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Suppression of HMGB1 release by stearoyl lysophosphatidylcholine: an additional mechanism for its therapeutic effects in experimental sepsis

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Abstract Stearoyl lysophosphatidylcholine (LPC) has recently been proven protective against lethal sepsis by stimulating neutrophils to eliminate invading pathogens through an H_2O_2-dependent mechanism. Here, we demonstrate that stearoyl LPC, but not caproyl LPC, significantly attenuates circulating high-mobility group box 1 (HMGB1) levels in endotoxemia and sepsis by suppressing endotoxin-induced HMGB1 release from macrophages/monocytes. Neutralizing antibodies against G2A, a potential cell surface receptor for LPC, partially abrogated stearoyl LPC-mediated suppression of HMGB1 release. Thus, stearoyl LPC confers protection against lethal experimental sepsis partly by facilitating the elimination of the invading pathogens and partly by inhibiting endotoxin-induced release of a late proinflammatory cytokine, HMGB1.—Chen, G., J. Li, X. Qiang, C. J. Czura, M. Ochani, K. Ochani, L. Ulloa, H. Yang, K. J. Tracey, P. Wang, A. E. Sama, and H. Wang. **Suppression of HMGB1 release by stearoyl lysophosphatidylcholine: an additional mechanism for its therapeutic effects in experimental sepsis. *J. Lipid Res.* 2005.** 46: **623–627.**

Supplementary key words high-mobility group box 1 • endotoxemia • tumor necrosis factor • bacterial endotoxin

"Severe sepsis" refers to an overwhelming systemic inflammatory response to infection and is defined by signs of organ dysfunction that include abnormalities in body temperature, heart rate, respiratory rate, and leukocyte counts. It is the most common cause of death in the intensive care unit, claiming ~225,000 victims annually in the United States alone. Although severe sepsis is generally accompanied by the inability to regulate the inflammatory response, an understanding of its basis is still unclear (1, 2).

Innate immune cells (e.g., macrophages, monocytes, and neutrophils) constitute the first line of defense against infection by ingesting and killing invading pathogens via various granular enzymes and reactive oxygen species (e.g., H_2O_2). If the invading pathogens are efficiently eliminated, the inflammatory response resolves normally to restore immunologic homeostasis (3). Inefficient pathogen clearance can lead to a rigorous, dysregulated, systemic inflammatory response manifested by the production of various proinflammatory cytokines [e.g., tumor necrosis factor (TNF), interleukin-1 (IL-1), and macrophage migration inhibitory factor (MIF)] (2–4).

We and others have recently demonstrated that a ubiquitous nucleosomal protein, high-mobility group box 1 (HMGB1), is released actively by macrophages/monocytes (5–8) and passively by necrotic cells (9) and that it subsequently prompts an inflammatory response (10–12). Suppression of HMGB1 activity or systemic accumulation confers protection against lethal endotoxemia (5) and sepsis (13–15), even when the first dose of an anti-HMGB1 agent is administered 24 h after the onset of disease. Together, these observations establish HMGB1 as an important "late" mediator with a wider therapeutic window in animal models of lethal sepsis and other diseases (3, 11, 12, 16, 17).

Recently, Yan et al. (18) demonstrated that stearoyl (but not caproyl or lauroyl) lysophosphatidylcholine (LPC) is protective in animal models of lethal sepsis, even when the first dose is given at 10 h after the onset of sepsis. They proposed that stearoyl LPC confers protection by stimulating neutrophils (but not macrophages) to destroy ingested bacteria in an H_2O_2-dependent mechanism (18), supporting the concept that failure to eliminate invading

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Abbreviations: HMGB1, high-mobility group box 1; LPC, lysophosphatidylcholine; LPS, lipopolysaccharide.

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pathogens contributes to the pathogenesis of severe sepsis (1). However, stearoyl LPC also confers protection against lethal endotoxemia (18), implying that it may exert protective effects through an additional, bactericidal-independent mechanism (3).

The aim of this study, therefore, was to identify additional, bactericidal-independent mechanisms by which stearoyl LPC protects against lethal sepsis. Here, we demonstrate that it also significantly attenuates circulating HMGB1 levels in endotoxemia and sepsis by suppressing endotoxin-induced HMGB1 release from macrophages/monocytes.

MATERIALS AND METHODS

Cell culture

Primary peritoneal macrophages were isolated from Balb/C mice (male, 7–8 weeks, 20–25 g) at 3 days after intraperitoneal injection of 2 ml of thioglycollate broth (4%) as described previously (6, 7). After several extensive washings, thioglycollate-elicited macrophages were resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum and immediately transferred onto six-well tissue culture plates (4×10^6 cells/2 ml/well). After preculture for 12 h, the adherent cells were gently washed with, and cultured in, serum-free OPTI-MEM I medium 2 h before lipopolysaccharide (LPS) stimulation.

Human peripheral blood mononuclear cells were isolated by density gradient centrifugation through Ficoll (Ficoll-Paque PLUS; Pharmacia, Piscataway, NJ) as described previously (6, 19) and cultured in RPMI 1640 medium, 10% heat-inactivated human serum, and 2 mM L-glutamine overnight. Nonadherent cells were subsequently removed, and adherent monocyte-enriched cultures were stimulated with LPS.

LPS stimulation

Macrophage/monocyte cell cultures were stimulated with LPS (200 ng/ml; Sigma-Aldrich, St. Louis, MO) in the absence or presence of various LPCs. For in vitro experiments, LPCs were dissolved in sterile water (5 mg/ml) and sonicated for 10 min immediately before use. At 16 h after LPS stimulation, HMGB1 levels in the culture medium were determined as described previously (5–7).

Animal models of endotoxemia and sepsis

Balb/C mice (male, 7–8 weeks, n = 10 mice/group) were subjected to endotoxemia [by intraperitoneal injection of LPS and *Escherichia coli* 0111:B4 (Sigma-Aldrich) at a median lethal dose of 10 mg/kg] or sepsis (by cecal ligation and puncture) as described previously (5, 13, 14). Various LPC species [caproyl (6:0), catalog no. L-3010; lauroyl (12:0), catalog no. L-5629; and stearoyl (18:0), catalog no. 2131; Sigma-Aldrich] were dissolved in saline (1× PBS, pH 7.4, 5 mg/ml) and sonicated for 10 min immediately before intraperitoneal administration (20 mg/kg) into mice at 0.5 and 12 h after the onset of endotoxemia and at 3, 15, and 27 h after the onset of sepsis. Serum HMGB1 levels were determined at 24 and 30 h after endotoxemia and sepsis, respectively (5, 14).

HMGB1 Western blot analysis

The levels of HMGB1 in the culture medium were assayed by Western blot analysis using rabbit polyclonal antibodies as described previously (5, 6). Western blots were scanned with a silver image scanner (Silverscaner II; Lacie Limited, Beaverton, OR), and the relative band intensity was quantified using NIH Image 1.59 software. The levels of HMGB1 were calculated with reference to standard curves generated with purified recombinant HMGB1 and expressed as means ± SEM of two experiments (n = 20).

TNF ELISA

The levels of TNF in the serum or culture medium were determined using a commercially available ELISA kit (catalog no. MTA00; R&D Systems, Minneapolis, MN) as described previously (6, 7). The levels of TNF were calculated with reference to standard curves of purified recombinant TNF at various dilutions.

HMGB1 immunostaining

Cellular HMGB1 was immunostained with antigen affinity-purified anti-HMGB1 polyclonal antibodies as described previously (6, 7). Briefly, macrophage cultures were fixed with phosphate-buffered formaldehyde (4%, pH 7.4, 15 min) and permeabilized with Triton X-100 (0.3%, pH 7.4, 10 min). After blocking the slides with 10% BSA (37°C, 1 h), cells were sequentially incubated with antigen affinity-purified anti-HMGB1 antibodies and FITC-conjugated anti-rabbit IgG (catalog no. F9887; Sigma-Aldrich), and images were acquired using a confocal microscope (Fluoroview; Olympus, Melville, NY).

Statistical analysis

Student's two-tailed *t*-test was used to compare means between groups. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Stearoyl LPC attenuated systemic accumulation of HMGB1 in vivo

Stearoyl LPC has recently been proven protective against lethal experimental sepsis by stimulating neutrophils (but

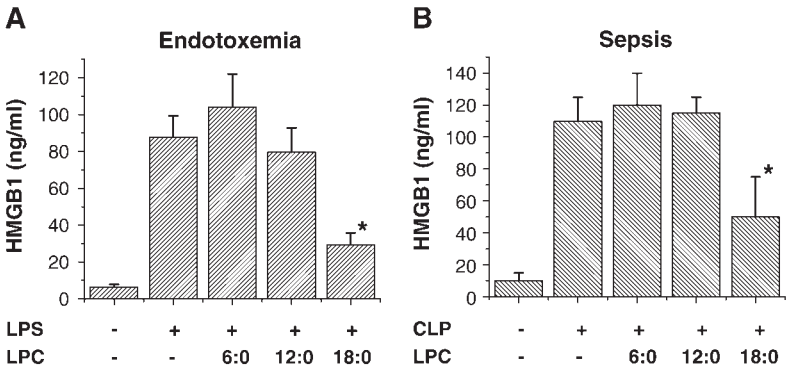

Fig. 1. Stearoyl lysophosphatidylcholine (LPC) attenuated systemic accumulation of high-mobility group box 1 (HMGB1) in endotoxemia and sepsis. Balb/C mice (male, 7–8 weeks, n = 10 mice/group) were subjected to endotoxemia or sepsis as described previously (5, 13, 14), and various LPC species [caproyl (6:0), lauroyl (12:0), and stearoyl (18:0) LPC] were intraperitoneally administered (20 mg/kg). Serum HMGB1 levels were determined at 24 and 30 h after endotoxemia and sepsis, respectively, and expressed as means ± SEM of two experiments (n = 20). Student's two-tailed *t*-test was used to compare the means between groups. * $P < 0.05$ versus controls [+LPS (lipopolysaccharide) or +CLP (cecal ligation and puncture) alone].

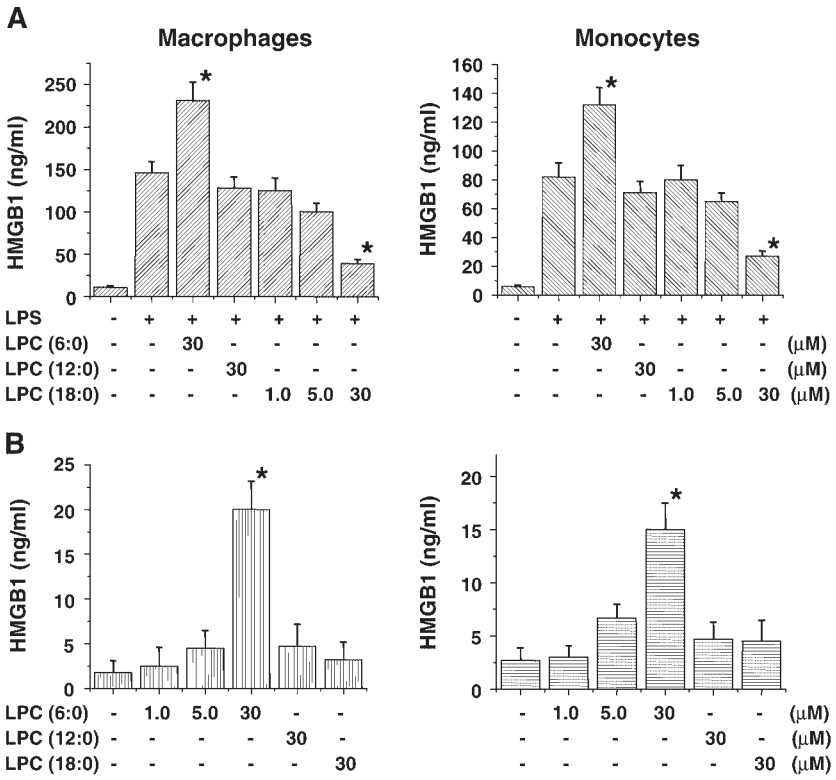

Fig. 2. Stearoyl LPC suppressed endotoxin-induced HMGB1 release in vitro. Thioglycollate-elicited peritoneal macrophages or peripheral blood mononuclear cells (monocytes) were stimulated with various LPCs at the indicated doses in the absence or presence of LPS (200 ng/ml). At 16 h after stimulation, HMGB1 levels in the culture medium were determined and expressed as means ± SEM of two independent experiments in triplicate (n = 6). * $P < 0.05$ versus controls (+LPS alone or −LPS).

not macrophages) to destroy ingested bacteria in an H_2O_2-dependent mechanism (18). However, stearoyl LPC also confers protection against lethal endotoxemia (18), implying that it may exert protective effects through an additional, bactericidal-independent mechanism (3). To gain further insight into the protective mechanisms of LPC action in systemic inflammatory diseases, we evaluated the effects of various LPC species on the systemic accumulation of HMGB1, a newly identified late mediator of endotoxemia and sepsis (3, 5, 13, 14). Repeated administration of stearoyl (but not caproyl or lauroyl) LPC significantly attenuated circulating HMGB1 levels in animal models of endotoxemia and sepsis (**Fig. 1**).

The effects of stearoyl LPC on the systemic accumulation of other proinflammatory cytokines in septic animals have been exhaustively investigated by Yan et al. (18). At an early stage of sepsis (4–8 h after onset), stearoyl LPC induced a modest and transient decrease in the levels of TNF and IL-1β and a contrasting increase in the levels of IL-2 and IFN-γ (18). Because of the modest and transient nature of these effects, these LPC-induced changes may not significantly contribute to stearoyl LPC-mediated protection against lethal sepsis (18). In agreement with the earlier report (18), we found that administration of stearoyl LPC did not significantly attenuate circulating TNF levels at a late stage of sepsis (24 h after onset) (TNF = 115 ± 25 pg/ml, control group receiving vehicle, n = 10 mice/group; versus TNF = 120 ± 32 pg/ml, experimental group receiving two doses of stearoyl LPC, 20 mg/kg, n = 10 mice/group). In light of the notion that immunosuppressive agents (e.g., ethyl pyruvate and nicotine) capable of inhibiting HMGB1 release rescue animals from lethal sepsis even when administered at 24 h after the onset of sepsis (14, 15), we propose that stearoyl LPC may confer

TABLE 1. Effect of different LPCs on endotoxin-induced TNF release

Cells	Control	LPS	LPS + 6:0 LPC (30 μM)	LPS + 12:0 LPC (30 μM)	LPS +18:0 LPC (30 μM)
Macrophage	0.3 ± 0.1	33.3 ± 5.6	41.7 ± 8.0	34.3 ± 4.6	30.1 ± 6.6
Monocyte	0.2 ± 0.1	12.3 ± 2.5	15.0 ± 3.1	11.3 ± 2.1	9.2 ± 1.9

LPC, lysophosphatidylcholine; LPS, lipopolysaccharide; TNF, tumor necrosis factor. Levels of TNF (ng/ml) in the culture medium are expressed as means ± SEM of two independent experiments in triplicate (n = 6).

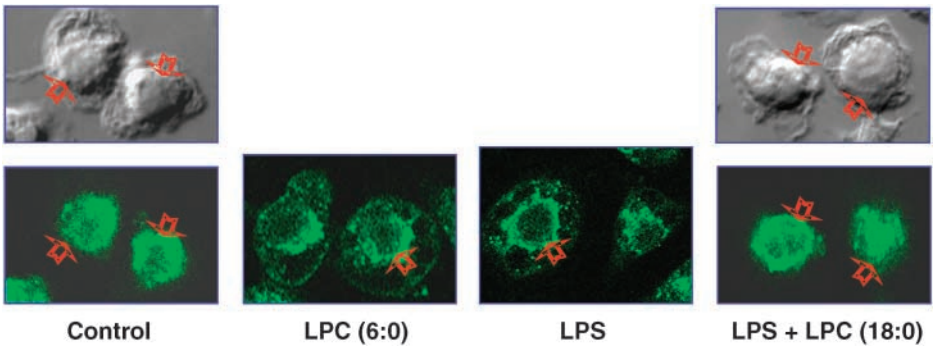

Fig. 3. Stearoyl LPC suppressed endotoxin-induced HMGB1 translocation. Human monocytes were stimulated for 16 h with various LPCs (30 μM) alone or in the presence of LPS (200 ng/ml) and immunostained with HMGB1-specific antibodies as described previously (6). Upper panels show light-translucent microscopic photographs of cells in the corresponding lower panels. Arrows indicate the nuclear regions of representative cells.

protection against lethal sepsis partly through attenuating excess accumulation of a late proinflammatory cytokine, HMGB1.

Stearoyl LPC suppressed endotoxin-induced HMGB1 release in vitro

Two distinct HMGB1 release pathways contribute to increases of its circulating levels: active release by endotoxin-stimulated macrophages/monocytes (5–7) and passive release by necrotic cells (9). To determine how LPC decreases circulating HMGB1 levels in endotoxemia and sepsis, we examined their effects on endotoxin-induced HMGB1 release. Stearoyl (but not caproyl or lauroyl) LPC, at relevant concentrations (1, 5, and 30 μM), dose-dependently decreased LPS-induced HMGB1 release from macrophages and monocytes (**Fig. 2A**), suggesting that stearoyl LPC attenuates circulating HMGB1 levels partly by inhibiting its active release from macrophages/monocytes. Notably, stearoyl LPC did not significantly suppress LPS-induced release of other proinflammatory cytokines (e.g., TNF-α) (**Table 1**), eliminating the possibility that stearoyl LPC exerts its suppressive effects by interfering with LPS activity through direct physical interaction.

Caproyl LPC enhanced endotoxin-induced HMGB1 release in vitro

In contrast, caproyl LPC dose-dependently and significantly enhanced endotoxin-induced HMGB1 release (Fig. 2A). In the absence of endotoxin, caproyl (but not lauroyl or stearoyl) LPC stimulated HMGB1 release in a dose-dependent manner (Fig. 2B). This induction of HMGB1 release was not attributable to cell death, because cell viability, as assessed by trypan blue exclusion, was not reduced by caproyl LPC (93 ± 4%, versus 95 ± 2% for control). Similarly, caproyl LPC induced other proinflammatory cytokines (such as TNF; data not shown) and triggered cytoplasmic translocation of nuclear HMGB1 in 40–50% monocytes (**Fig. 3**).

The cytokine-inducing activities of caproyl LPC are consistent with the predominant proinflammatory properties of most endogenous LPC molecules (20). For instance, Gardella et al. (20) demonstrated that endotoxin-induced

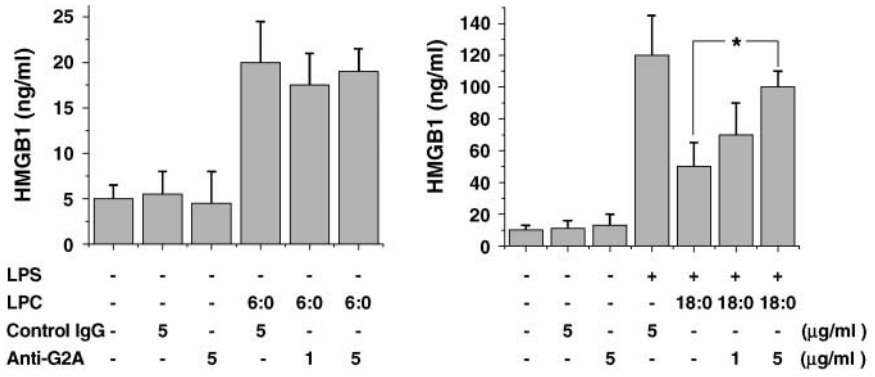

Fig. 4. Effect of anti-G2A antibodies on HMGB1 release in vitro. Macrophages were stimulated with caproyl (6:0) or stearoyl (18:0) LPC (30 μM) alone or in the presence of LPS (200 ng/ml), irrelevant goat immunoglobulin (Control IgG), or G2A-neutralizing antibodies (Anti-G2A; catalog no. sc-9692; Santa Cruz Biotechnology). At 16 h after stimulation, HMGB1 levels in the culture medium were determined and expressed as means ± SEM of two independent experiments in triplicate (n = 6). * $P < 0.05$.

HMGB1 release is dependent on the activation of phospholipase A_2 and the accompanying production of LPC. Consistent with these observations, we found that a mixture of various endogenous LPC species (generated from bovine brain; catalog no. L-1381; Sigma-Aldrich) induced a dose-dependent HMGB1 release in macrophage and monocyte cultures (data not shown).

Stearoyl LPC suppressed HMGB1 release by interfering with its cytoplasmic translocation

Despite the proinflammatory properties of many endogenous LPC species, purified stearoyl LPC attenuates increases in systemic HMGB1 levels and protects against lethal endotoxemia and sepsis (18). To investigate the mechanisms of stearoyl LPC-mediated suppression of HMGB1 release, we determined its effect on endotoxin-induced HMGB1 translocation, an essential step for HMGB1 release (6, 20). Consistent with previous reports (6, 20), quiescent human monocytes were shown to maintain an intracellular pool of HMGB1 in the nucleus. After endotoxin stimulation, nuclear HMGB1 was translocated to the cytoplasm in 85–95% of the cells (Fig. 3). Pretreatment with stearoyl LPC (30 μM) abrogated HMGB1 translocation in 55–65% of the endotoxin-stimulated cells, indicating that stearoyl LPC inhibits HMGB1 release by interfering with its cytoplasmic translocation.

Stearoyl LPC enhances neutrophil bactericidal activities through G2A (18), a potential cell surface receptor for LPC species with longer fatty acid acyl chains (e.g., stearoyl LPC). G2A-specific neutralizing antibodies did not significantly affect the caproyl LPC-induced HMGB1 release (**Fig. 4**). However, these anti-G2A antibodies significantly, in a dose-dependent manner, attenuated stearoyl LPC-mediated suppression of HMGB1 release (Fig. 4). Thus, it will be interesting to determine if stearoyl LPC inhibits endotoxin-induced HMGB1 release via G2A in future studies.

In conclusion, the capacity of various LPCs to attenuate endotoxin-induced HMGB1 release correlates with their different efficacy in protecting against lethal endotoxemia and sepsis (3, 18). The parallel capacity of stearoyl LPC to simultaneously enhance neutrophil bactericidal activities (18) and attenuate systemic HMGB1 accumulation strengthens the notion that the pathogenesis of sepsis is attributable to both invading pathogens and excessive accumulation of late proinflammatory cytokines. Thus, the potential therapeutic stearoyl LPC confers protection against lethal sepsis partly by facilitating the elimination of the invading pathogens and partly by attenuating the excess accumulation of late proinflammatory cytokines (e.g., HMGB1) in lethal sepsis. It is thus important to use LPC as a unique molecular tool to define the underlying causes of lethal sepsis and other inflammatory diseases.

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