethal sepsis (n = 30 mice/group) (Fig. 6B), significantly increasing animal survival rate from 33% to 73% (p < 0.05).

**TSNIIA-SS attenuates sepsis-induced systemic HMGB1 accumulation**

To gain insight into its protective mechanism, we evaluated the effects of TSNIIA-SS on the systemic accumulation of TNF, NO, and HMGB1. Consistent with an early report (31), systemic TNF was barely detectable at late stage of sepsis. Delayed administration of TSNIIA-SS did not attenuate circulating TNF levels at 52 h after the onset of sepsis (TFN = 65 ± 15 pg/ml, vehicle control group (n = 10 mice/group) vs TNF = 85 ± 23 pg/ml, TSNIIA-SS group (n = 10 mice/group); p > 0.05). Similarly, delayed administration of TSNIIA-SS did not attenuate circulating NO levels at 52 h after the onset of sepsis (18.0 ± 4.5 μM, vehicle control group vs 15.5 ± 3.3 μM, TSNIIA-SS group (n = 3 mice); p > 0.05). In contrast, repeated administration of TSNIIA-SS dose dependently and significantly attenuated (p < 0.05) circulating HMGB1 levels in septic mice (Fig. 7), indicating that TSNIIA-SS confers protection against lethal sepsis partly by attenuating systemic HMGB1 accumulation.
FIGURE 7. TSNIIA-SS attenuates sepsis-induced systemic HMGB1 accumulation. BALB/c mice were subjected to lethal sepsis by CLP, and i.p. administered with control saline (0.2 ml/mouse) or TSNIIA-SS (at indicated doses) at +24 and +48 h after CLP. At 52 h after the onset of sepsis, serum HMGB1 levels were determined and expressed as the mean ± SD (n = 10 mice). *p < 0.05 (by ANOVA or Tukey test).

TSNIIA-SS protected against sepsis-induced cardiac dysfunction

Because TSNIIA-SS has been successfully used for patients with cardiovascular disorders (23), we also evaluated whether it improves cardiovascular function in septic animals. Despite the lack of myocardial injury (36), there is a hypodynamic change in cardiovascular function manifested by a significant decrease in cardiac output in the late stage (e.g., 20 h after the onset) of sepsis (30). Administration of TSNIIA-SS did not significantly affect the mean arterial blood pressure (106.1 ± 4.7 mm Hg, CLP group vs 96.4 ± 8.7 mm Hg, CLP with TSNIIA-SS (n = 6 mice); p > 0.05), but slightly reduced the heart rate (378.3 ± 25.1 beats/minutes, CLP group vs 334.1 ± 25.8 beats/minutes, CLP with TSNIIA-SS, 15 mg/kg (n = 6 mice); p < 0.05). More importantly, it dose dependently reduced total peripheral vascular resistance (Fig. 8A), and yet significantly increased cardiac stroke volume (Fig. 8B) and cardiac output (Fig. 8C). Taken together, these data indicate that TSNIIA-SS, an effective pharmacologic agent used for patients with cardiovascular disorders in China, appears to be protective against sepsis-induced cardiovascular dysfunction in an animal model of sepsis.

Discussion

The pathogenesis of lethal sepsis remains obscure, but is associated with dysregulated inflammatory response, tissue injury, and multiple organ dysfunction. The inflammatory response is mediated in part by bacterial endotoxin (37), which stimulates macrophages/monocytes to sequentially release early (e.g., TNF and IL-1) and late (e.g., HMGB1) proinflammatory cytokines. Although early cytokines may be protective against infection (38), dysregulated inflammatory response sustained by late-acting mediators (such as HMGB1) may contribute to the development of tissue injury and organ dysfunction at the late stage of lethal sepsis. Therefore, agents capable of selectively attenuating systemic HMGB1 accumulation may hold potential in the treatment of lethal sepsis.

Many NSAIDs (e.g., aspirin, ibuprofen, and indomethacin) fail to protect against lethal sepsis (31), and consistently fail to significantly inhibit LPS-induced HMGB1 release (32, 33). In contrast, the Chinese herb Danshen (Salvia miltiorrhiza) contains medicinal substances (such as tanshinone I, tanshinone IIA, and cryptotanshinone) that effectively attenuate endotoxin-induced HMGB1 release in macrophage/monocyte cultures. However, due to poor solubility and bioavailability, tanshinones I and IIA failed to rescue mice from lethal sepsis, even after repeated administration at 24, 48, 72, and 96 h after the onset of sepsis (survival rate = 50%, control vehicle group vs survival rate = 57%, tanshinone I group, 12 mg/kg; survival rate = 61%, tanshinone IIA group, 12 mg/kg (n = 14 mice/group); p > 0.05), forcing us to explore other water-soluble derivatives as potential therapeutic agents.

TSNIIA-SS, a clinically approved drug for patients with cardiovascular disorders, completely abrogates endotoxin-induced HMGB1 release in macrophage/monocyte cultures. The mechanism by which TSNIIA-SS inhibits endotoxin-induced HMGB1 release remains elusive, but is partly attributable to its ability to interfere with LPS-induced HMGB1 cytoplasmic translocation. Although containing similar backbone chemical structure with glucocorticoids, TSNIIA-SS does not appear to use the glucocorticoid receptor to inhibit HMGB1 release because specific glucocorticoid receptor antagonist fails to abolish TSNIIA-SS-mediated inhibition of HMGB1 release. Interestingly, tanshinone I can inhibit phospholipase A2 (26), an enzyme that enhances endotoxin-induced HMGB1 release by generating lysophosphatidylcholine (19). Similarly, cryptotanshinone can inhibit acetylcholinesterase (34), an enzyme that eliminates the HMGB-1-inhibiting neurotransmitter, acetylcholine (13, 39, 40). It will thus be important to determine whether TSNIIA-SS inhibits HMGB1 release by inhibiting secretory phospholipase A2 or acetylcholinesterase in future studies.

At concentrations that completely abrogated LPS-induced HMGB1 release, TSNIIA-SS does not affect the release of most (58 of 62) other cytokines, indicating selectivity for inhibiting HMGB1 over most other cytokines. Even when given several hours after LPS stimulation, TSNIIA-SS is still effective in blocking HMGB1 release, distinguishing itself from all previously known HMGB1 inhibitors (including ethyl pyruvate, nicotine, and stearoyl lysophosphatidylcholine) (13, 19, 21). These unique properties enable us to strategically administer TSNIIA-SS in a delayed...
Inhibition of inflammatory cytokine production with tanshinone-II A. Sama, and D. Chen are coinventors of a patent application.

Disclosures

Acknowledgments

References


