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Selective histone deacetylase 6 inhibition prolongs survival in a lethal two-hit model

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ABSTRACT

Background: Hemorrhagic shock (HS) followed by a subsequent insult ("second hit") often initiates an exaggerated systemic inflammatory response and multiple organ failure. We have previously demonstrated that valproic acid, a pan histone deacetylase inhibitor, could improve survival in a rodent "two-hit" model. In the present study, our goal was to determine whether selective inhibition of histone deacetylase 6 with Tubastatin A (Tub-A) could prolong survival in a two-hit model where HS was followed by sepsis from cecal ligation and puncture (CLP).

Methods: C57Bl/6J mice were subjected to sublethal HS (30% blood loss) and then randomly divided into two groups ($n = 13$ per group) such as Tub-A group (treatment) and vehicle (VEH) group (control). The Tub-A group was given an intraperitoneal injection of Tub-A (70 mg/kg) dissolved in dimethyl sulfoxide (DMSO). The VEH group was injected with DMSO (1 μl/g body weight). After 24 h, all mice were subjected CLP followed immediately by another dose of Tub-A or DMSO. Survival was monitored for 10 d. In a parallel study, peritoneal irrigation fluid and liver tissue from Tub-A- or DMSO-treated mice were collected 3 h after CLP. Enzyme-linked immunosorbent assay was performed to quantify activity of the myeloperoxidase and concentrations of tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6) in the peritoneal irrigation fluid. RNA was isolated from the liver tissue, and real-time polymerase chain reaction was performed to measure relative messenger RNA levels of TNF-α and IL-6.

Results: Treatment with Tub-A significantly improved survival compared with that of the control (69.2% *versus* 15.4%). In addition, Tub-A significantly suppressed myeloperoxidase activity (169.9 ± 8.4 ng/mL *versus* 70.4 ± 17.4 ng/mL; $P < 0.01$) and reduced levels of cytokines TNF-α and IL-6 in the peritoneal fluid (TNF-α: 105.7 ± 4.7 *versus* 7.4 ± 2.4 pg/mL; IL-6: 907.4 ± 2.3 *versus* 483.6 ± 1.6 pg/mL; $P < 0.01$) compared with those in the VEH control. Gene expression measured by real-time polymerase chain reaction confirmed that Tub-A inhibits transcription of TNF-α and IL-6.

Conclusions: Tub-A treatment significantly improves survival, attenuates inflammation, and downregulates TNF-α and IL-6 gene expression in a rodent two-hit model.

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1. Introduction

Hemorrhagic shock (HS) is a major cause of morbidity and mortality among trauma patients, whereas septic shock (SS) is a leading cause of mortality in the intensive care units [1]. Patients who survive the acute episode of blood loss often exhibit a systemic inflammatory response syndrome, which can be further complicated by immune dysfunction [2]. The combination of hemorrhage and subsequent sepsis in trauma patients (two-hit insult) is considered to be a major reason for the development of multiple organ failure and death in trauma patients [3]. Despite advances in supportive treatments, the mortality and morbidity remain high with a substantial burden on the health care system [4].

Sepsis is classically attributed to hyperinflammatory responses that result in excessive production of cytokines, which can lead to cellular injury and organ dysfunction [5]. It has been shown that shock decreases the acetylation of nuclear and cytoplasmic proteins, which in turn impairs gene transcription and the function of multiple pathways that are involved in cell survival [6]. Inhibition of histone deacetylase (HDAC) can induce protein acetylation. It has been reported that histone hyperacetylation results in upregulation of cell cycle inhibitors (p21Cip1, p27Kip1, and p16INK4), repression of inflammatory cytokines (interleukin [IL]-1, IL-8, tumor necrosis factor-alpha [TNF-α]), and downregulation of immune stimulators (IL-6, IL-10, and CD154) [7]. Our team has previously demonstrated that treatment with valproic acid (VPA), a pan HDAC inhibitor (HDACI), results in improved survival in a rodent two-hit (HS followed SS) model [8]. However, class and isoform selective inhibition of HDAC is now gaining favor as it limits the toxicity that has been observed with pan-HDACIs. HDAC6, a member of the HDAC family, whose major substrate is α-tubulin, is being increasingly implicated in the pathogenesis of inflammatory disorders. In the present study, we tested the hypothesis that specific HDAC6 inhibition with Tubastatin A (Tub-A) would improve survival in a rodent two-hit model as follows: HS followed by SS from cecal ligation and puncture (CLP).

2. Material and methods

All the research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations related to animal experimentation. The study adhered to the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, and was approved by the Institutional Animal Care and Use Committee. Male C57BL/6J mice (18–26 g) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed for 3 d before the experiment to ensure good health.

2.1. Animal model and experimental design

Hemorrhage was induced as previously described by Liu *et al.* [8]. Mice were anesthetized with 0.7%–1.2% isoflurane (Abbott Laboratories, North Chicago, IL) mixed with air, which was administered via a nose cone scavenging system and allowed to breathe spontaneously, using a veterinary multichannel anesthesia delivery system and vaporizer (Kent Scientific Corporation, Torrington, CT). The bilateral femoral artery was cannulated with polyethylene 10 catheters (Clay Adams, Sparks, MD). The left femoral artery cannula was used for hemorrhage and fluid resuscitation, whereas the right arterial catheter was connected to the Ponemah Physiology Platform (Gould Instrument Systems, Valley View, OH) for continuous hemodynamic monitoring. To induce HS, baseline arterial blood samples were obtained, and then additional blood was withdrawn to a target of 30% of the estimated total blood volume (total blood volume [mL] = weight [g] $\times$ 0.07 [mL/g] over 10 min. After 30 min of unresuscitated shock, the animals were randomly assigned to three groups (n = 7–13 per group), and treatment was administered via intraperitoneal injection as follows: (a) sham animals, instrumentation and anesthesia but no hemorrhage and no CLP (sham; n = 7); (b) Dimethyl sulfoxide (DMSO [1 μL/g]) vehicle-treated animals (VEH; n = 13), and (c) Tub-A (70 mg/kg) treated animals (Tub-A; n = 13). After 1 h of observation, catheters were removed, vessels were ligated, and skin incisions were closed. Animals were recovered from anesthesia and returned to their cages. Twenty-four hours later, these mice were reanesthetized with isoflurane, and polymicrobial sepsis was induced by CLP as described by Rittirsch *et al.* [9]. In brief, the peritoneal cavity was opened under inhaled isoflurane anesthesia. Cecum was eviscerated, ligated at the designated position (75%) using a 5-0 suture, and punctured through and through (2 holes) with a 21 gauge needle. The punctured cecum was squeezed to expel a small amount of fecal material and returned to the peritoneal cavity. The abdominal incision was closed in two layers with 4-0 silk suture. A second (same as previous) dose of DMSO and Tub-A was given via intraperitoneal administration, and animals were woken from anesthesia and transferred to their cages for observation. They were monitored for 10 d to document survival. A second experiment was designed to measure the concentration of selected proinflammatory cytokines in the peritoneal fluid and their messenger RNA (mRNA) expression in the liver. In this experiment, a different set of mice were subjected to the two-hit (same as experiment 1) and sacrificed 3 h (n = 3 per group) after CLP. At the time of sacrifice, the abdominal cavity was opened and irrigated with 1 mL of isotonic sodium chloride solution, and this fluid was collected, liver tissues were harvested for further assay.

2.2. Myeloperoxidase assay

Myeloperoxidase (MPO) activity in the peritoneal irrigation fluid was determined using the MPO Assay Kit (Cell Sciences Inc, Canton, MA) according to the manufacturer's instructions. The peritoneal cavity was irrigated with 1 mL normal saline, the fluid was centrifuged at 1500 g at 4°C for 10 min, and supernatants were saved for analysis.