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Abstract

Honokiol is a biphenolic isolate extracted from the bark of the magnolia tree that has been used in traditional Chinese and Japanese medicine, and has more recently been investigated for its anti-inflammatory and anti-bacterial properties. Honokiol has previously been demonstrated to improve survival in sepsis models that have rapid 100% lethality. The purpose of this study was to determine the impact of Honokiol on the host response in a model of sepsis that more closely approximates human disease. Male and female C57BL/6 mice underwent cecal ligation and puncture (CLP) to induce polymicrobial intraabdominal sepsis. Mice were then randomized to receive an injection of either Honokiol (120mg/kg/day) or vehicle and were sacrificed after 24 hours for functional studies or followed 7 days for survival. Honokiol treatment after sepsis increased the frequency of CD4+ T cells and increased activation of CD4+ T cells as measured by the activation marker CD69. Honokiol also increased splenic dendritic cells. Honokiol simultaneously decreased frequency and number of CD8+ T cells. Honokiol decreased systemic TNF without impacting other systemic cytokines. Honokiol did not have a detectable effect on kidney function, lung physiology, liver function or intestinal integrity. In contrast to prior studies of Honokiol in a lethal model of sepsis, Honokiol did not alter survival at seven days (70% mortality for Honokiol vs. 60% mortality for vehicle). Honokiol is thus effective in modulating the host immune response and inflammation following a clinically relevant model of sepsis but is not sufficient to alter survival.

Keywords

Honokiol; sepsis; lymphocyte; inflammation; TNF; intestine; survival; activation
INTRODUCTION

Sepsis is life threatening organ dysfunction caused by a dysregulated host response to infection (1). Between 230,000 and 370,000 people die in the United States annually from sepsis (2). Despite significant advances in the understanding of the pathobiology of sepsis, treatment is generally supportive in nature (3). Early administration of antibiotics and fluids are associated with improved outcome in sepsis (4-6), but when these fail, mortality from septic shock is still over 40% (7). As such, identifying potential adjunctive therapies for septic patients is of the utmost importance.

Honokiol is a natural biphenolic chemical found in the bark of the magnolia tree that has anti-inflammatory and anti-bacterial properties (8-11). Honokiol reduces expression of TNF at baseline and in models of rheumatoid arthritis, cancer, ischemia/reperfusion, and endotoxemia (12-14). Notably, Honokiol improves survival following a lethal model of CLP in male ICR mice and male Sprague-Dawley rats, in which all animals die within 90 hours (15;16). This is associated with improvements in kidney function, lung physiology, liver injury and systemic inflammation. However, rapid mortality does not approximate what is seen in human patients (17) and may result from different mechanisms. Further, novel preclinical therapeutics that are beneficial in high mortality models of sepsis frequently have different efficacies in more clinically relevant models (18-20), and the use of highly lethal models to study sepsis has been proposed to be one reason (of many) why preclinical studies in animals have failed to translate into therapeutic gain in patients (21).

We therefore compared the impact of Honokiol administration versus vehicle in a model of sepsis whose mortality more closely approximates that seen in human septic shock. Analysis of multiple organs demonstrated that Honokiol impacted both the immune system and systemic inflammation (but not the kidney, liver, lungs or intestine) yet failed to alter survival after CLP.

MATERIALS AND METHODS

Animals

Six week old male and female C57BL/6 mice obtained from the Jackson Laboratory (Bar Harbor, ME) were used for all experiments. Mice were kept on a 12-hour light cycle and had free access to water and chow. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Emory University School of Medicine (Protocol DAR-2002717-042217GN).

Sepsis model

Mice underwent CLP to induce polymicrobial intraperitoneal sepsis. Surgery was performed under isoflurane anesthesia using aseptic technique. Through a small midline incision, the cecum was identified. The cecum was ligated at approximately 50% of its length without causing intestinal obstruction and was punctured twice with a 25 gauge needle. A small amount of stool was extruded before the intestine was returned to the abdomen, which was subsequently closed in layers. For functional studies, mice were euthanized 24 hours after...
CLP. A separate cohort of animals was followed for 7 days for survival studies. All mice received buprenorphine (0.1mg/kg, McKesson Medical, San Francisco, CA) immediately pre-operatively to minimize animal suffering. In addition, animals were re-dosed with buprenorphine post-operatively when deemed appropriate by the staff of the Division of Animal Resources at Emory University, who were blinded to whether an animal received Honokiol. To mimic the clinical situation where fluid resuscitation and antibiotics are standard of care in all septic patients (3), insensible fluid losses were replaced with 1mL of normal saline given subcutaneously. In addition, ceftriaxone (50mg/kg, Sigma-Aldrich, St. Louis, MO) and metronidazole (30mg/kg, Apotex Corp, Weston, FL) were administered subcutaneously twice daily following surgery. Animals that were sacrificed 24 hours after CLP received antibiotics immediately after surgery and 12 hours post-operatively while animals followed for survival received antibiotics every 12 hours for a total of 48 hours. All animals were checked twice daily to determine if they were moribund which was identified as follows: a) surgical complications unresponsive to immediate intervention (wound dehiscence, bleeding), b) medical conditions unresponsive to treatment including self-mutilation, severe respiratory distress, icterus, or intractable diarrhea, or c) clinical or behavioral signs unresponsive to appropriate intervention persisting for 1 day. Animals that were identified as being moribund were immediately sacrificed.

**Honokiol administration**

Honokiol extract was prepared as described previously by co-author JLA (13). Honokiol was dissolved in 100% ethanol combined with 20% intralipid (Sigma, St. Louis, MO), used as vehicle. Mice were randomized to receive either a subcutaneous injection of either Honokiol (250 μL, total daily dose 120mg/kg) or an equivalent volume of vehicle. Animals received the first injection of Honokiol 30 minutes after CLP and then daily until sacrifice, up to a total of 5 days.

**Phenotypic flow cytometric analysis**

At the time of sacrifice, spleens were collected and single-cell suspensions were prepared from each animal. Samples were stained with anti-CD3-Alexa 700, anti-CD4-PB, anti-Gr-1-FITC, anti-B220-PerCP (BD Bioscience, San Jose, CA), anti-CD8-PO (Life Technologies, Carlsbad, CA), anti-NK1.1-APC-Cy7, anti-CD69-PE (Biolegend, San Diego, CA), anti-CD11b-APC, and anti-CD11c-PE-Cy7 (eBioscience, San Diego, CA). CountBright absolute counting beads (Life Technologies) were added to cell suspensions and were used according to manufacturer’s instructions to determine absolute cell counts. Samples were run on an LSR II flow cytometer (BD Biosciences). Resulting flow cytometry standard files were analyzed using FlowJo software (version 10.0.7, TreeStar, Ashland, OR).

**Serum cytokines**

Serum cytokines levels were determined using a multiplex cytokine assay kit (Bio-Rad, Hercules, CA) according to manufacturer instruction. Levels of IL-1β, IL-6, IL-10, MCP-1, and TNF are reported in pg/mL. All samples were run in duplicate.
Renal function, liver function and complete blood count

Whole blood was collected at the time of sacrifice. A total of 20 μL of whole blood was analyzed for hemoglobin, white blood cell count and platelet count with a veterinary hematology analyzer (Hematrue Hematology Analyzer, Heska, Loveland CO). The remaining blood was centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting serum was analyzed for aspartate and alanine aminotransferase (AST, ALT) levels using a Beckman AU480 serum chemistry analyzer (Beckman Coulter, Brea, CA). Determinations of blood urea nitrogen (BUN) and serum creatinine were performed using a commercially available kit according to manufacturer specifications (Arbor Assays, Ann Arbor, MI). All samples were run in duplicate.

Lung weight, histology and myeloperoxidase (MPO) activity

The right lung was excised at time of sacrifice and weighed to determine a “wet” weight. After drying in an oven at 65°C for 24 hours, the tissue was re-weighed to establish a “dry” weight. A ratio of weight to dry weight was then calculated. Animals also had left lung tissue excised and either sectioned and stained for H&E or snap frozen in liquid nitrogen. Slides were evaluated for histopathology by a blinded observer (ABF). MPO activity was performed on frozen lung sections. Each sample was weighed and recorded. Substrate buffer containing o-dianisidine (0.167 mg/mL, Sigma) and 7.5% H₂O₂ was added to each sample. After addition of substrate, absorbance at 460 nm was measured every 30 seconds for each sample over 6 minutes (Synergy HT, Bio-Tek). MPO activity was calculated as ΔOD/minute (U) per mg of lung tissue and all samples were run in duplicate (14).

Liver histology

A segment of liver was excised at sacrifice and immediately fixed in 10% formalin. Sections were subsequently paraffin embedded, sectioned and stained with H&E. The slides were evaluated for histopathology by a blinded observer (ABF).

Blood and peritoneal fluid cultures

Whole blood samples (~50 μL) were collected into EDTA-lined tubes at the time of sacrifice. In addition, a total of 3 mL of sterile PBS was lavaged into the peritoneum and then aspirated at the time of sacrifice. Both blood and peritoneal fluid samples were serially diluted and plated on sheep blood agar plates and incubated at 35°C in 5% CO₂. After 24 hours, colony counts were determined.

Intestinal integrity

Apoptotic cells were quantified in 100 contiguous well-oriented crypt-villus units by an examiner blinded to sample identity (NJK). Apoptotic cells were identified by morphologic criteria on H&E-stained sections where cells with characteristic nuclear condensation and fragmentation were considered to be apoptotic (22,23).

Crypt proliferation was assayed by quantititating S-phase cells stained with 5-bromo-2′-deoxyuridine (BrdU, 120 mg/kg) which was injected 90 minutes before sacrifice. Jejunal sections were deparaffinized, serially rehydrated in ethanol, and incubated in 1%
H$_2$O$_2$ for 10 minutes. Slides were then immersed in citrate-based antigen decloaker (Biocare Medical, Concord, CA), heated in a steamer for 45 minutes at ~98°C to facilitate antigen retrieval, blocked with Dako Protein Block (Dako, Carpinteria, CA) for 30 minutes at room temperature before being incubated with rat monoclonal anti-BrdU (1:500; Accurate Chemical & Scientific, Westbury, NY) overnight at 4°C. Samples were then incubated with goat anti-rat secondary antibody (1:200; Vector Laboratories) for 30 minutes at room temperature, followed by streptavidin-horseradish peroxidase (1:500; Dako) for 30 minutes at room temperature and finally developed with diaminobenzidine followed by hematoxylin counterstaining. S-phase cells were quantified in 100 contiguous crypts.

In vivo intestinal permeability was measured by gavaging mice with 0.5mL of FITC conjugated-dextran (FD-4, 22mg/ml, molecular mass 4.4 kDa, Sigma) five hours prior to sacrifice and sampling the serum at the time of sacrifice (24;25). Blood was centrifuged at 10,000 rpm at 4°C for 10 min. The resultant plasma (50 μl) was diluted 1:2 with PBS (pH 7.4) and the concentration of FD-4 measured using fluorescence intensity of FD-4 (Synergy HT, BioTek, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. All samples and standards were run in duplicate.

**Statistical analysis**

Data were analyzed using the statistical analysis software Prism 6.0 (GraphPad, San Diego, CA) and are presented as mean ± SEM. Each data set was tested for normality using the D’Agostino-Pearson omnibus normality test. If data were normally distributed, comparisons were performed with a Student’s t-test. If data did not meet normality definitions, comparisons were done with a Mann-Whitney test. For the survival study, data were analyzed using the log-rank test. A p-value of <0.05 was considered to be statistically significant for all experiments.

**RESULTS**

**Effect of Honokiol on adaptive immune system following sepsis**

Honokiol increased the frequency of splenic CD4$^+$ T cells in septic mice compared to animals that received vehicle. This was not associated with a change in absolute CD4$^+$ T cell number (Fig. 1A and 1D). In contrast, Honokiol administration decreased both frequency and absolute number of CD8$^+$ T cells (Fig. 1B and 1D). There were no differences in the frequency or absolute number of splenic B220$^+$ B cells (Fig. 1C and 1E).

CD4$^+$ and CD8$^+$ lymphocytes were then stained for the early activation marker CD69. Honokiol treated animals had a greater frequency of activated CD4$^+$ T cells compared to animals treated with vehicle alone (Fig. 2A and 2C). Honokiol did not affect CD8$^+$ T cell activation (Fig. 2B and 2D).

**Effect of Honokiol on innate immune system following sepsis**

Honokiol increased the frequency and absolute number of dendritic cells (CD11c+, Fig. 3A and 3D). No difference was detected in the frequency or absolute count of granulocytes.
(CD11b+ Gr1+, Fig. 3B and 3E). Honokiol also decreased frequency of NK cells (NK1.1+, Fig. 3C and 3F); however, no change in absolute number of cells was identified.

**Effect of Honokiol on serum cytokines following sepsis**
Honokiol decreased serum TNF concentration compared to animals given vehicle (Fig. 4A). In contrast, Honokiol did not induce statistically significant differences in serum IL-1β, IL-6, MCP-1, G-CSF, or IL-10 (Fig. 4B-F).

**Effect of Honokiol on local and systemic bacterial burden following sepsis**
Despite alterations in both the adaptive and innate immune systems and cytokines, Honokiol did not alter either local (peritoneal) or systemic (blood) bacterial counts (Fig. 5A, B).

**Effect of Honokiol on the kidneys, lung, liver, blood counts and intestine following sepsis**
Honokiol did not alter renal function as measured by BUN and creatinine after CLP (Figure 6A, B). Honokiol also had no detectable effect on neutrophil activity in the lung as measured by MPO activity (Fig. 6C), pulmonary wet: dry ratio (Fig. 6D) or lung histology (data not shown). Further, Honokiol did not alter serum AST or ALT levels (Fig. 6E, F) or alter liver histology (data not shown). Hemoglobin and platelet counts were not statistically different following Honokiol (Fig. 6 G, H) although Honokiol treatment decreased total white blood cell count (Fig. 6I). Honokiol also did not alter intestinal integrity as assayed by permeability, apoptosis, and proliferation (Fig. 7).

**Effect of Honokiol on survival following sepsis**
Honokiol administration did not alter survival after CLP compared to vehicle (30% vs. 40% survival 7 days after CLP, Fig. 8).

**DISCUSSION**
Honokiol administration following CLP augmented the frequency of CD4+ T cells as well as their activation. Further, Honokiol induced an upregulation of antigen-presenting dendritic cells. Honokiol also decreased systemic TNF levels. While a more robust immune response coupled with a decreased early pro-inflammatory state might have been predicted to result in alterations in bacterial burden and survival, Honokiol failed to improve either bacterial clearance or survival following CLP.

Our results contrast with previous work in septic ICR mice and Sprague-Dawley rats in a number of key ways. First, prior studies demonstrated improved survival when Honokiol was given after the onset of CLP (15;16), whereas we did not detect a difference in survival. In addition, Weng et al. found that Honokiol therapy after CLP ameliorated lung injury in male ICR mice as measured by wet: dry ratio and histology and reduced sepsis-induced elevation in the liver enzymes AST and ALT (15). In contrast, we did not find a beneficial effect of Honokiol on any of these outcomes. Similarly, Li et.al found Honokiol administration after CLP reduced renal oxidative stress and improved kidney histologic features in rats whereas we did not identify changes in renal function as assayed by BUN.
and creatinine (16). Further, Li et al. found that Honokiol decreased TNF, IL-1β and IL-6, whereas we found a decrease in TNF without alterations in other cytokines.

There are numerous potential mechanisms that may underlie these profound differences. The first relates to the mortality of the model. Both prior studies used a highly lethal model of CLP in which all mice given vehicle were dead within 4 days, as opposed to 50% mortality at the same timepoint in mice given vehicle in this study. Importantly, our goal was to use a model with a 7-day mortality that approximates mortality seen in human septic shock (7) since the identical treatment can have widely differing efficacy (ranging from benefit to harm) when used in models of sepsis with varying mortality. This is because the mechanism of mortality is likely different between high, moderate and low mortality models of sepsis. While it is not entirely clear why rodents (or people) die of sepsis, rapidly lethal models of sepsis typically have a more significant pro-inflammatory component and the anti-inflammatory benefits of Honokiol may play a more important role in this setting. Further, previous studies used ICR mice or Sprague-Dawley rats, whereas C57BL/6 mice were used in these experiments. While each of these studies utilized a single genetic strain, genetic susceptibility to sepsis is highly strain dependent (26,27). Next, both of the previous studies on Honokiol and sepsis used only male mice whereas this study used both genders. The host response to sepsis is highly dependent upon gender, and this may therefore account for some of the difference (28). Finally, neither of the previous published papers using Honokiol mentioned giving either antibiotics or fluid resuscitation to mice or rats following CLP whereas this study gave both in order to mimic standard of care in sepsis management (3).

Our results are consistent with numerous studies demonstrating that Honokiol decreases TNF levels. The importance of TNF in the pathophysiology of sepsis is complex. TNF blockade and TNF knockout have widely varying effects depending on the timing and model of sepsis used (29-31). Further, while TNF blockade has not been a successful strategy in treating human sepsis in multiple randomized controlled trials, a meta-analysis of these trials shows a small benefit of TNF blockade, suggesting it may be beneficial in select patient populations (32). Notably, the decrease in TNF levels in this study did not alter a number of physiologic outcomes typically impacted by TNF such as intestinal epithelial permeability or apoptosis. The absence of a survival benefit with Honokiol despite the decrement in could be due to a) TNF not having a major role in mediating mortality, b) the fold change of TNF being too small to impact outcome, or c) other detrimental changes induced by Honokiol counteracting the beneficial effects of decreased TNF. Notably, a review of 12 clinical trials of TNF in sepsis suggested that the efficacy of anti-TNF in human sepsis is related to risk of mortality, with the highest benefit seen in those with the highest severity of illness and risk of dying, with decreasing efficacy as risk of mortality decreased (33). This is consistent with Honokiol decreasing mortality in rapidly lethal models of CLP (where TNF potentially plays a key role in mediating mortality) but not altering survival in a lower mortality model of CLP, where the importance of TNF might potentially be lower.

Sepsis induces multiple immunological derangements, with a known decrease in circulating T cells (34). In theory, augmentation of CD4+ T cell frequency and activation as well as augmentation of antigen-presenting dendritic cells might have been predicted to improve outcome by improving the host’s ability to clear infection. However, this was not the case as
Honokiol did not alter bacterial burden. This contrasts with Honokiol’s capacity to clear monomicrobial infections in vitro (35;36). It is possible that the decrease in CD8+ T cell frequency and number counteracted what might have otherwise been beneficial effects. Of note, based upon published literature, the dose of Honokiol used in this study would be considered to be high dose. High dose Honokiol has been demonstrated to inhibit the proliferative capacity of CD8+ T cells through an IL-2 dependent fashion (8) which may have contributed to the findings herein.

This study has a number of limitations. First, we looked at only a single timepoint, and it is likely that valuable insights would have been obtained by examining the impact of Honokiol at multiple timepoints. Further, we did not study the impact of Honokiol on unmanipulated mice since there are multiple publications on this. In addition, both previous papers examining Honokiol in sepsis used dimethyl sulfoxide as the vehicle for Honokiol injected intraperitoneally. In contrast, this study used 20% intralipid injected subcutaneously. We chose this approach because of extensive experience of one of the authors (JLA). However, the presence of intralipid has been shown to augment inflammation and decrease bacterial clearance (37;38), and it is unknown if this played a role in our outcomes. In addition, we did not vary the time after CLP that Honokiol was initiated. The rationale for starting Honokiol 30 minutes after CLP was to mimic the two prior studies of Honokiol in sepsis, which both initiated the therapy at this timepoint. However, we cannot rule out that Honokiol may have been more effective if started later. Further, we cannot rule out that a different dose of Honokiol might have been more effective in the less severe model of CLP used in this study. Finally, while the goal was to mimic survival seen in patients with septic shock (42% according to recent estimates (7)), mortality was slightly higher than expected with 12 out of 20 mice dying in the vehicle group as opposed to the goal of 8-9. We therefore cannot know what effect Honokiol would have in a sepsis model with an even lower mortality, although we speculate that it would not be beneficial. Finally, in figures 1-3, more control animals died than Honokiol mice prior to planned 24 hour sacrifice, which resulted in 5 control mice and 7 mice that received Honokiol for the analysis. Even though mice were gender matched at the beginning of the experiment, the increased death in the control group were more likely male mice (since male mice have a higher mortality from sepsis) meaning the data analyzed at 24 hours may not have been gender balanced.

Despite these limitations, this study demonstrates that Honokiol augments the immune response and decreases TNF but is unable to change survival in a clinically relevant model of sepsis. These results directly contrast with the survival benefit induced by Honokiol in two other studies of highly lethal sepsis in different animal strains/species. Further experiments are needed to determine whether Honokiol represents a potential adjunct to sepsis management by altering the immune system and inflammation or whether future studies with Honokiol should instead target other diseases.

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Reference List


FIG. 1. Effect of Honokiol on the adaptive immune system
Honokiol increased the frequency of CD4$^+$ T cells but did not alter absolute cell count (A, frequency, p=0.002; absolute count, p=0.60). CD8$^+$ T cell frequency and absolute counts were both decreased in animals receiving Honokiol (B, frequency, p=0.002; absolute count, p=0.005). B cell frequency and absolute cell count were unaffected by Honokiol (C, frequency, p=0.72; absolute count, p=0.63). Representative flow cytometry plots are shown for CD4$^+$ and CD8$^+$ T cells (D) and B220$^+$ B cells (E), n=5-7 for all groups.
FIG. 2. Effect of Honokiol on T cell activation
Honokiol administration increased CD4⁺ T cell activation as measured by expression of CD69 (A, p=0.005), however, there was no difference in CD8⁺ T cell activation (B, p=0.26). Representative flow cytometry plots are shown for CD4⁺ T cells (C) and CD8⁺ T cells (D), n=5-7 for all groups.
FIG. 3. Effect of Honokiol on the innate immune system
Honokiol increased the frequency and absolute count of splenic dendritic cells (A, frequency, p=0.01; absolute count, p=0.006). No difference was detected in frequency or absolute cell count of granulocytes (B, frequency, p=0.15; absolute count, p=0.34). Honokiol also decreased the frequency of NK cells without changing their absolute cell count (C, frequency, p=0.03; absolute count, p=0.20). Representative flow cytometry plots are shown for dendritic cells (D), granulocytes (E), and NK cells (F), n=5-7 for all groups.
FIG. 4. Effect of Honokiol on systemic cytokines
Honokiol decreased levels of TNF (A, p=0.03) but did not change levels of IL-1β (B, p=0.51), IL6 (C, p=0.87), MCP-1 (D, p>0.99), G-CSF (E, p=0.90) or IL-10 (F, p=0.24), n=8-9/group for all cytokines except n=6 for G-CSF in Honokiol group.
FIG. 5. Effect of Honokiol on bacterial burden
Honokiol did not change bacterial burden in either the peritoneal fluid (A, p=0.30) or blood (B, p=0.48), n=12/group.
FIG. 6. Effect of Honokiol on renal function, lung physiology, liver function and complete blood count
Honokiol did not change BUN (A, p=0.37, n=10-12), creatinine (B, p=0.47, n=7-10), lung neutrophil activity (MPO enzyme activity, C, p=0.76, n=9-10) wet:dry lung weight, a proxy for pulmonary edema (D, p=0.86, n=12-13), AST (E, p=0.05, n=12-13), ALT (F, p=0.17, n=12-13), hemoglobin (G, p=0.55, n=12) or platelets (H, p=0.47, n=12). In contrast, Honokiol decreased total white blood cell count (I, p=0.04, n=11).
FIG. 7. Effect of Honokiol on gut integrity
Honokiol did not change intestinal permeability (A, p=0.82), apoptosis (B, p=0.68), or proliferation (C, p=0.34), n=11-12/group.
FIG. 8. Effect of Honokiol on survival
Honokiol treatment did not alter mortality following CLP (p=0.98, n=20/group). Animals in both experimental group and control group were gender matched for this and all experiments.