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THE EFFECT OF *EX VIVO* CDDO-ME ACTIVATION ON NUCLEAR FACTOR ERYTHROID 2–RELATED FACTOR 2 PATHWAY IN WHITE BLOOD CELLS FROM PATIENTS WITH SEPTIC SHOCK

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Abstract

Nuclear factor erythroid 2–related factor 2 (NRF2) has been shown to protect against experimental sepsis in mice and lipopolysaccharide (LPS)-induced inflammation in *ex vivo* white blood cells from healthy subjects by upregulating cellular antioxidant genes. The objective of this study was to test the hypothesis that *ex vivo* methyl 2-cyano-3, 12-dioxoolean-1,9-dien-28-oate (CDDO-Me) activates NRF2-regulated antioxidant genes in white blood cells from patients with septic shock and protects against LPS-induced inflammation and reactive oxidative species production. Peripheral blood was collected from 18 patients with septic shock who were being treated in medical and surgical intensive care units. Real-time polymerase chain reaction was used to quantify the expression of NRF2 target genes (*NQO1*, *HO-1*, *GCLM*, and *FTL*) and *IL-6* in peripheral blood mononuclear cells (PBMCs), monocytes, and neutrophils after CDDO-Me treatment alone or after subsequent LPS exposure. Superoxide anion (O_2^-) was measured to assess the effect of CDDO-Me pretreatment on subsequent LPS exposure. Treatment with CDDO-Me increased the gene expression of *NQO1* ($P = 0.04$) and decreased the expression of *HO-1* ($P = 0.03$) in PBMCs from patients with septic shock. Purified monocytes exhibited significant increases in the expression of *NQO1* ($P = 0.01$) and *GCLM* ($P = 0.003$) after CDDO-Me treatment. Levels of other NRF2 target genes (*HO-1* and *FTL*) remained similar to those of vehicle-treated cells. Peripheral blood mononuclear cells showed a trend toward increased *IL-6* gene expression after CDDO-Me treatment, whereas purified monocytes showed a trend toward decreased *IL-6*. There was no discernible trend in the *IL-6* expression subsequent to LPS treatment in either vehicle-treated or CDDO-Me–treated PBMCs and monocytes. Treatment with CDDO-Me significantly increased O_2^- production in PBMCs ($P = 0.04$). Although CDDO-Me pretreatment significantly attenuated O_2^- production to subsequent LPS exposure ($P = 0.03$), the change was comparable to that observed in vehicle-treated PBMCs. Pretreatment with CDDO-Me followed by LPS exposure had no significant effect on O_2^- levels in purified monocytes. These data suggest that the NRF2 pathway is differentially responsive to CDDO-Me activation in

peripheral blood cells from patients with septic shock and results in increased O_2^- production. The data may also suggest a suppressed NRF2 pathway in white blood cells from critically ill patients.

Keywords

Critical illness; septic shock; NRF2; CDDO-Me; antioxidants; reactive oxygen species

INTRODUCTION

Critically ill patients in intensive care units (ICUs) are predominantly those with sepsis, septic shock, and systemic inflammatory response syndrome (SIRS). The state of shock is characterized by refractory hypotension and multiple organ failure, which occurs mostly as a consequence of uncontrolled host response to microbial infection. The septic response accelerates the activation of immune cells and release of large amounts of cytokines and reactive oxygen species (ROS). Reactive oxygen species have been shown to play a significant role in the pathogenesis of sepsis by modulating toll-like receptor 4 signaling (1, 2) and priming immune cells (3). Reactive oxygen species also cause oxidative damage to endothelial and epithelial cells, leading to tissue damage and organ failure (4). Nuclear factor erythroid 2Yrelated factor 2 (NRF2), a basic leucine zipper oxidant-responsive transcription factor, regulates important cytoprotective genes, including antioxidants, and hence maintains cellular redox homeostasis. Animal studies have established the critical role of NRF2 in improving survival during sepsis (5). Insufficient NRF2 activity leads to a decline in constitutive expression of antioxidant genes and greatly impairs the capacity to mount a protective stress response (6, 7).

Triterpenoid analogs such as methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oate (CDDO-Me) are potent activators of NRF2 in cell culture and *in vivo* (8, 9). Recent preclinical *ex vivo* evaluation of triterpenoid derivatives showed promising results for activating NRF2-regulated genes in peripheral blood mononuclear cells (PBMCs) and neutrophils isolated from healthy human subjects (10). Furthermore, an initial activated immune response during shock is subsequently followed by immune suppression. However, the possibility of activating the NRF2 pathway in critically ill patients to restore redox balance and immune cell function has not been studied. This type of translational medical research is important because genomic responses in mouse models may or may not mimic human inflammatory diseases (11). In this study, we evaluated the responsiveness of PBMCs of patients with septic shock to CDDO-Me, an NRF2 activator. We tested the hypothesis that CDDO-Me treatment is capable of activating NRF2-regulated antioxidant genes in white blood cells isolated from patients with septic shock. We also hypothesized that NRF2 activation with CDDO-Me pretreatment is protective against subsequent lipopolysaccharide (LPS)-induced inflammation and ROS production.

MATERIALS AND METHODS

Subjects

Critically ill patients from medical and surgical ICUs at a tertiary care center aged 18 years or older were prospectively enrolled. Inclusion criteria were the onset of shock within the previous 72 h (as defined by a systolic blood pressure of <90 mmHg, despite adequate fluid replacement, or a need for vasopressors for at least 1 h) and hypoperfusion or organ dysfunction attributable to sepsis. Enrolled patients were followed to confirm clinical evidence of infection and systemic response to infection using the definitions of the Surviving Sepsis Campaign by the Society of Critical Care Medicine (12). Using these simple and easily reproducible diagnostic criteria, we were able to define a specific study population within the continuum of sepsis syndrome. The study population represents the sickest patients with sepsis syndrome. If, during the clinical course, no evidence of infection was demonstrated, then the diagnosis was changed from presumed septic shock to SIRS. The principal investigator (R.J.F.) obtained informed consent from the patients or their respective power of attorney if the patients themselves were not able to participate in the process of informed consent. Patients with previous ICU admission were excluded from the study. Blood from healthy controls (n = 4) was collected and used to compare the effect of CDDO-Me treatment on NRF2 activation. This study was approved by the Johns Hopkins University Institutional Review Board (IRB Study No. NA_00008804), and all subjects signed a written informed consent.

Blood collection and isolation of cells

Blood samples were collected in cell preparation tubes with sodium citrate (BD, Franklin Lakes, NJ). Peripheral blood mononuclear cells and polymorphonuclear cell fractions were separated by density-gradient centrifugation as described elsewhere (13, 14). The PBMC fraction was washed with phosphate-buffered saline and resuspended in RPMI 1640. Monocytes were isolated from PBMCs with magnetically labeled CD14 microbeads, and neutrophils were negatively selected from the polymorphonuclear cell fraction with magnetically labeled CD16 microbeads according to the manufacturer's instructions (Milenyi Biotech, Auburn, Calif). Binding of magnetically labeled CD14 beads on monocytes does not trigger any signal transduction, as the beads lack the cytoplasmic domain required to induce signal transduction. Their use has been shown not to affect CD80, CD86, or CD83 expression when compared with the plate adherence method (15). The cell viability was assessed by trypan blue dye exclusion test and cell number determined with a hemocytometer. Cells were plated (2×10^6) and cultured at 37°C in a 5% CO₂ atmosphere for 1 h and then treated with CDDO-Me or vehicle.

CDDO-Me and LPS treatment

Peripheral blood mononuclear cells and monocytes were treated with vehicle (1% dimethyl sulfoxide in RPMI medium) or CDDO-Me (20 nM) for 20 h before being harvested for gene expression studies. In a separate experiment, cells pretreated with CDDO-Me were stimulated with LPS (100 ng/mL; *Escherichia coli* serotype 055:B5; Sigma, St. Louis, Mo). Reactive oxygen species were measured in the culture medium 1 h after LPS treatment, and

NRF2 target and *IL-6* mRNA levels were measured in PBMCs and monocytes 3 h after LPS treatment.

Antioxidant gene expression analysis

Expression of NRF2-regulated antioxidant genes and inflammatory genes was measured in PBMCs and monocytes with an ABI 7000 quantitative real-time polymerase chain reaction. Total RNA was extracted from the cells with the RNeasy mini kit (Qiagen, Valencia, Calif) according to the manufacturer's instructions. Quantitative polymerase chain reaction was carried out with 50 ng of cDNA, exon-specific Taqman primers, and probe sets (Life Technologies, Grand Island, NY) for analysis of selected genes: *NRF2* and its target genes NADPH-quinone oxidoreductase (*NQO1*), heme oxygenase 1 (*HO-1*), glutamate cysteine ligase modifier subunit (*GCLM*), and ferritin (*FTL*), and interleukin 6 (*IL-6*). Gene expression data were normalized with β actin, and relative fold change for each gene was calculated using the 2^{-CT} method as described elsewhere (16).

Measurement of ROS

Generation of superoxide anion was assessed by luminol-derived horse-radish peroxidase-dependent chemiluminescence. One hundred microliters of cell culture medium was carefully removed and incubated in a test tube containing 10 μ M luminol and 0.5 units horseradish peroxidase at 37°C. The tube was immediately placed in the luminometer (LB 960; Berthold Co., Wildbad, Germany) to record chemiluminescence for 5 min.

Statistical analysis

A paired two-tailed *t* test was used to compare NRF2 targets and inflammatory markers between the groups as means of relative fold change (STATA 11.2; StataCorp LP, College Station, Tex). Statistical significance was two-tailed and accepted at $P < 0.05$.

RESULTS

Demographic and clinical characteristics of the patients

The eight female and 10 male patients had a mean age of 54 (range, 20 – 88) years. In terms of race, 14 were white, two were African American, and two were considered Bother. In 14 of the 18 patients, evidence of infection later during the ICU course confirmed the diagnosis of septic shock. Two patients developed a SIRS of noninfectious origin (one had acute renal failure and the other had liver failure). Care was withdrawn in two patients with a poor prognosis. The severity of illness as measured by Acute Physiology and Chronic Health Evaluation II (APACHE II) score (mean, 23; range 7 Y 41) and the mortality rate of 33% (six of 18) were typical of a common ICU population (Table 1).

Effect of CDDO-Me treatment on NRF2 target gene expression in white blood cells of healthy controls

We observed a significant increase in the gene expression of *NQO1* ($P = 0.02$), *HO-1* ($P = 0.05$), *GCLM* ($P = 0.01$), and *FTL* ($P = 0.04$) after CDDO-Me-treatment. However, NRF2 mRNA expression did not differ between CDDO-Me-treated and vehicle-treated PBMCs

from healthy subjects. These observations are in agreement with previously described findings by Thimmulappa et al. (10). We then exposed CDDO-Me- and vehicle-treated PBMCs isolated from healthy subjects to LPS and observed its effect on NRF2 target gene expression. After LPS treatment, the expression of *NQO1* ($P = 0.02$) and *GCLM* ($P = 0.001$) remained significantly higher in CDDO-Me-treated PBMCs than in vehicle-treated PBMCs. *HO-1* and *FTL* had higher expression levels after LPS treatment but were not statistically different from levels in vehicle-treated PBMCs. Lipopolysaccharide treatment had no effect on *NRF2* gene expression in either the vehicle-treated or the CDDO-Me-treated PBMCs (Fig. 1).

Effect of CDDO-Me treatment on NRF2 target gene expression in white blood cells of patients with septic shock

We tested the hypothesis that NRF2-regulated antioxidant response can be activated *ex vivo* with CDDO-Me in PBMCs from patients with septic shock. We found a significant increase in the gene expression of *NQO1* ($P = 0.04$) in CDDO-Me-treated PBMCs, whereas the expression levels of *GCLM* ($P = 0.11$) and *FTL* ($P = 0.22$) were not significantly different from those of vehicle-treated PBMCs. Interestingly, *HO-1* mRNA levels showed a significant decline ($P = 0.03$) after CDDO-Me treatment. Furthermore, CDDO-Me treatment had no effect on *NRF2* gene expression (Fig. 2). Because monocytes are considered the primary ROS-producing cells in PBMCs, we treated purified monocytes from the same critically ill patients with CDDO-Me ($n = 8$). Purified monocytes showed a significant increase in the expression of *NQO1* ($P = 0.01$) and *GCLM* ($P = 0.003$) genes after CDDO-Me treatment, but *FTL* ($P = 0.08$) gene expression levels did not change significantly as compared with those in vehicle-treated monocytes (Fig. 3). *HO-1* gene expression showed a borderline decreasing trend ($P = 0.09$) compared with levels in vehicle-treated monocytes. We did not observe any significant difference in *NQO1*, *HO-1*, *GCLM*, or *FTL* gene expression levels in CDDO-Me-treated or vehicle-treated neutrophils (data not shown).

CDDO-Me pretreatment does not provide a sustained antioxidant response after subsequent LPS treatment

In simultaneous experiments, we treated some of the vehicle- and CDDO-Me-treated cells with LPS to test the hypothesis that NRF2 activation with CDDO-Me pretreatment is protective against subsequent LPS-induced inflammation and ROS production in PBMCs and monocytes from patients with septic shock. Although the PBMC gene expression levels of *NQO1* ($P = 0.09$), *GCLM* ($P = 0.06$), and *HO-1* ($P = 0.11$) were lower after treatment with vehicle and LPS than after treatment with vehicle alone, the differences did not reach statistical significance (Fig. 4). Similarly, gene expression of *NQO1* ($P = 0.11$) and *HO-1* ($P = 0.07$) did not differ significantly between PBMCs treated with CDDO-Me plus LPS and PBMCs treated with CDDO-Me alone. In contrast, *GCLM* mRNA level was significantly lower ($P = 0.03$) in CDDO-Me-treated PBMCs after LPS treatment than in PBMCs treated with CDDO-Me alone. No statistically significant difference was observed in the expression of any NRF2 target gene between vehicle- and CDDO-Me-treated PBMCs after LPS treatment, indicating no particular advantage of CDDO-Me pretreatment (Fig. 4).

In monocytes treated with vehicle or CDDO-Me followed by LPS, gene expression levels of *NQO1*, *HO-1*, and *GCLM* decreased significantly compared with levels in monocytes treated with vehicle alone ($P = 0.03$, $P = 0.04$, $P = 0.03$, respectively) or CDDO-Me alone ($P = 0.05$, $P = 0.05$, $P = 0.04$, respectively) (Fig. 5). However, none of the NRF2 targets differed when we compared monocytes treated with vehicle and LPS with monocytes treated with CDDO-Me and LPS.

Effect of CDDO-Me treatment on IL-6 expression

To understand the effect of CDDO-Me treatment on inflammation, we measured *IL-6* mRNA levels in cells after LPS treatment. Vehicle-treated PBMCs showed an expected but statistically nonsignificant overall increase in *IL-6* expression after LPS treatment ($P = 0.6$). The CDDO-MeYtreated PBMCs showed a trend toward increased *IL-6* expression after LPS treatment, but the increase was not statistically significant ($P = 0.3$). In contrast, purified monocytes showed a trend toward decreased *IL-6* expression after LPS treatment. None of the relationships were statistically significant, suggesting insufficient protection by CDDO-Me (Fig. 6).

Effect of CDDO-Me treatment on ROS production in white blood cells from patients with septic shock

We measured superoxide anion levels in vehicle- and CDDO-Me-treated PBMCs and monocytes after exposing them to LPS. The CDDO-Me-treated PBMCs had significantly higher ROS levels than did vehicle-treated PBMCs ($P = 0.04$; Fig. 7). The CDDO-Me-treated PBMCs that were subsequently exposed to LPS had significantly lower ROS levels than did PBMCs treated with CDDO-Me alone ($P = 0.03$). However, there was no difference in ROS production between vehicle-treated and CDDO-MeYtreated PBMCs after LPS treatment. Purified monocytes showed a trend toward an increase in ROS production after treatment with CDDO-Me alone or CDDO-Me followed by LPS exposure as compared with monocytes treated with vehicle or vehicle followed by LPS, respectively. However, these differences were not statistically significant.

DISCUSSION

A dysfunctional NRF2 pathway has been identified as a critical defect in various diseases and animal models of sepsis and septic shock; however, it has not been extensively explored in critically ill patients (4, 17–19). In this study, we attempted to upregulate NRF2 activity in white blood cells from patients with septic shock in an *ex vivo* experiment. This study was based on previously published preclinical studies in mouse and normal human cells that showed that CDDO-Me and CDDO-Im are potent NRF2 activators that produce an overall increase in antioxidant gene response and attenuate LPS-induced inflammation (10, 16). Pretreatment of peritoneal neutrophils and macrophages from wild-type mice with CDDO-Im induced antioxidative genes (*Ho-1*, *Gclc*, *Gclm*, and *Nqo1*) and attenuated LPS-induced ROS generation as well as expression of proinflammatory cytokines such as *Il-6* and *Tnf-α*. In PBMCs and neutrophils of healthy volunteers, CDDO-Im and CDDO-Me increased nuclear levels of NRF2 and upregulated the expression of NRF2 targets *NQO1*, *GCLC*, *GCLM*, and *HO-1*; CDDO-Im and CDDO-Me suppressed LPS-induced inflammatory

response in PBMCs as measured by IL-6 and TNF- α gene expression; and CDDO-Im attenuated LPS-induced ROS generation and the expression of IL-6 and TNF- α in neutrophils. However, with a similar experimental approach, the major findings of this study indicate that, in white blood cells of patients with septic shock, NRF2 is differentially responsive to CDDO-Me activation, as indicated by *NQO1*, *HO-1*, *GCLM*, and *FTL* mRNA levels. Although we observed significant increases of *NQO1* expression in PBMCs and of *NQO1* and *GCLM* expression in purified monocytes, there was no change in other NRF2 target genes that we examined. Interestingly, in contrast to healthy controls in this study and previous studies, we observed a significant decline in *HO-1* expression in both PBMCs and purified monocytes after CDDO-Me activation. Because of the huge variability in the data, we performed sensitivity analysis by removing outliers. Interestingly, sensitivity analysis further increased the statistical significance of these findings possibly owing to a reduction in data variability. Nevertheless, the overall conclusion of the study did not change. Although it will be difficult to predict from this study how this inverse *HO-1* expression may affect patients with septic shock, these findings may be important because NRF2-regulated *HO-1* gene expression has been shown to protect against experimental sepsis by upregulating IL-10 and IL-1 levels and increasing mitochondrial biogenesis (19, 20). Furthermore, in a recent study, Noel et al. (21) found significant opposing effects of *NQO1* and *HO-1* mRNA levels on asthma symptoms in humans. A higher *NQO1* expression correlated with worse asthma symptoms, whereas the reverse was true for *HO-1* expression.

The response of CDDO-Me-pretreated PBMCs to subsequent LPS treatment was different in this study from the response of PBMCs from healthy subjects reported by Thimmulappa et al. (10). First, we did not observe a significant change in *IL-6* gene expression between vehicle- and CDDO-Me-treated PBMCs, although higher *IL-6* mRNA levels were found in CDDO-Me-treated PBMCs after LPS exposure. Second, the responses of PBMCs and purified monocytes appear to be different, but it is difficult to conclude with certainty because none of our results pertaining to *IL-6* gene expression are statistically significant. These observations are in contrast to previously known effects of LPS, which was shown to increase the expression of NRF2 target genes *NQO1* and *HO-1* in normal human monocytic cell lines and mouse models (16, 22).

Furthermore, CDDO-Me treatment resulted in a significant increase in ROS production and did not protect cells against subsequent LPS exposure. This was evident by diminishing antioxidant gene expression and high ROS levels after LPS treatment. Higher ROS levels in CDDO-Me-treated cells may also indicate CDDO-Me-specific effects in addition to inadequate Nrf2 activity. Methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oate has been shown to induce ROS levels in various cancer cells, and pretreatment with *N*-acetylcysteine ameliorates CDDO-Me-induced ROS production (23, 24). More recently, CDDO-Me has been found to have severe adverse effects in a clinical trial of patients with chronic kidney disease (25). Another confusing aspect of the ROS study was that monocytes did not produce significant ROS. Hence, a question remains regarding the source of ROS in PBMCs. The only probable source of ROS in these circumstances could be T cells, which become activated and undergo rapid apoptosis in critically ill patients. It is known that ROS can activate T cells, along with many other immune cells, and recent studies showed that T-

cell activation can also result in ROS production (26, 27). However, whether this hypothesis holds true in critically ill patients requires further examination. Therefore, use of triterpenoid-based NRF2 activation needs to be understood in more detail before safer clinical alternatives can be developed for use in critically ill patients.

We observed considerably high interindividual variations in our data, a result that is not unexpected in human studies. It may be caused partly by heterogeneity of the study population. The range of illness severity was wide, as measured by APACHE II score. Future studies with clinical outcome measures need to include stratified analysis by severity of illness. This wide range of illness severity could also be a reason that we did not see more significant differences between the groups. However, most of the critically ill patients in our study were in septic shock. These findings are supported by a recent study conducted by The Inflammation and Host Response to Injury Large Scale Collaborative Research Program to determine the genomic responses in white blood cells to systemic inflammation (trauma, burns, endotoxemia) in patients and mouse models. That study found that genomic responses to different types of acute inflammatory stress are highly similar in humans despite heterogeneities in the patient population (11). As a future direction, studies should be undertaken to determine the expression levels of the *NRF2* gene and target genes in patients with septic shock and the association between these gene expression levels and mortality early in the disease process.

Although our study is limited by a small sample size, it provides important and clinically relevant information regarding CDDO-MeY dependent activation of NRF2 in patients with septic shock. Our data showed partial activation (*NQO1* in *PBMCs*; *NQO1* and *GCLM* in purified monocytes) in patients with septic shock. Moreover, we did not observe any particular advantage of CDDO-Me pretreatment on subsequent LPS exposure. Although previous studies postulated that activation of the NRF2 pathway may be useful in attenuating oxidative stress and inflammation (10, 22), major findings of this study provide a more realistic understanding of how CDDO-Me might affect patients with septic shock and further emphasize the need for prioritizing translational medical research. Also, more clinically relevant animal models are urgently needed for preclinical evaluation of candidate drug molecules. Our study illustrates this need by showing that the effect of CDDO-Me differs in *PBMCs*, depending on whether they are from critically ill humans, healthy humans, or mice. A recent comparative study questioned whether mouse models mimic human inflammatory diseases (11). It appears that NRF2 activity in vasopressor-dependent critically ill patients is legitimately low and incapable of mounting a continuous protective effect in the presence of subsequent LPS exposure. This finding may have important clinical implications because critically ill patients, especially those with sepsis and septic shock, have high levels of LPS in the systemic circulation at any given time. Achieving a clinically relevant NRF2 response in critically ill patients may also require a higher dose; however, dose-related adverse effects may be a concern especially because NRF2 may have differential effects on some of the protective genes, such as *HO-1*, as seen in our study. Nonetheless, it is fundamentally important to restore antioxidant response in critically ill patients, and future studies should focus on identifying and screening novel NRF2-activating

compounds. Alternate methods to salvage NRF2 activity using siRNA and antioxidant supplementation to restore antioxidant response are also highly desirable.

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ABBREVIATIONS

CDDO-Me	methyl 2-cyano-3,12-dioxoolean-1,9-dien-28-oate
FTL	ferritin
GCLM	glutamate cysteine ligase modifier
HO-1	heme oxygenase 1
IL-6	interleukin 6
LPS	lipopolysaccharide
NQO1	NADPH-quinone oxidoreductase
NRF2	nuclear factor erythroid 2–related factor 2
PBMC	peripheral blood mononuclear cell
ROS	reactive oxygen species

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FIG. 1. Effect of CDDO-Me treatment on NRF2 target gene expression in white blood cells of healthy controls

Treatment with CDDO-Me resulted in significant increases in the gene expression of *NQO1* ($P = 0.02$), *HO-1* ($P = 0.05$), *GCLM* ($P = 0.01$), and *FTL* ($P = 0.04$) in PBMCs isolated from healthy controls. However, CDDO-Me treatment had no effect on NRF2 mRNA levels.

Subsequent LPS exposure to CDDO-Me-treated PBMCs caused a sustained and significant expression of *NQO1* ($P = 0.02$) and *GCLM* ($P = 0.001$) as compared with that in vehicle-treated PBMCs. Heme oxygenase 1 and FTL expression was higher in CDDO-Me-treated PBMCs after LPS exposure but was not statistically significant. Lipopolysaccharide treatment had no effect on NRF2 gene expression in either the vehicle-treated or CDDO-Me-treated PBMCs. Each bar represents mean $\pm$ SEM of relative fold change in mRNA. * P 0.05, ** P 0.01, *** P 0.001.

FIG. 2. Effect of *ex vivo* CDDO-Me treatment on NRF2 target gene expression in PBMCs from patients with septic shock

Nuclear factor erythroid 2-related factor 2 mRNA levels were comparable between vehicle-treated and CDDO-Me-treated PBMCs from critically ill patients. Peripheral blood mononuclear cells from critically ill patients ($n = 9$) showed a significant increase in *NQO1* ($P = 0.04$) and a decrease in *HO-1* ($P = 0.03$) gene expression after CDDO-Me treatment. No significant change was observed in NRF2 target genes *GCLM* ($P = 0.11$) and *FTL* ($P = 0.22$). * $P < 0.05$.

FIG. 3. Effect of *ex vivo* CDDO-Me treatment on NRF2 target gene expression in monocytes from patients with septic shock

Magnetically purified monocytes from critically ill patients ($n = 9$) treated with CDDO-Me showed a significant increase in *NQO1* ($P = 0.01$) and *GCLM* ($P = 0.003$) gene expression. *Ferritin* ($P = 0.08$) gene expression remained comparable to that of vehicle (Veh)-treated monocytes. *Heme oxygenase 1* gene expression showed a decreasing trend similar to that in PBMCs, but expression was not statistically different from that of vehicle-treated monocytes ($P = 0.09$). ** $P < 0.01$; *** $P < 0.001$.

FIG. 4. CDDO-Me pretreatment fails to sustain NRF2 transcriptional activity in PBMCs from patients with septic shock after subsequent LPS exposure

Antioxidant gene expression did not differ between vehicle- and CDDO-Me-treated PBMCs after LPS exposure, indicating that CDDO-Me pretreatment does not result in sustained NRF2 activation ($n = 4$). Gene expression for *NQO1*, *HO-1*, and *GCLM* was lower in PBMCs treated with LPS and vehicle than in PBMCs treated with vehicle alone, but the differences were not significant ($P = 0.09$, $P = 0.11$, $P = 0.06$, respectively). Exposure of PBMCs to LPS after CDDO-Me pretreatment resulted in a nonsignificant decline in *NQO1* ($P = 0.11$) and *HO-1* ($P = 0.07$) gene expression and a significant decline in *GCLM* ($P = 0.03$) expression. Each bar represents mean $\pm$ SEM of relative fold change (RFC) in mRNA. $*P = 0.05$.

FIG. 5. Antioxidant gene expression in CDDO-Me-pretreated monocytes and response to subsequent LPS exposure

Purified monocytes had a highly suppressed antioxidant response after LPS exposure, and pretreatment with CDDO-Me offered no advantage over pretreatment with vehicle ($n = 4$). Exposure of monocytes to LPS after initial treatment with vehicle or CDDO-Me produced statistically significant declines in *NQO1*, *HO-1*, and *GCLM* gene expression compared with vehicle or CDDO-Me treatment alone. Each bar represents mean $\pm$ SEM of relative fold change (RFC) in mRNA. * $P < 0.05$.

FIG. 6. IL-6 gene expression in CDDO-Me-treated PBMCs and monocytes from patients with septic shock

The CDDO-Me-treated PBMCs ($n = 4$) showed a trend toward increased *IL-6* expression after LPS treatment ($P = 0.10$, veh + LPS versus CDDO-Me + LPS). In contrast, purified monocytes ($n = 4$) showed a trend toward decreased *IL-6* expression after LPS treatment ($P = 0.22$, veh + LPS versus CDDO-Me + LPS). RFCVrelative fold change.

FIG. 7. CDDO-Me treatment aggravates ROS production in PBMCs and monocytes from patients with septic shock

The CDDO-Me-treated PBMCs ($n = 4$) had significantly higher ROS levels than did vehicle-treated PBMCs ($P = 0.04$). Pretreatment of PBMCs with CDDO-Me significantly attenuated ROS production in response to subsequent LPS exposure ($P = 0.03$). There was no difference in ROS production between vehicle- and CDDO-Me-treated PBMCs after LPS treatment. Purified monocytes ($n = 4$) showed a trend toward increased ROS production after CDDO-Me treatment alone ($P = 0.12$) and after CDDO-Me treatment followed by LPS exposure ($P = 0.24$) as compared with monocytes treated with vehicle or vehicle plus LPS, respectively, but the increase was not statistically significant. Each bar represents mean $\pm$ SEM of relative luminescence units ([RLUs] counts) calculated by the integral of the area under the curve for 5 min. * $P < 0.05$.

Table 1

Demographic and clinical characteristics of patients

No. patients	18
Age, years	54 (20 – 88)
Sex	
Female	8
Male	10
Race	
White	14
Black	2
Other	2
APACHE II	23 (7 – 41)
Final diagnosis	
Septic shock	14
SIRS (noninfectious)	2
Acute renal failure	1
Liver failure	1
Mortality	
Alive	12
Dead	6
Withdrawal of care	2

APACHE II—Acute Physiology and Chronic Health Evaluation II; a severity of disease classification system; sepsis—SIRS caused by severe infection; SIRS—systemic inflammatory response syndrome.