LOW-VOLUME BINARY DRUG THERAPY FOR THE TREATMENT OF HYPOVOLEMIA

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ABSTRACT—The selective regulation of total peripheral circulation in hypovolemic crisis offers a unique approach for treating and preventing hemorrhagic shock. Ideally, such a therapeutic intervention would require targeting of the striated muscle vascular beds without altering the vascular resistance in vital organ vascular beds. We discovered that a combination of cannabinoid receptor agonist, THC ($\Delta^8$-tetrahydrocannabinol), and cyclooxygenase 2 inhibitor, NS-398, caused selective microvascular constriction in the mouse cremaster muscle manifested by a pronounced and significant 27.4% ± 7.9% decrease in vessel diameter relative to control (P < 0.01). This observation, and the reported lack of microvascular response in the mesentery and brain, led us to hypothesize that the drug combination could favorably redistribute blood volume in hypovolemia and prolong survival. To test the hypothesis, male Sprague-Dawley rats were subjected to a pressure-controlled hemorrhage (mean arterial pressure reduced to 30 ± 13.73 mmHg) then randomly assigned to one of six treatment groups (n = 6 per group). The untreated, NS-398-treated, and THC-treated groups manifested an insignificant difference in survival between groups after shock. The group treated with a combination of THC and NS-398 manifested a significant increase in mean survival from 53 ± 12 to 227 ± 23 min after shock (P < 0.001). The drug combination significantly reduced IL-1$\alpha$, IL-1$\beta$, IFN-$\gamma$, and IL-10 production compared with the group resuscitated with normal saline. In addition, histological evaluation indicated that the therapy protects the lungs and liver against hemorrhagic shock–induced damage. The combination of cannabinoid receptor agonist and cyclooxygenase 2 inhibitor represents a potentially new approach to low-volume therapeutic intervention for hypovolemia.

KEYWORDS—Hypovolemia, hemorrhagic shock, cannabinoid, cyclooxygenase 2 (COX-2), microcirculation, rat, mice

INTRODUCTION

Hypovolemia caused by traumatic injury from automobile accidents, penetrating wounds, and falls, resulting in the onset of hemorrhagic shock (HS), is one of the leading causes of death for individuals younger than 45 years in the United States (1). The clinical syndrome of HS is characterized by a reduction in hydrodynamic volume causing inadequate tissue perfusion and a subsequent loss of normal metabolic function. The body’s initial response to early HS is to divert peripheral blood to the vital organs via alterations in the permeability of the resistance microvasculature (2, 3). Concurrently, the reduced perfusion initiates an organized set of programmed neuroendocrine and inflammatory-mediated responses that are unique to HS (4, 5). The condition manifests increased levels of proinflammatory cytokines (6), nitric oxide (7), reactive oxygen species (8), and peroxynitrite. If left unchecked, the biochemical mediators ultimately cause the failure of the microcirculation characterized by peripheral vasodilation, neutrophil adhesion, nonresponsiveness to catecholamines, and capillary leakage (9). This collapse of the microcirculation is believed to be directly related to the heterogeneous responses of the arterioles (A0-A4) and the venules (V0-V4) during the decomposition phases of HS (10).

The basis of our hypothesis was that we could regulate the microvascular tone in the peripheral striated muscle, thus preferentially redistributing the remaining blood volume in HS. Moreover, an earlier study by Wagner and coworkers (11) demonstrated that pretreatment of animals with $\Delta^8$-tetrahydrocannabinol (THC), before inducing HS, doubled the survival time relative to control animals. The selective regulation of the microvascular components combined with the modulation of inflammatory responses to HS provides a novel target for HS treatment. The concept of treating HS via vascular tone regulation is not new, and peptide hormones are often clinically used in the treatment of HS (12). However, the development of an efficacious HS therapy using small organic drug molecules remains elusive. In the process of studying the mechanism(s) of the beneficial effects of THC in HS (11), we unexpectedly discovered that a combination of THC and a cyclooxygenase 2 (COX-2) inhibitor triggered a pronounced microvascular constriction in the mouse cremaster, a striated muscle model. Based on this discovery, in combination with the lack of microvascular response in the mesentery and brain using a similar combination (5), we hypothesized that the selective striated muscle microvascular response could be exploited to treat HS. In addition, anti-inflammatory pharmacology of COX-2 inhibition and cannabinoid receptor (CBR) activation was predicted to add additional protection against the sequelae of HS.

We now report the development of a novel HS therapy composed of a selective COX-2 inhibitor (NS-398) combined with CBR 1 and 2 (CB-1 and CB-2) agonist/partial agonist THC. Our results indicate that (a) this combination causes prolonged...
vasoconstriction in the resistance vessels in the mouse striated muscle and (b) this effect translates to stabilization of the mean arterial pressure (MAP) in hemorrhaged rats leading to a statistically significant (P < 0.001) increase in survival time compared with untreated hemorrhaged rats. The combination therapy protects the lung and liver from HS-induced damage compared with the group receiving normal saline resuscitation. This novel drug combination may offer a new approach for the low-volume resuscitation of HS.

MATERIALS AND METHODS

All the animals were used in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All procedures were performed on animals upon approval from the University of Tennessee Health Science Center, Institutional Animal Care and Use Committee, C57BL/male mice (6–8 weeks old; 18–20 g) and male Sprague-Dawley rats (4 months of age; 180–220 g) were obtained from Harlan (Indianapolis, Ind.). NS-398 was obtained from Sigma (Sigma-Aldrich, St Louis, Mo). 6,6,9-Trimetethyl-3-pentyl-6α,7,10,10a-tetrahydro-6H-benzo[c]chromen-1-ol (THC) and N-arachidonylethanolamine (anandamide [AEA]) were synthesized in our laboratory. Solutions of the CB-1 ligands and NS-398 were prepared by dissolving the drug(s) in ethanol, followed by the addition of Stepanex (nonionic surfactant), and isotonic saline (final ratio 5:5:90 ethanol/Stepanex/saline). Drug concentrations were adjusted such that a 200-μL injection delivered 12 mg/kg CB-1 ligand and/or 1 mg/kg COX-2 inhibitor. The 1.4F Micro-Tip catheter transducer and the pressure control unit (model TCB-600) were obtained from Millar Instruments Inc. (Houston, Tex). Blood pressure data were analyzed using Acquaknowledge III software (BIOPAK Systems, Inc, Santa Barbara, Calif).

Experimental design

Animals used for the mouse cremaster muscle model were housed ad libitum in cages, each containing at least four mice. The animals were housed at approximately 25°C and a 12-hour-light–12-hour-dark cycle in the animal facility. Random selection of animals for the mouse cremaster study was performed from different cages. Mice were divided in to five groups (n = 6 in each group): (a) no treatment, (b) AEA only, (c) NS-398 only, (d) THC only, and (e) NS-398 + THC. A minimum criterion for inclusion of animals after the cremaster muscle preparation was based on evaluation of the physiological tone of the A3/A4 arterioles. A 0.1 μM of adenosine solution was administered topically, and only those vessels that demonstrated at least 20% dilation were considered for the experiment.

In the rat hemorrhagic model, male Sprague-Dawley rats (180–220 g) were housed ad libitum with two animals to a cage. The animals were housed at approximately 25°C and a 12-hour-light–12-hour-dark cycle in the animal facility. Subjects were selected at random from different cages and were assigned to one of the following treatment groups: (a) hemorrhaged no treatment control, (b) hemorrhaged vehicle, (c) hemorrhaged THC, (d) hemorrhaged NS-398, (e) hemorrhaged NS-398 + THC, and (f) hemorrhaged then maintained at 40 mmHg with normal saline. Tissue samples for histopathology were collected from the three groups: THC/NS-398–treated group, hemorrhaged then maintained at 40 mmHg with normal saline group, and normotensive untreated control group.

Cremaster muscle preparation—The cremaster muscle procedure was carried out as previously described (13). Six- to 8-week-old C57BL mice (n = 6 per group) weighing 18 to 20 g were anesthetized by i.p. injection of ~50 μL of anesthetic (87 mg/mL ketamine + 13 mg/mL xylazine). The dose of the anesthetic was dependent on the weight of the animal. The core body temperature of the animals was thermostatically controlled using heating pads maintained at 37°C. Each mouse was prepared for surgery by shaving the ventral thoracic area. A midline incision was made at the ventrocervical region, and the underlying tissue was bisected laterally, and the testis retracted. The animal was then intubated with PE50 tubing to facilitate ventilation, and the thorax was closed after completion of intubation. Next, the left hind limb was shaved, and an incision made to expose the femoral neurovascular bundle. Venous catheters were implanted by surgical isolation of the femoral vein using PE10 tubing. The animal was then placed on a specially designed surgery board in such a way that the right testicle was oriented over the glass transillumination window. A constant drip of warmed (37°C) physiological saline (4.3 mM NaHCO₃, 126.4 mM NaCl, 0.9 mM KCl, 0.4 mM CaCl₂, 0.2 mM MgSO₄) was used to irrigate the preparation. The scrotum was incised, and the underlying fascia blunt dissected to expose the testicle. The distal apical end of the cremaster muscle was pinned to the distal end of the transillumination window. A longitudinal incision was made along the long axis of the cremaster muscle, taking care not to damage any of the major vessels and the testis. A transverse incision was made, and the testicle to the cremaster muscle was cut by first forming a clot using a pair of forceps and then detaching it from the cremaster muscle. The testicle was then placed back into the inguinal canal. The cremaster muscle was pinned evenly over the transillumination window. Upon completion of the experiments, the animals were killed by i.v. injection of a saturated KCl solution. The end point of these studies was defined as an irreversible event characterized by a decrease in microvascular sensitivity due to drying of the muscle on the transillumination window, euthanasia when the animal was in severe distress, or after completion of the study. Microvascular responses and blood pressure were recorded throughout the course of the experiment.

Intravital microscopy—The experiments were carried out using an industrial grade microscope (Nikon MM-11, Japan) and were viewed on a monitor and recorded on a Panasonic digital video recorder for off-line processing. The physiological saline was aerated with a nitrogen/carbon dioxide mixture (N₂/CO₂ = 95%/5%). The tissue was allowed to stabilize and equilibrate for 30 min before any data were collected. The A1–A4 arterioles were identified for further examination, tested for response to a 0.1 μM adenosine solution, and then allowed to return to its normal tone (~10 min). The microvascular response to increasing i.v. (femoral venous catheter) doses of THC (2–12 mg/kg) was first examined. A dose of 12 mg/kg was required to produce a significant microvessel response (P = 0.0395) relative to controls. A subsequent cohort was administered NS-398 (2 mg/kg) i.v. with no further treatment or injected with 12 mg/kg THC 3 min after NS-398 administration. Evaluated cohorts were then administered either THC with or without NS-398 (2 mg/kg) pretreatment. The preparation was viewed under ×20 magnification and was recorded for off-line data analysis. The video images (AVI format) were first converted to digital image files and then analyzed using MetaMorph software (Molecular Devices Corporation, Downingtown, Pa).

Blood pressure experiments in mice—Six- to 8-week-old C57BL mice were anesthetized and prepared for surgery following the same procedure as described above for the cremaster preparation. The animal was then intubated using PE50 tubing followed by a femoral catheterization to administer the drug. The carotid artery was exposed by blunt dissection and then ligated at three different positions: proximal from the heart, distal from the heart, and one in between the two aforementioned ligatures. The distal and the proximal ends were tied to stem the blood flow from the heart. A small incision was made between the two tied ligatures and a 1.4F pressure transducer catheter (Millar Micro-Tip Catheter Transducer) was inserted toward the heart. The proximal ligature was then untied, and the catheter transducer was inserted further toward the heart until it reached the ventricle by holding the catheter in place by the ligature between the proximal and the distal ligatures. The transducer was connected to a computer via a transducer control unit for data collection. The data were analyzed using Acquaknowledge III software.

Hemorrhagic shock model in rats—A pressure-controlled rat model was used to test the drug combination (12 mg/kg THC + 2 mg/kg NS-398) that mimics the reactive vasoconstriction effect. Animals were divided into control cohorts. Male Sprague-Dawley rats, 180 to 220 g, 4 months of age (n = 6 per group), were first anesthetized using isoflurane, followed by an anesthetic i.p. injection of 1 μL/g of animal weight (87 mg ketamine/mL + 13 mg xylazine/mL). The combination of anesthetics was used throughout the experiment to maintain the anesthetic plane. Body temperature was maintained using a thermostatically controlled feedback heating blanket at ~37°C. The animal was restrained in the supine position by affixing the hind legs to the mounting board. The groin area was shaved on the left limb, which was incised to expose the femoral neurovascular bundle. The left femoral vein and artery were cannulated with PE50 tubing and tied firmly into place using 4-0 silk thread. A midline incision was made in the ventrocervical region, and the underlying tissue was bisected laterally and away from the testis. The animal was then intubated using a double-lumen endotracheal tube. The catheter was then placed into the right atrium, and the blood pressure catheter was inserted into the artery to monitor blood pressure and heart rate. The catheter was firmly tied into place using 4.0 silk thread. The left femoral vein was used for drug injections, and the left femoral artery was used for blood withdrawal.

Animals underwent a stepwise bleeding following the surgical procedures using a 100-μL syringe, set at a withdrawal rate of 100 μL/min in a tapering withdrawal method. In this method, the animal underwent sequential blood draws at 750, 500, and 250 μL and then a final bleeding to stabilize the MAP at 40 mmHg. Each bleeding step was followed by an equilibration period to allow compensatory vascular responses to stabilize. Drug injections were administered based on the milligram-per-kilogram dosing determined in the intravital microscopy studies 10 min after the induction of shock. The end point of these studies was defined as an irreversible event characterized by a decrease in
MAP to less than 20 mmHg, euthanasia when the animal was in severe distress, or after completion of the study. Survival time and blood pressure were recorded throughout the course of the experiments. Successful experiments were defined as those that at least doubled the survival time of the animals relative to controls that were either untreated, injected with a vehicle solution, or injected with a solution containing only one drug. The positive controls for these studies were animals maintained at 40 mmHg using normal saline resuscitation for 4 h (untreated controls). Successful responses were monitored for a maximum of 4 h.

**Cytokine analysis**—After termination of the studies on the binary therapy and fluid resuscitated cohorts, 500 µL of blood was collected and centrifuged, and the plasma collected and frozen at −80°C for future analysis. A multiplexed cytokine assay FlowMetrix system (Lumixin, Austin, Tex) was used to measure multiple cytokines levels in the rat serum. The principal advantage of this assay is its multiplexing capability, which allows for accurate, simultaneous measurement of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor, IFN-γ, and TNF-α.

**Histopathology**—After termination of the studies, the intact proximal, mid, and distal small intestine and the large intestine lumens were infused with formalin to prevent autolysis of the villi. The pancreas was removed first followed by the spleen, liver, lungs, thymus, kidney, heart, intestines, and brain. The tissues were prepared for routine histological evaluation by being fixed in 10% neutral-buffered formalin, paraffin embedded, sectioned 4 to 6 µm in thickness, and stained with hematoxylin-eosin. Treatment groups were unmasked after evaluation. Scoring of the pathologic findings used the standard 0 to +4 grading system. No changes were assigned 0; mild changes were assigned 0 to +1; moderate changes were assigned +1 to +2; marked changes were assigned +2 to +3; and severe changes were assigned +3 to +4. The findings that were to be scored included congestion, edema, hemorrhage, leukocyte influx, hypoxia, apoptosis, and necrosis. However, not all of these were observed.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism (GraphPad Prism Software, La Jolla, Calif.). All subjects were considered independent, and end points consistently defined.

The responses considered from the microvascular experiments were from control (vehicle administration only), AEA alone, THC alone, NS-398 alone, and NS-398 + THC binary administration. The responses considered from the HS experiments were from control (untreated, vehicle, and saline resuscitated), THC alone, NS-398 alone, and NS-398 + THC binary therapy administration. Results are expressed as mean ± SEM. The groups in the study have been compared by using one-way ANOVA and using Bartlett post hoc test (P < 0.05). Survival curves for each treatment were analyzed by the Kaplan-Meier survival analysis using a log-rank test to assess the equality of survival functions.

**RESULTS**

**Effect on the microvasculature in C57BL mice**

The cremaster muscle arterioles (A3-A4 resistance vessels ranging from 15- to 30-µm diameter) were considered for analysis. Control experiments using only the vehicle (Stepanetex/ethanol/saline in the ratio 5:5:90) revealed no significant change in the vessel diameter (Fig. 1A) or MAP (Fig. 1B), thus indicating that the vehicle or volume of injection does not influence the A3-A4 resistance vessels. The administration of THC had little or no effect on vessel diameter at doses ranging from 0.8 to 10 mg/kg (data not shown). However, at a dose of 12 mg/kg, a transient but significant (P < 0.05) mean constriction of 4.9% ± 2.4% (Fig. 1A) was observed, which returned to basal dimensions after ~80 s. The constriction was accompanied by a persistent 6.9 ± 1.9 increase in Δ MAP (Fig. 1B). In contrast, 5 mg/kg of the endocannabinoid AEA elicited a significant 14.8 ± 2.4 increase (P < 0.001) in vessel diameter relative to vehicle with a 1.7 ± 1.8 decrease in Δ MAP (Fig. 1, A and B). Vasodilation from AEA exposure is consistent with literature reports; therefore, the results suggest that the THC affects striated muscle microvasculature via a mechanism distinct from AEA.

The unexpected lack of microvascular response to THC at doses less than 12 mg/kg prompted us to investigate differences in downstream signaling of the endocannabinoid system using pharmacological probes. Cyclooxygenases have previously been reported to affect brain microvascular responses to THC by inhibiting dilation (14). To determine if a COX-2/CBR coupled pathway was involved in the striated muscle microvessels, animals were pretreated with the selective COX-2 inhibitor, NS-398 (15). The groups studied were the control (vehicle administration) group, NS-398–treated group, and the THC group pretreated with NS-398. Administration of NS-398 (2 mg/kg) resulted in a significant vasodilatation of 7.8% ± 0.7% (P < 0.001) and an insignificant decrease in Δ MAP of 2.8 ± 3.8 (Fig. 1, A and B). In contrast, administration of NS-398 at 3 min before THC administration resulted in a pronounced and significant 27.4% ± 7.9% decrease in vessel diameter relative to the control (P < 0.01), NS-398–treated (P < 0.001), and THC-treated (P < 0.05) groups (Fig. 1, all panels). This response was accompanied by insignificant 9.8 ± 4.4 rises in Δ MAP. The observed striated muscle microvascular contraction led to our hypothesis that the effect could result in an advantageous redistribution of blood flow to treat HS.

**Effect on survival time of hemorrhaged rats**

The HS model used in the current study was first validated by comparing the overall survival time of hemorrhaged untreated animals to the treated animals. It was critical from the
onset of the experiments to assess the effects of the ketamine/xylazine anesthetic, although this combination has been previously used in HS models (16). In the absence of intervention, animals subjected to severe hypovolemia remained stable for 35 to 40 min (Fig. 2) followed by a rapid decline, which is consistent with existing literature data (17).

Control experiments using the vehicle or the single agents, THC or NS-398, were conducted to obtain baseline values before binary therapy studies. In our studies, the mean total shed blood was equal to 3.35 ± 0.27 mL, and the total injected volume of vehicle or drug solution was 200 μL (Table 1). Injection of NS-398 (2 mg/kg) solution 10 min after shock maintained the MAP at an average of 40 mmHg for 80 min after injection, after which 67% of the cohort rapidly declined (Fig. 2). Although 33% of the animals survived past 200 min, the total survival was not statistically significant relative to untreated controls. It has been reported that NS-398 has no beneficial effects on the outcome of HS (18), consistent with our experimental outcome. Administration of THC (12 mg/kg) 10 min after shock alone triggered an increase in MAP to within 70% of the basal MAP for approximately 30 min after injection followed by a rapid decline in MAP (Fig. 2). The responses observed in our experiments are consistent with a previous report on HS and CB-1 agonist (11). The injection of THC alone did not significantly increase the survival of shocked animals relative to untreated animals. The treatment resulted in 100% mortality in 110 min.

Injection of the vehicle neither altered the MAP nor significantly increased survival times relative to untreated controls, with one animal surviving the duration of the experiment (Fig. 3). In contrast, injection of 200 μL of the binary therapy consisting of THC (12 mg/kg) and NS-398 (2 mg/kg) 10 min after shock significantly increased survival time relative to other cohorts. The results from the Kaplan-Meier curve analysis (log-rank test) manifested in a 4.7-fold ($P < 0.001$) increase in survival time after binary drug therapy relative to untreated controls (Fig. 3). The increased survival was accompanied by increase in MAP to within 65% of the basal level during the entire course of the experiment. This increase in MAP was statistically significant against vehicle controls between 40 and 120 min ($P < 0.001$) (Fig. 3).

Cytokine analysis

The circulating levels and tissue levels of proinflammatory and anti-inflammatory cytokines can be used as markers of the severity of HS, as well as the outcome prognosis of resuscitated HS victims (4, 5). The host defense mechanism that ultimately leads to autocatabolism in stage IV HS is triggered early in HS by the production of the proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α and the anti-inflammatory cytokine IL-10. The upregulation of the proinflammatory cytokines combined with the expression of endothelial cell adhesion molecules (P selectin) initiates the chemotactic cascade leading to neutrophil activation, migration, and adhesion. The process is regulated by IL-10, which functions as an inhibitor to the inflammatory response. The results of the cytokine analysis on serum samples revealed that a statistically significant reduction in the proinflammatory cytokines (IL-1α, IL-1β, and IFN-γ) and the anti-inflammatory cytokine (IL-10) occurs with binary therapy. The absence of significant differences in TNF-α may reflect the fact that it is an early modulator of the inflammatory response and its levels may subside at 4 h after shock induction. Table 2 summarizes the effects of the binary therapy on serum cytokine levels at the end of the experimental procedure.

Histopathology

Prolonged hypovolemia, the ensuing decompensation phase of HS, and the collapse of the microcirculation lead to tissue damage and multiple organ failure. The ability of the binary therapy in reducing or preventing HS-associated organ damage is a critical marker of the efficacy of the intervention. Organs were harvested from all treatment cohorts; however, because

### Table 1. Blood hemorrhage volume in experimental groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. animals</th>
<th>Hemorrhage volume, mean (±SD), mL</th>
<th>Volume injected, μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binary therapy (NS-398 + THC)</td>
<td>6</td>
<td>3.45 (±1.1)</td>
<td>200</td>
</tr>
<tr>
<td>THC control</td>
<td>6</td>
<td>3.70 (±0.30)</td>
<td>200</td>
</tr>
<tr>
<td>NS-398 control</td>
<td>6</td>
<td>3.45 (±0.96)</td>
<td>200</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>6</td>
<td>3.18 (±1.2)</td>
<td>200</td>
</tr>
<tr>
<td>Untreated control</td>
<td>6</td>
<td>3.00 (±0.8)</td>
<td>0</td>
</tr>
</tbody>
</table>

### Fig. 2. Experimental results of the rat HS model comparing control groups to the binary showing the percent survival (A) and MAP (B). The treatments are identified by binary therapy consisting of THC/NS-398 (THC, 12 mg/kg; NS-398, 2 mg/kg) ( ), untreated control ( ), 12 mg/kg THC control ( ), and 2 mg/kg NS-398 control ( ). The shock induction period is designated by H (dark gray region), and the 10-min hold time is designated by S (light gray shading). Drug injections were carried out at the end of the 10-min hold period S. Data points in panel B are shown in 10-min intervals.

### Fig. 3. Experimental results of the rat HS model comparing control groups to the binary showing the percent survival (A) and MAP (B). The treatments are identified by binary therapy consisting of THC/NS-398 (THC, 12 mg/kg; NS-398, 2 mg/kg) ( ), untreated control ( ), vehicle control ( ), and resuscitation with normal saline ( ). The shock induction period is designated by H (dark gray region) and the 10-min hold time is designated by S (light gray shading). Drug injections were carried out at the end of the 10-min hold period S. Data points in panel B are shown in 10-min intervals. Panel B shows the time interval where the MAP between the binary therapy and other groups was statistically significant ($P < 0.01$ and $P < 0.05$).
of the short survival time of the control groups, there was insufficient time for manifestations of pronounced tissue damage. Therefore, a group consisting of animals maintained at 40 mmHg via infusion of normal saline, a clinically used therapeutic intervention, was used for a comparative analysis. This intervention was effective in preventing mortality up to 170 min after HS, after which a 33% mortality was observed (Fig. 3). The organs from this control group were compared with the binary therapy–treated group and a nonshocked group.

The most consistent findings in the control group were a sequestration of leukocytes (mostly neutrophils) within the pulmonary vasculature, leukocyte pavementing, and intra-alveolar edema (Fig. 4). These lesions were not observed in the animals treated with the binary therapy; the lungs of which were comparable to nonshocked animals. These findings are extremely significant in that neutrophil destruction of lung tissue is a major component of the acute respiratory distress syndrome, which is often a consequence of HS and resuscitation.

Lesions were also observed in the livers and kidneys of the untreated animals; however, the lesions in the kidneys were not consistently observed in the control cohort. The liver lesion was hepatocellular hypoxia, suggested by the differential staining quality to the hepatocytes in the centrilobular zone (Fig. 5). Within the kidneys of the control animals, there was evidence of congestion and flattening of the proximal tubular epithelial cell lining with resulting dilation of the lumen. The changes in the control kidneys did not involve all tubules, and the changes were often segmental. Histological scoring of the tissue changes is presented in Figure 6.

**DISCUSSION**

The primary objective of the study was to test the hypothesis that regulation of the microvascular tone in the striated muscle could be exploited for a novel therapy in the treatment of HS. The basis of our hypothesis arose from intravital microscopy studies directed at understanding tissue-specific responses to endocannabinoids and synthetic cannabinoids. Wagner et al. (11) had suggested that AEA, an endocannabinoid; THC; and HU-210 (CBR agonist) may be efficacious in HS because of cardiac output redistribution or favorable microcirculation vasodilation. Although vasodilation in vital organs in HS should improve organ perfusion, a concomitant decrease in systemic blood pressure would be predicted to accompany this effect. Therefore, we focused on a potential compensatory mechanism for a closed system, wherein the microcirculation of the striated muscle compartments constricts to compensate for vital organ resistance vessel dilation.

To evaluate the effects of endocannabinoids and synthetic cannabinoids on the striated muscle resistance vessels, we selected the mouse cremaster muscle as the model system. In this model, the real-time effects of drugs on the diameter of A3-A4 arterioles in striated muscle were studied. The endocannabinoid, AEA, is the prototypical agent that has previously been evaluated in resistance vessels (19–21). In our system, AEA administration resulted in vessel dilation of 14.8% ± 2.4%. The dilation is generally consistent with published data in other vascular beds. In contrast, synthetic cannabinoids manifest vascular bed–dependent effects on the resistance vessels. It has been reported that THC, HU-210, and WIN-55212-2 (CBR agonist) do not elicit vasodilation (22, 23), which is generally consistent with our data using up to 10 mg/kg THC. However, at 12 mg/kg, the administration of THC results in a mean constriction of 4.9% ± 2.4% returning to basal dimensions after ~80 s. The disparity in vascular responses to endocannabinoids and synthetic cannabinoids has been extensively reviewed (21, 24). Although not evaluated in these studies, the effect of AEA versus THC may reflect drug activation of the vanilloid receptor 1 versus the CB-1/CB-2 receptors, respectively.

The mechanism of AEA and THC action in the striated muscle resistance vessels remains unclear; however, we initiated preliminary screening of previously reported signaling pathways. In the course of these studies, we unexpectedly discovered that pretreatment with the selective COX-2 inhibitor before THC administration resulted in a pronounced and significant 27.4% ± 7.9% decrease in vessel diameter. This
response was in contrast to the study by Ellis and coworkers (14), wherein nonselective COX inhibition, i.e., pretreatment with indomethacin, abolished vasodilation in the cerebral microvasculature. The differences in these results prompted us to hypothesize that the effects could be exploited to favorably redirect blood volume in hypovolemia. We therefore shifted our research effort to obtain proof of principle that a THC–COX-2 inhibitor combination would manifest efficacy in HS.

The protocol used to evaluate the potential of a THC–COX-2 binary therapy was the pressure-controlled rat model of HS. The mean resting MAP of 120 mmHg was reduced stepwise until a MAP of 40 mmHg was achieved followed by a 10-min holding period. Injection of 200 μL of the binary therapy consisting of THC (12 mg/kg) and NS-398 (2 mg/kg) resulted in an overall survival time of 240 min after shock induction. This was a significant increase relative to the 55-min survival time reported using THC alone (11). Compared with untreated controls, the survival time was increased 4.7-fold and was highly significant (P < 0.001) as determined by a log-rank test of the Kaplan-Meier curve. The binary therapy also increased the MAP of shocked animals to within 65% of the basal level over the course of the experiment.

To assess the requirement of the THC–COX-2 combination, as well as volume administered, in stabilizing hypovolemia, the individual agents and vehicle were tested in the rat HS model. The volume of vehicle, 200 μL, shifted the Kaplan-Meier curve to the right, increasing the survival time 2.6-fold, wherein 2 subjects manifested a significant pressor effect at 120 min. The vehicle injection did not affect the MAP, thus suggesting that the increased survival was not due to volume replacement. However, the effect on survival was not statistically significant, relative to untreated controls, or to the single drug treatments. The COX-2 inhibitor, NS-398 (2 mg/kg), provided some increase in survival time for 33% of the subjects; however, the overall survival was not statistically different from untreated controls. The inhibition of COX-2 did not improve the overall MAP throughout the course of the experiment. Administration of THC (12 mg/kg) 10 min after shock caused a significant pressor effect lasting 40 min after injection, after which the MAP rapidly declined. The pressor effect was not sufficient to significantly increase the survival time relative to untreated controls. In all cases, the binary therapy significantly (P < 0.001) increased the survival outcomes of the cohort relative to all other treatment groups. In addition, the binary therapy manifested a significant pressor effect throughout the course of the experiments but was significant over the first 120 min. Combined, the data clearly demonstrate that the drug combination, not volume replacement or either single agent, is required for maximum benefit in subjects subjected to HS.

The second measure of the efficacy of the binary therapy was the analysis of pathophysiological changes associated with HS in the presence and absence of binary therapy. The comparison was made between nonshocked, resuscitation with normal saline, and treatment with the binary therapy. All the organs were harvested and evaluated by histopathology, wherein significant changes were manifested in the lungs primarily and also in the liver. The binary therapy had pronounced effects in the lungs preventing neutrophil sequestration within the pulmonary vasculature, neutrophil pavementing, and edema, which were noted in the normal saline resuscitation cohort. These findings are consistent with results reported in similar rat models of HS (25, 26). The lung lesions were not present in the binary therapy–treated group. The binary therapy also protected against hepatocellular hypoxia and, probably subsequently, hepatocyte necrosis. These lesions have previously been reported in fluid resuscitation models using the rat (27). The selective striated muscle vasoconstriction, although beneficial to supporting the function of vital organs, may pose a potential risk of capillary “no-reflow” in the peripheral muscle tissue. The no-reflow in striated muscle capillaries has been reported to manifest in ischemia/reperfusion injury; however, in our studies, striated muscle tissue collected from hind quarters showed no evidence of muscle injury (28).

![Fig. 5. Representative light microscopic section of liver from nonshocked (A), resuscitation with normal saline (B), and the binary therapy consisting of THC/NS-398 (THC, 12 mg/kg; NS-398, 2 mg/kg)-treated (C) cohorts. The zonal regions between the hepatic triad (HT) and the central vein (CV) are identified as 1, 2, and 3, with 1 having the highest oxygen tension and 3 the lowest. B demonstrates the differential staining between zones 1 and 3 in the untreated control suggestive of early centrilobular hypoxia (hematoxylin-eosin stain, original magnification ×40).](Image 46x627 to 295x733)

![Fig. 6. Bar graph representation of organ scoring in histopathological samples of the lung, liver, and kidney. The scoring scale ranges from 0 to +4, with no changes assigned 0; mild changes were assigned 0 to +1; moderate changes were assigned +1 to +2; marked changes were assigned +2 to +3; and severe changes were assigned +3 to +4. The binary therapy treated cohort is illustrated by black bars and the saline resuscitated cohort represented by gray bars.](Image 326x110 to 554x312)
The protective effects of the combined drugs may extend beyond the stabilization of the MAP. Specifically, the potent anti-inflammatory effects of cannabinoids, via activation of the CB-2 receptor (29) and/or COX-2 inhibitors (30), may provide polypharmacological effects that protect inflammation-mediated organ damage in HS. This is borne out in the nine-panel cytokine analysis of plasma taken from binary therapy and resuscitated cohorts. The inflammatory mediators IL-1α, IL-1β, IFN-γ, and IL-10 are significantly downregulated in the drug-treated cohort, the reduction of which may reduce presentation of endothelial cell adhesion molecules and subsequent leukocyte accumulation in the vasculature. The underlying mechanism of this observation remains to be determined; however, the effects of the combined drugs offer an intriguing area for future investigation.

In summary, the overall objective of the study was to evaluate a binary therapy, consisting of a CBR agonists and COX-2 inhibitor, and its effect on survival in the rat model of HS. We have demonstrated that the MAP of hypovolemic rats can be increased and stabilized by the injection of a drug solution equaling one-tenth the total volume of shed blood without fluid or blood resuscitation. In addition, our research has also demonstrated that the binary drug mixture has significant anti-inflammatory and protective effects on the lungs and liver. Taken collectively, the results of these experiments clearly indicate the future potential of this HS intervention therapy. The binary therapy is currently being refined using clinically relevant drugs and excipients.

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