Sophorolipids block lethal effects of septic shock in rats in a cecal ligation and puncture model of experimental sepsis*

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Objective: Sophorolipids, a family of natural and easily chemoenzymatically modified microbial glycolipids, are promising modulators of the immune response. The potential of the therapeutic effect of sophorolipids was investigated in vivo in a rat model of sepsis and in vitro by analysis of nitric oxide and cytokine production.

Design: Prospective, randomized animal study.

Setting: Experimental laboratory.

Subjects: Male Sprague-Dawley rats, 200–240 g.

Interventions: Intra-abdominal sepsis was induced in vivo in 166 rats via cecal ligation and puncture (CLP); 60 rats were used to characterize the model. The remaining rats were treated with sophorolipids or vehicle (dimethylsulfoxide [DMSO]/physiologic saline) by intravenous (iv) tail vein or intraperitoneal (IP) injection immediately post-CLP (25/group). Survival rates were compared at 36 hrs after surgery. In vitro, macrophages were cultured in lipopolysaccharide (LPS) ± sophorolipid and assayed for nitric oxide (NO) production and gene expression profiles of inflammatory cytokines. In addition, splenic lymphocytes isolated from CLP rats ± sophorolipid treatment (three per group) were analyzed for cytokine production by RNase protection assay.

Measurements and Main Results: CLP with 16-gauge needles optimized sepsis induction and resultant mortality. Sophorolipid treatment improved rat survival by 34% (iv) and 14% (IP) in comparison with vehicle controls (p < .05 for iv treatment). Sophorolipids decreased LPS-induced macrophage NO production by 28% (p < .05). mRNA expression of interleukin (IL)-1β was downregulated by 42.5 ± 4.7% (p < .05) and transforming growth factor (TGF)-β1 was upregulated by 11.7 ± 1.5% (p < .05) in splenocytes obtained 6 hrs post-sophorolipid treatment. LPS-treated macrophages cultured 36 hrs with sophorolipids showed increases in mRNA expression of IL-1α (51.7%), IL-1β (31.3%), and IL-6 (66.8%) (p < .05).

Conclusions: Administration of sophorolipids after induction of intra-abdominal sepsis significantly decreases mortality in this model. This may be mediated in part by decreased macrophage NO production and modulation of inflammatory responses. (Crit Care Med 2006; 34:E188)

Key Words: sepsis; sophorolipid; mortality; glycolipid; nitric oxide; cytokines; lipopolysaccharide

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epic shock is a common and frequent cause of death in hospitals. Estimates of the incidence of septic shock in the United States range from 300,000 to 500,000 per annum, and mortality rates ascribed to refractory hypotension from septic shock approach 90%. The overall estimated crude mortality rate from septic shock is 35%, and the annual health care cost is estimated at $5 billion to $10 billion (1). In patients with Gram-negative sepsis, bacterial components including DNA and endotoxin, specifically cell wall lipopolysaccharide (LPS), are believed to be causative factors of septic shock via induction of cytokine cascades (2–6). Pro-inflammatory cytokines, specifically monocyte-macrophage derived tumor necrosis factor (TNF) and interleukin (IL)-1 (6), are now known to play an important role in the inflammatory response. Septic shock can result in activation of the coagulation cascade and apoptosis, causing further organ damage and disseminated intravascular coagulation (DIC) (7).

Intra-abdominal sepsis is directly related to delivery of endotoxin-producing Gram-negative pathogens into the peritoneal cavity. This can occur directly from spillage of fecal matter or by translocation of endogenous bacteria from the gastrointestinal tract. Despite significant improvements in antibiotic therapy and aggressive surgical management, septic shock following peritonitis remains a difficult clinical situation to manage (8, 9). Thus, identifying agents that could interfere with septic shock is of great clinical importance.

Sophorolipids are glycolipids having disaccharide sophorose linked glycosidically to the hydroxyl group at that penultimate carbon of C16 to C19 chain fatty acids (Fig. 1). They are fermentatively produced by yeasts such as Candida bombicola, Yarrowia lipolytica, Candida apicola, and Candida boyorizensis when fed with carbohydrates, fatty acids, or their mixture thereof. First described in 1961, sophorolipids occur as a mixture of macrolactone and free acid structures that

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*See also p. 258.

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Inhibit NO production have been considered to be responsible for sepsis-related acute mortality and to act, as demonstrated here in both in vivo and in vitro models, through modulation of inflammatory responses.

MATERIALS AND METHODS

Sophorolipid Synthesis

Sophorolipids were synthesized by fermentation of C. bombicola (11, 27). The fermentation media contained glucose (100 g), yeast extract (10 g), urea (1 g), and oleic acid (40 g) per 1000 mL of water. After 7 days of fermentation, sophorolipid was extracted three times with ethyl acetate. The extracts were pooled and the solvent was removed. The obtained product was washed with hexane to remove residual fatty acids. Liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) analysis was carried out to verify the purity of the compounds. No residual fatty acids or media components were found in the sophorolipids.

In Vivo Sepsis Induction

To optimize a rat model of sepsis-related mortality and test the effect of sophorolipids in an in vivo CLP model of sepsis (36), 60 male adult Sprague-Dawley rats (200–240 g; Taconic, Germantown, NY) were anesthetized with an intraperitoneal injection of Nembutal (40 mg/kg; Abbott Laboratories, North Chicago, IL). The abdomen of each animal was shaved and scrubbed with Betadine. A midline laparotomy was performed and the cecum was ligated just below the ileocecal valve with a 3–0 silk ligature. To determine optimal sepsis induction, the anesthesized rat was punctured with a 14, 16, 18, or 20-gauge needle proximal to the ligature (15 rats/needle gauge). The abdominal incision was then closed in two layers with 2.0 silk (36, 37). All subsequent experiments, in the presence or absence of sophorolipid, employed 16-gauge needle puncture.

Sophorolipid Treatment

The CLP-treated animals (n = 25/group) were treated with sophorolipid (5 mg/kg rat weight, in 4% DMSO-saline) following CLP, by either intravenous (iv; tail vein) or intraperitoneal (IP) injection. The two control groups (n = 25/group) received a similar volume of placebo (4% DMSO in saline) either iv (tail vein) or IP at the end of the operation. All animals were housed singly in standard cages and had access to Chow and water throughout the experiment. Animals were monitored over 36 hrs, and the survival rate was compared between the experimental and control groups. The study was approved by the Animal Care and Use Committee at SUNY-Downstate Medical Center.

Gene Profiles

The gene expression profiles of specific inflammatory cytokines (tumor necrosis factor [TNF]-α, IL-1α, IL-1β, IL-6, IL-10, tumor growth factor [TGF]-β1, and macrophage inhibitory factor [MIF]) were measured in splenic lymphocytes isolated from sophorolipid- or vehicle-treated rats 6 hrs after CLP and in a rat alveolar macrophage cell line, NR8383 (CRL-2192, ATCC, Manassas, VA), which were left untreated; treated (activated) with LPS (from Salmonella typhimurium; 6511, Sigma-Aldrich, St. Louis, MO) to create a cellular model of sepsis (28, 38); treated with LPS and subsequently with sophorolipid; or treated with sophorolipid only.

Splenic Lymphocytes. Spleens were harvested from animals killed 6 hrs post-CLP with or without sophorolipid treatment (iv route; three per group) and placed in cold culture media (RPMI-1640, GIBCO, NY). Lymphocytes were obtained by gently grinding spleens between the frosted edges of two glass slides (Fisher Scientific, Suwannee, GA). Cell suspensions were poured through Nitex (nylon) mesh (Tetko, Elmsford, NY) to remove stroma. Splenic lymphocytes were rinsed twice with ice-cold phosphate-buffered saline (PBS) and quickly scraped into TRIzol (Invitrogen, Carlsbad, CA) for isolation of total RNA. Cytokines were assayed with the RNase protection assay system, as previously described (39), with the BD/Pharmingen Biosciences (San Diego, CA) RiboQuant Multi-Probe Template Sets rCK-1 and rCK-3, according to the manufacturer’s recommendations (rCK-1: IL-1α, IL-1β, TNFβ, IL-3, IL-4, IL-5, IL-6, IL-10, TNFα, IL-2, interferon...
from each group was loaded, in duplicate, onto a vertical polyacrylamide gel electrophoresis (PAGE) apparatus (IBI, Standard Thermoplate Sequencer, New Haven, CT), along with the appropriate controls and probes, and run as per the manufacturer’s instructions. The gel was then adsorbed to filter paper, dried under vacuum, placed in a cassette with X-AR film (Kodak, Rochester, NY) and an intensifying screen, developed at −80°C, and visualized with a medical film processor (SRX-101A, Konica, Taiwan). radioactive bands were analyzed and quantitated with the Gel Doc 2000 System with specific software (The Discovery Series: Quantity One, BioRad). To adjust for differences in sample processing, hybridization signals in each sample were divided by the signal for the housekeeping ribosomal protein mRNA (L32). Data are represented as final adjusted volume (area of band [mm²] x pixel intensity units [INT]).

Effect of Sophorolipid on Macrophage Production of NO

To further test the in vitro effect of sophorolipids, mouse macrophages (RAW 264.7, ATCC TIB-71) were cultured with LPS (50 ng/mL), with or without sophorolipids (25–100 ng/mL). Aliquots of culture supernatants were collected at 5 days and NO content was determined by measuring nitrite (modified Griess reaction), as previously described (28, 40). In brief, triplicate 50-μL aliquots of culture supernatant were mixed in wells of a 96-well microtiter plate with 100 μL of Greiss reagent containing a 1:1 (vol/vol) mixture of 1% (wt/vol) sulfanilamide in 30% acetic acid and 0.5% (wt/vol) of N-(1-Naphthyl) ethylenediamine dihydrochloride in 60% acetic acid. The chromophore generated by the reaction with nitrite was detected spectrophotometrically with a microtiter plate reader at 550 nm (ELX 800, BioTek Instruments, Winooski, VT). The concentration of nitrite was calculated from a standard curve calibrated with known concentrations of NaNO₂.

RESULTS

Sepsis Induction

Preliminary studies in our laboratory revealed average mortality rates of 39%, 42%, 21%, and 27% when CLP was induced with 14-, 16-, 18-, and 20-gauge needles, respectively, for all cumulative time points over the course of 36 hrs (Fig. 2). We chose 16 gauge for the remainder of our experiments because this yielded the highest overall mortality.

Effect of Intravenous or Intraperitoneal Sophorolipid Treatment on Survival after CLP

Intravenous Administration. Following CLP and iv administration of vehicle, the survival rate at 36 hrs in the control group was 47.8%. The survival rate increased to 81.8% among animals given iv injections of sophorolipid immediately after CLP (p < .05) (Fig. 3).

Intraperitoneal Administration. Following CLP and IP administration of vehicle, the survival rate at 36 hrs in the control group was 42%, 21%, and 27% when CLP was induced with 14-, 16-, 18-, and 20-gauge needles, respectively, for all cumulative time points over the course of 36 hrs (Fig. 2). We chose 16 gauge for the remainder of our experiments because this yielded the highest overall mortality.

Figure 2. Cumulative mortality rates in experimental animal sepsis (celiac ligation and puncture) after 12, 24, and 36 hrs with use of 14-, 16-, 18-, and 20-gauge needles. Data represent average of 15 animals per group. Sixteen-gauge needle puncture yielded the highest mortality (42%) when averaged across all time points; there was an average of 19%, 48%, and 60% mortality at 12, 24, and 36 hrs, respectively.

Figure 3. Survival rate in intravenous natural sophorolipid mixture–treated and control animals (Kaplan-Meier); experiments ceased at 36 hrs. Vehicle: celiac ligation and puncture (CLP) + dimethylsulfoxide/saline, SL; CLP + sophorolipid (SL) treatment. CLP was induced with a 16-gauge needle, as described in Materials and Methods.

Statistical Analysis

Survival data were compared by Kaplan-Meier analysis with log-rank test to compare survival function between conditions. Values other than survival data are expressed as mean ± SEM. Significance was determined by either Student’s t-test or analysis of variance (ANOVA) with Tukey posthoc analysis for p value adjustments, with use of SPSS (SPSS, Chicago, IL). In each case, significance was set at p ≤ .05.
control group was 53%. The survival rate increased to 67% among animals given injections of sophorolipid immediately after CLP (p = .08; data not shown).

Effect of Sophorolipid on NO Production

When macrophage cells (RAW 264.7) were treated with 50 ng/mL LPS, increased levels of NO production were observed (34.8 ± 3.9 μM, Fig. 4). In contrast, when sophorolipid was added to LPS-treated cultures, NO production was significantly reduced by 25% to 26.3 ± 0.4 μM in comparison with LPS alone (Fig. 4). Cells cultured with or without sophorolipid in the absence of LPS generated little to no NO production. (Fig. 4)

Effect of Sophorolipid on Cytokine Production

mRNA derived from splenocytes, obtained from rats 6 hrs after CLP and sophorolipid treatment, had a 42.5% ± 4.7% (p < .05) reduction in IL-1β expression in comparison with vehicle-treated controls (Fig. 5A). In contrast, mRNA derived from splenocytes, obtained from rats 6 hrs after CLP and sophorolipid treatment, showed an 11.7 ± 1.5% (p < .05) increase in expression of TGF-β1 (Fig. 5B). There was no difference in cytokine expression for IL-1α, TNF-α, IL-6, IL-10, and MIF from mRNA obtained from rats 6 hrs after CLP/vehicle vs. CLP/sophorolipid treatment (data not shown).

Rat alveolar macrophage cell lines ± LPS activation were subsequently cultured in the presence or absence of sophorolipid and assessed for cytokine mRNA production by RNase Protection Assay (Fig. 6).

High levels of IL-1α and IL-1β, IL-6, IL-10, TNF-α, and MIF were observed in the LPS-treated cultures in comparison with nontreated cells (Fig. 7). When sophorolipid was added to LPS-treated cultures, expression of IL-1α, IL-1β, and IL-6 was significantly increased (by 51.7%, 31.3%, and 66.8 %, respectively; p < 0.05, ANOVA) (Fig. 7). There were no differences in IL-10, TNFα, or MIF when LPS-treated cells were cultured in the presence or absence of sophorolipids (Fig. 7). In addition, there were no differences in TGF-β1 mRNA expression in LPS-treated, LPS-sophorolipid-treated cells, or only-sophorolipid-treated cells in comparison with untreated cells (data not shown). In contrast, addition of sophorolipid to non-LPS-treated cells did not elicit changes in mRNA expression in comparison with cells alone (Fig. 7).

DISCUSSION

Despite advancements in the treatment of sepsis, mortality remains high (1). Gram-negative infections result in endotoxin-induced upregulation of plasma cytokine levels (41–45), which induces septic shock. Current treatments for septic shock caused by Gram-negative bacteria include antibiotic therapy and intensive care to support aberrations in cardiovascular, endocrine, and other organ systems. Antibiotics may be harmful when given in the setting of Gram-negative sepsis (9, 46, 47) because of further release of endotoxin and promotion of life-threatening complications. Therefore, there is a great interest in identifying novel strategies to treat not only infections but also the underlying inflammatory responses. For this reason, agents that can modulate inflammatory responses, in addition to having direct antimicrobial activity, would be advantageous.

The multifactorial nature of septicemia from an intra-abdominal source creates a difficult environment for novel and effective therapeutic intervention (48). Attempts to use immunomodulation to control sepsis have met with mixed results (49). Although experimental studies in vitro and in animals with the anti-endotoxin antibody (50) showed promise, application in the clinical world met with failure (51).

The only approved drug on the market for sepsis is recombinant protein C (Drotrecogin alfa [activated], Xigris, Lilly, Indianapolis, IN), an antithrombotic agent whose mechanism is poorly understood. It has been shown to increase survival, through mechanisms which include reduction of serum d-Dimer and IL-6 levels (52), and has a favorable benefit/risk ratio for septic surgical patients (53, 54). Despite a mild increase in risk of bleeding (3.5% vs. 2.0%; p = .06) (52), it is listed as contraindicated for patients with recent or active bleeding/coagulopathy, making it unsuitable for many septic, traumatic, or surgical patients. Therefore, development of additional pharmaceutical agents that could be administered to treat sepsis would be valuable.

Sphorolipids are a unique class of natural microbial glycolipids that can be chemoenzymatically modified (11). Glycolipids have been shown to modulate several disorders (12–16, 18–22). Therefore, the therapeutic potential of sphorolipids, a novel class of modified glycolipids, was investigated in sepsis. Along with conferring the immunomodulatory effect during septic shock, sphorolipids could also be demonstrating an antibacterial effect. Sphorolipids are biosurfactants that mediate antibacterial effects through mechanisms involving membrane destabilization and increased permeability (25). Although the concentration of sphorolipids in the bloodstream is far less than the minimal inhibitory concentrations for most of the organisms, the microbicidal property of sphorolipids may reduce the bacterial load in the bloodstream through cell lysis (25).

A number of models for inducing intra-abdominal sepsis exist. A single inoculum of one Gram-negative bacterial species has been the model of sepsis used for the screening of antimicrobial drugs in vitro (9). We prefer the cecal ligation and puncture model of experimental sepsis, which is far less than the minimal inhibitory concentration of sophorolipids in the bloodstream through mechanisms which include reduction of serum d-Dimer and IL-6 levels (52), and has a favorable benefit/risk ratio for septic surgical patients (53, 54). Despite a mild increase in risk of bleeding (3.5% vs. 2.0%; p = .06) (52), it is listed as contraindicated for patients with recent or active bleeding/coagulopathy, making it unsuitable for many septic, traumatic, or surgical patients. Therefore, development of additional pharmaceutical agents that could be administered to treat sepsis would be valuable.

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as it reproduces the clinical situation of bowel perforation and subsequent mixed bacterial infection and polymicrobial peritonitis. This well described and widely used animal model allows a consistent septic insult with predictable mortality, cytokine production, and alteration of physiologic parameters (36, 41). High mortality in the cecal ligation and puncture model can be elicited, and in our experiments optimal mortality approached 60% at 36 hrs with use of a 16-gauge needle. Similar findings with use of this gauge needle have been reported by others (36). We felt that reduction of mortality by administration of sophorolipid could be effectively monitored in this setting.

In the current study, polymicrobial sepsis was induced with the previously established cecal ligation and puncture model. We found that sophorolipid administered soon after the insult decreased mortality at 36 hrs when administered iv. It is possible that sophorolipid treatment can be administered after the development of sepsis, making it an attractive potential therapy. Because treatment of sepsis generally requires iv administration, investigation of sophorolipid treatment using this route was paramount, and IP instillation was used as a comparative process. Although it is unclear why only iv treatment yielded a statistically significant decrease in mortality, it may be due to differences in the pharmacokinetics of sophorolipid, which are route-dependent. These aspects of sophorolipid administration are currently not known.

Sphorolipid dose and animal number selection were predicated on preliminary data from an investigation in which mice underwent CLP ± sophorolipid treatment (data not shown). In these experiments, maximum mortality decrease was obtained with 5-mg/kg sophorolipid treatment. Furthermore, this dose represents the current maximum solubility for sophorolipids in DMBSO for subsequent in vivo administration.

The dose of sophorolipids was well within the reported safety range. The lethal dose (LD) of 50% of animals (LD50) of naturally occurring sophorolipid or its derivatives in rodents is minimally 5.8 g/kg when administered subcutaneously and 10 g/kg when ingested orally (55), and the oral no-adverse-effect level (NOAEL) is reported to be approximately 200 mg/kg/day (56). Others have observed no measurable consequences with 12.5 g/kg sophorolipid in rats or 6 g/kg in mice (57). Although the therapeutic range and metabolism of sophorolipid treatment in sepsis are unknown, the relatively low dose used in our studies was 5 mg/kg, which nonetheless demonstrated an effect of decreasing sepsis-related mortality. This dose approximated 1/1000 of that with reportable side effects.

NO production is a useful marker of macrophage response to bacterial LPS (28, 38). The ability of sophorolipid to moderate macrophage NO production when exposed to LPS was investigated in a tissue culture model. Addition of sophorolipid decreased NO production when it was cultured with LPS-treated macrophages. Although induction of NO has a reported benefit in certain nonsepsis disease states such as parasitic infections (55, 58, 59), such is not the case for sepsis. Furthermore, when NO was administered as therapy in acute lung injury, there was no observed decrease in mortality (60), and it may in fact worsen the patient’s condition. Indeed, NO production is responsible for sepsis-related acute lung injury (29) and central neural apoptosis (30). Furthermore, agents that inhibit NO production have been considered as therapeutic options for sepsis (31–33, 61, 62) with varying results (34, 35). Our data show that addition of sophorolipid did not cause an increase in NO production when it was cultured with healthy cells. This is in critical contrast to other putative therapeutic candidates, which, although promising in the treatment of sepsis, appear to increase NO production (63). It is likely that sophorolipid-mediated mortality reduction acts through NO modulation in conjunction with additional mechanisms. However, it is also possible that NO production may have little if anything to do with our observed reduction in sophorolipid-mediated mortality and may represent a bystander effect. Additional mechanisms responsible for the sophorolipid effect, including inflammatory cytokine modulation, receptor-ligand and second messenger interactions, and other metabolic processes, warrant further investigation.

Septic shock results, in part, from alteration of plasma cytokine levels (42–45), which can cause fever, hypotension, and prostration. Our data show that sophorolipids modulate the inflammatory response to sepsis. Splenocyte cytokine

Figure 5. A, interleukin (IL)-1β production, and B, tumor growth factor (TGF)-β1 production in splenic lymphocytes obtained from rats 6 hrs after cecal ligation and puncture (CLP) ± sophorolipid (SL) treatment. Data are expressed as percent control (CLP + vehicle) ± SEM; p < .05 compared with control, Student’s t-test.

Figure 6. RNase protection assay; A, rCK-1, and B, rCK-3 template cytokine analysis as described under Materials and Methods. Lanes 1 and 14, untreated controls; 2 and 3, protected CK probes; 4 and 5, LPS (100 ng/mL), 6 and 7, LPS (100 ng/mL) plus sophorolipid (200 ng/mL), 8 and 9, sophorolipid (200 ng/mL); 10 and 11, yeast tRNA background control; 12 and 13, CK series RNA control.
profiles were determined at 6 hrs in order to evaluate any sophorolipid-mediated changes during the early stages of sepsis and identify key mediators of the inflammatory process that are required or present disease progression. Similarly, the transcription factor early growth response (EGR)-1 was identified as a key regulator of another inflammatory disease process, pancreatitis, and was found to be upregulated in the early stages of the disease (64).

Splenocytes obtained from rats treated with sophorolipid immediately after CLP had decreased production of IL-1β and increased production of TGF-β1 in comparison with rats treated with DMSO/saline vehicle. IL-1, including IL-1α and IL-1β, is a well-established proinflammatory cytokine that is elevated in sepsis (65), and the reduction of IL-1 is an established marker for evaluating the mechanisms of therapeutic candidates in sepsis treatment (28). Increases in the plasma levels of the anti-inflammatory cytokine TGF-β1 are also considered beneficial in sepsis, but its role is less clear owing to widely reported ranges (66, 67) and differences in active vs. total forms (68). It is possible that the observed decrease in sepsis mortality in the sophorolipid group is due to its direct effect on cytokine synthesis, making it similar to other therapies (46, 47, 19). It is more likely that additional factors, including NO and others as yet unidentified, are part of the sophorolipid-mediated protective mechanisms.

Using an in vitro model of sepsis, LPS treatment of rat macrophages, we observed significant increases in expression of IL-1α, IL-1β, and IL-6 36 hrs after sophorolipid treatment. The IL-1β observation is the opposite of what we observed in splenocytes. Although these two systems are not intended to linearly translate into mechanisms that explain our observed sophorolipid-mediated reduction in sepsis-related mortality in vivo, there are several possible explanations for the observed differences in cytokine expression in these two in vitro systems. First, it may simply be due to the fact that the macrophage cell line is a single population, whereas the splenocytes comprise many different cell populations. Similar discrepancies in cytokine (69) and matrix metalloproteinase (70) expression have been observed during organ-derived cell mixtures and malignant clonal cell lines. Differences in cytokine-activated mononuclear cell cytotoxic activity in cells obtained from cord blood vs. peripheral blood have also been reported (71), demonstrating variability in organ-derived cell responses. Finally, there may be a difference in the expression of these cytokines at different times following sepsis induction. Similar changes in IL-6, inducible NO synthase, and other genes have been observed in early vs. late gene expression in two experimental models of acute pancreatitis (64). Either way, cytokines, including but not limited to IL-1, are involved in the response to sophorolipid therapy.

CONCLUSIONS

Sophorolipid treatment after CLP potentially reduced mortality in experimental sepsis and may exert its effects through reduction of NO and the modulation of inflammatory responses. Further investigation is warranted to better understand this novel candidate for use in treating sepsis and reducing sepsis-related mortality.

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