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Carbenoxolone Blocks Endotoxin-Induced Protein Kinase R (PKR) Activation and High Mobility Group Box 1 (HMGB1) Release

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The pathogen- and damage-associated molecular patterns (for example, bacterial endotoxin and adenosine 5′-triphosphate (ATP)) activate the double-stranded RNA-activated protein kinase R (PKR) to trigger the inflammasome-dependent high mobility group box 1 (HMGB1) release. Extracellular ATP contributes to the inflammasome activation through binding to the plasma membrane purinergic $P2X_7$ receptor ($P2X_7R$), triggering the opening of $P2X_7R$ channels and the pannexin-1 (panx-1) hemichannels permeable for larger molecules up to 900 daltons. It was previously unknown whether panx-1 channel blockers can abrogate lipopolysaccharide (LPS)-induced PKR activation and HMGB1 release in innate immune cells. Here we demonstrated that a major gancao (licorice) component (glycyrrhizin, or glycyrrhizic acid) derivative, carbenoxolone (CBX), dose dependently abrogated LPS-induced HMGB1 release in macrophage cultures with an estimated $IC_{50} \approx 5$ μmol/L. In an animal model of polymicrobial sepsis (induced by cecal ligation and puncture (CLP)), repetitive CBX administration beginning 24 h *after* CLP led to a significant reduction of circulating and peritoneal HMGB1 levels, and promoted a significant increase in animal survival rates. As did $P2X_7R$ antagonists (for example, oxidized ATP, oATP), CBX also effectively attenuated LPS-induced $P2X_7R$/panx-1 channel activation (as judged by Lucifer Yellow dye uptake) and PKR phosphorylation in primary peritoneal macrophages. Collectively, these results suggested that CBX blocks LPS-induced HMGB1 release possibly through impairing PKR activation, supporting the involvement of PKR in the regulation of HMGB1 release.

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INTRODUCTION

Sepsis is an overwhelming systemic inflammatory response to severe infections, and remains the primary cause of mortality in medical intensive care units. It afflicts approximately 750,000 Americans each year, and costs the United States healthcare system nearly $17 billion annually (1). Current treatments are predominantly supportive and often ineffective, necessitating the continued search for effective therapies. The pathogenesis of sepsis is partly mediated by pathogen- and damage-associated molecular patterns (for example, endotoxins, adenosine 5′-triphosphate [ATP] or high mobility group box 1 [HMGB1]) (2), which activate innate immune cells via binding to various pattern-recognition receptors (for example, toll-like receptor 4 [TLR4], $P2X_7R$, CD24, or Siglec-10) (3–5). For instance, crude endotoxins (containing trace amounts of bacterial proteins and nucleic acids) can stimulate macrophages to sequentially release early (for example, tumor necrosis factor [TNF], interleukin [IL]-1β and interferon [IFN]-γ) and late (for example, HMGB1) proinflammatory mediators (6,7). In animal models of endotoxemia or sepsis, circulating HMGB1 levels plateau between 24 and 36 hours (6,8), distinguishing HMGB1 from other early cytokines (9). Moreover, HMGB1-neutralizing antibodies confer protection against lethal endotoxemia (6) and sepsis (8,10) even when given 24 hours after the onset of sepsis, establishing HMGB1 as a late mediator of lethal inflammatory diseases (11).

The mechanisms underlying the regulation of endotoxin-induced HMGB1 release remain poorly elucidated. Emerging evidence has suggested an essential role for the inflammasome in the regulation of LPS/ATP-induced HMGB1 release (7,12), because genetic disruption of key inflammasome components (for example, caspase 1 or Nalp3) impaired the LPS/ATP-induced HMGB1 release. Recently, we discovered that the double-stranded RNA-activated protein kinase R (PKR) functions as the key regulator of

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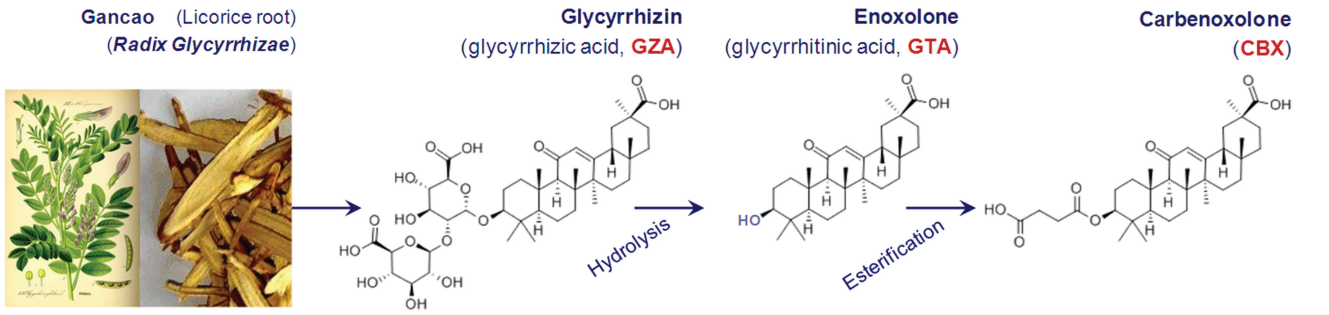

Figure 1. Chemical structures of a major gancao component and derivatives. The major gancao component GZA can be metabolized into glycyrrhitinic acid (GTA) via the glycaronidase-mediated hydrolysis *in vivo*. GTA (also called enoxolone) can be chemically derivatized by esterification into a succinate ester termed "carbenoxolone."

inflammasome activation and HMGB1 release (7). It has been suggested that LPS activates the inflammasome signaling pathways partly through eliciting the passive ATP leakage (13). Indeed, ultrapure LPS (free from contaminating bacterial proteins and nucleic acids) fails to trigger HMGB1 release unless the initial LPS (10 μg/mL) priming is accompanied by a second stimulus (for example, ATP) (7,12). Similarly, ATP itself is unable to induce HMGB1 release without prior LPS exposure (12), although it can induce PKR phosphorylation (7) and inflammasome activation (14–16).

It has been suggested that ATP activates the inflammasome through binding to the purinergic $P2X_7$ receptor ($P2X_7R$) (5), which triggers an immediate (within milliseconds) opening of the ATP-gated $P2X_7R$ channel permeable for small cationic ions. Subsequently, the pannexin-1 (panx-1) hemichannels are recruited and activated, allowing passage of larger anionic molecules up to 900 Da (for example, ATP) (17–19). This feed-forwarding ATP-mediated ATP release contributes to the LPS-stimulated inflammasome activation (20) and subsequent inflammasome-dependent cytokine release (for example, IL-1β and IL-18) (14–16,21,22). It is not known whether agents capable of inhibiting the panx-1 hemichannel can inhibit LPS-induced PKR activation and HMGB1 release, thereby conferring protection against lethal sepsis.

Gancao (*Radix Glycyrrhizae*; licorice) has been used traditionally in the clinical management of various inflammatory ailments including peptic ulcer, hepatitis and pulmonary bronchitis for many centuries. Its antiinflammatory properties are attributable to a major component, glycyrrhizin (glycyrrhizic acid, GZA) (Figure 1), which has been proven beneficial in animal models of influenza (23), hepatitis (24), endotoxemia (25), lung inflammation (26) and colitis (27). The replacement of the glucuronic acid in GZA by succinic acid gives rise to a new compound, carbenoxolone (CBX), a licensed drug prescribed in the UK for esophageal ulceration and inflammation (28). Unlike GZA, CBX (10 μmol/L) can effectively inhibit the panx-1 hemichannel–mediated ATP release in response to hypoxia (29), sheer stress (30) and low oxygen tension (31). Furthermore, CBX can inhibit LPS-induced dye uptake (17,32), and confer protection against LPS-induced acute lung injury (33) or cerebral ischemic injury (34). It was previously unknown whether CBX can inhibit endotoxin-induced HMGB1 release and protects animals against lethal sepsis. In this study, we demonstrated that CBX remarkably inhibited endotoxin-induced HMGB1 release possibly through blocking $P2X_7R$-gated panx-1 channels, and rescued mice from lethal sepsis. In addition, the blockade of panx-1 channels by CBX and $P2X_7R$ antagonists correlated with an inhibition of LPS-induced PKR activation, suggesting a critical role of $P2X_7R$-gated panx-1 channels in the regulation of PKR-mediated inflammasome activation and inflammatory responses.

MATERIAL AND METHODS

Materials

Bacterial endotoxin (lipopolysaccharide, LPS, *E. coli 0111:B4*), CBX (C4790), KN-62 (I2142), adenosine 5′-triphosphate-2′,3′-dialdehyde (oxidized ATP, oATP, A6779), brilliant blue G (BBG, B0770), Lucifer Yellow (L0144), paraformaldehyde (P6148) and mouse anti-β-actin antibodies (A1978) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Calcein-AM (C3099), CM-DiI (C7001), Dulbecco's modified Eagle medium (DMEM, 11995-065), penicillin/streptomycin (cat. 15140-122), fetal bovine serum (FBS, 26140079) and trypan blue (15250-061) were from Invitrogen/Life Technologies (Carlsbad, CA, USA). Anti-LC3 antibody (sc-16755), anti-PKR antibody (sc-6282), and HRP conjugated goat anti-mouse IgG (sc-2060) were from Santa Cruz Biotechnology Inc., Dallas, TX, USA. Anti-phosphorylated PKR antibody (07-886) was from Millipore (Billerica, MA, USA). HRP conjugated donkey anti-rabbit IgG was from GE Healthcare (NA934; Port Washington, NY, USA).

Cell Culture

Murine macrophagelike RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Primary peritoneal macrophages were isolated from BALB/c mice (Taconic, Germantown, NY, USA;

male, 7–8 wks, 20–25 g) at 2–3 d after intraperitoneal injection of 2 mL thioglycollate broth (4%) as described previously (35,36). Both RAW 264.7 cells and primary macrophages were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% FBS. Adherent macrophages were gently washed with, and cultured in, DMEM before stimulating with LPS (0.5 μg/mL) in the absence or presence of CBX or purinergic $P2X_7R$ antagonists (oATP, 50 μmol/L; BBG, 0.5 μmol/L; KN62, 0.5 μmol/L) for 16 h. Subsequently, the cell-conditioned culture media were analyzed for levels of HMGB1, nitric oxide (NO) and other cytokines by Western blotting analysis, the Griess reaction, ELISA, and cytokine antibodies arrays as described previously (36–38).

Cell Viability Assay

Cell viability was assessed by the trypan blue exclusion assay as described previously (39,40). Briefly, trypan blue was added to cell cultures at a final concentration of 0.08%. After incubation for 5 min at room temperature, the cell viability was assessed by the percentage of dye-excluding cells in five 40× microscope fields.

Western Blotting

The levels of HMGB1 in the culture medium, serum or peritoneal lavage fluid were determined by Western blotting analysis as described previously (6,41,42). The levels of total or phosphorylated PKR in primary macrophage cell lysates were determined by Western blotting analysis with reference to β-actin. Briefly, equal amounts of cellular proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% nonfat milk, the membrane was incubated with respective antibodies (anti-PKR, 1:2000; anti-phospho-PKR, 1:1000; anti-β-actin. 1:5000) overnight. Subsequently, the membrane was incubated with the appropriate secondary antibody, and the immunoreactive bands were visualized by chemiluminescence technique.

TNF ELISA

The levels of TNF in the culture medium or serum were determined using commercial enzyme linked immunosorbent assay (ELISA) kits (MTA00, R&D Systems, Minneapolis, MN, USA) with reference to standard curves of purified recombinant TNF at various dilutions as described previously (37,38,42–44).

Nitric Oxide Assay

The levels of nitric oxide in the culture medium were determined indirectly by measuring the NO^{2-} production with a colorimetric assay based on the Griess reaction (35,40). NO^{2-} concentrations were determined with reference to a standard curve generated with sodium nitrite at various dilutions.

Cytokine Antibody Array

Murine cytokine antibody arrays (M0308003, RayBiotech Inc., Norcross, GA, USA), which detect 62 cytokines on one membrane, were used to determine serum cytokine levels as described previously (35,40). Briefly, the membranes were sequentially incubated with equal volumes of murine serum (after 1:10 dilution), primary biotin-conjugated antibodies, and horseradish peroxidase–conjugated streptavidin. After exposing to X-ray film, the relative signal intensity was determined using the Scion Image software.

Lucifer Yellow Dye Uptake Assay

The Lucifer Yellow dye uptake was used to measure the $P2X_7R$-gated channel activities as described previously (17,45,46). Briefly, RAW 264.7 cells were stimulated with LPS in the absence or presence of CBX or other $P2X_7R$ receptor antagonists (KN-62, BBG, or oATP) for 16 h. Subsequently, cell cultures were incubated with Lucifer Yellow (LY, 1 mg/mL) for 15 min, and fixed with 2% paraformaldehyde following three extensive washes with 1 × PBS. The number of cells with diffused fluorescent signals was counted under a fluorescence microscope. The cells containing punctuate fluorescent signals were excluded, as the punctuate signals likely resulted from phagocytosis (rather than passive diffusion through panx-1 hemichannels) of the LY dye.

Animal Model of Polymicrobial Sepsis

This study was approved and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research, Manhasset, New York, USA. To evaluate the therapeutic potential of CBX, a clinically relevant animal model of sepsis induced by cecal ligation and puncture (CLP) was employed (37,38,47). Briefly, the cecum of BALB/c mice was ligated at 5.0 mm from the cecal tip, and then punctured once with a 22-gauge needle. CBX was administered intraperitoneally into mice at indicated doses and time points, and animal survival rates were monitored for up to 2 wks. In parallel experiments, mice were euthanized to collect blood or peritoneal lavage fluid at 50 h (2 h after the second dose of CBX) after CLP, and assayed for serum levels of TNF, HMGB1 and other cytokines, as described previously (48).

Statistical Analysis

Data are expressed as mean (SEM of two independent experiments in triplicates (n = 2). One-way analyses of variance (ANOVA) followed by the Tukey test for multiple comparisons were used to compare between different groups. The Kaplan-Meier method was used to compare the differences in mortality rates between groups. A P value <0.05 was considered statistically significant.

RESULTS

CBX Dose Dependently Attenuated Endotoxin-Induced HMGB1 Release

To elucidate the mechanisms underlying the endotoxin-mediated HMGB1 release, we evaluated the antiinflammatory properties of a major gancao component (GZA) and its derivative (CBX) *in vitro*. Consistent with previous reports (25,49), GZA dose dependently inhibited endotoxin-induced release of nitric oxide and TNF in murine macrophagelike RAW

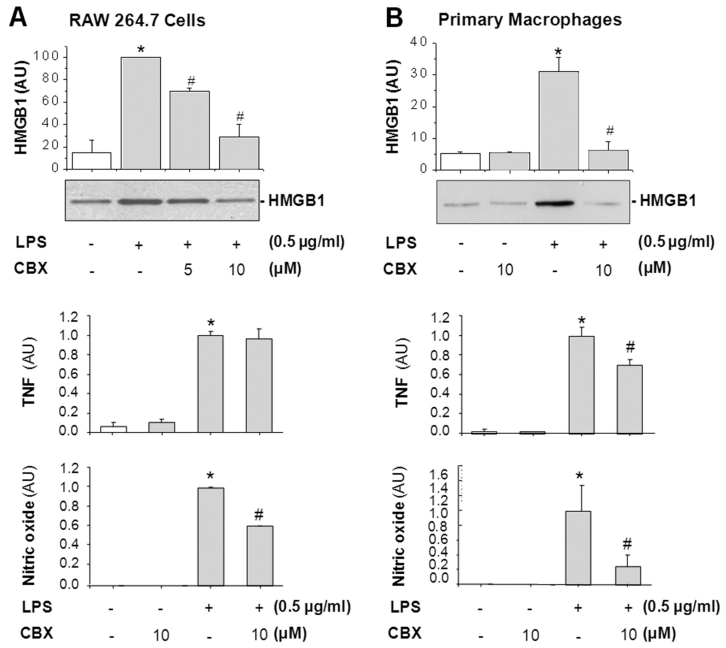

Figure 2. CBX effectively abrogated LPS-induced HMGB1 release in macrophage cultures. Murine macrophagelike RAW 264.7 cells (panel A) or primary peritoneal macrophages (panel B) were stimulated with LPS in the absence or presence of CBX at indicated concentrations. At 16 h after stimulation, the levels of HMGB1, TNF and nitric oxide in the culture medium were respectively determined by Western blotting, ELISA and Griess reactions, and expressed in arbitrary units (AU). *$P < 0.05$ versus negative control (– LPS); #$P < 0.05$ versus positive control (+ LPS alone).

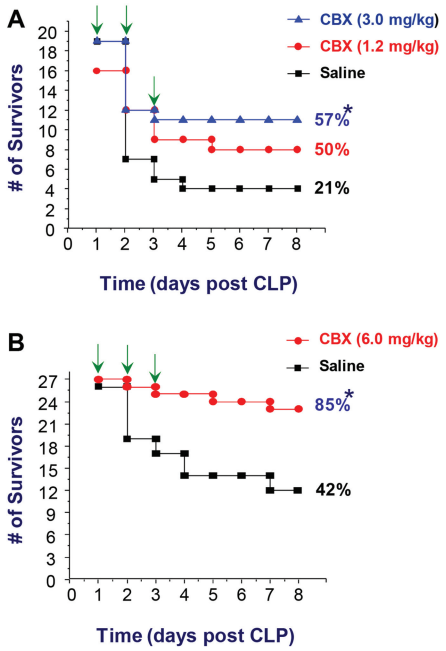

Figure 3. Delayed administration of CBX rescued mice from lethal sepsis. BALB/c mice were subjected to lethal sepsis (induced by CLP), and intraperitoneally administered with saline (0.2 mL/mouse) or CBX at indicated doses at +24, +48 and +72 h after CLP. Animal survival rates were monitored for 2 wks, and the Kaplan-Meier method was used to compare the differences between groups. *$P < 0.05$ versus saline control group.

264.7 cells (data not shown). Similarly, CBX dose dependently inhibited LPS-induced HMGB1 release in both RAW 264.7 cells (Figure 2A) and primary peritoneal macrophage cultures (Figure 2B), with an estimated IC_{50} and IC_{100} approximately 5 μmol/L and 10 μmol/L, respectively. At the concentrations effective for abrogating HMGB1 release, CBX only partly (by ~40%) attenuated LPS-induced nitric oxide production without significantly affecting TNF secretion in RAW 264.7 cells (see Figure 2A). Similarly, CBX only partially inhibited LPS-induced TNF secretion and nitric oxide production in primary peritoneal macrophage cultures (see Figure 2B), suggesting CBX as an effective HMGB1 inhibitor in macrophage cultures.

The CBX-mediated inhibition of HMGB1 release was not dependent on its cytotoxic activities, as CBX did not reduce cell viability at concentrations up to 20 μmol/L (data not shown). At higher concentrations (50 μmol/L), however, CBX did exhibit noticeable cytotoxicity to RAW 264.7 cells, but not to primary peritoneal macrophages. These observations were consistent with previous findings that prolonged incubation with CBX resulted in cytotoxicity in other tumor cell lines (50). Accordingly, we chose to use nontoxic doses of CBX (10 μmol/L) for most experiments in the present studies unless otherwise noted.

CBX Rescued Mice from Lethal Polymicrobial Sepsis

In light of the pathogenic role of HMGB1 in lethal sepsis (8), we explored the therapeutic potential of CBX using a clinically relevant animal model of polymicrobial sepsis induced by CLP. The first dose of CBX was given 24 h after CLP, a time point at which mice developed clear signs of sepsis including lethargy, diarrhea and piloerection. Repeated administration of CBX beginning 24 h after the onset of sepsis (followed by additional doses at 48 and 72 h after CLP) conferred a dose-dependent and significant protection against lethal sepsis (Figures 3A, B), supporting a therapeutic potential for CBX in the treatment of sepsis.

CBX Selectively Attenuated Sepsis-Induced Local and Systemic HMGB1 Accumulation

To gain insight into its protective mechanism, we evaluated the effects of CBX on the systemic accumulation of various cytokines by cytokine antibody arrays. At late stages of experimental sepsis (for example, 50 h after CLP), most early cytokines (including IL-1β and TNF) were no longer detectable in the circulation of septic mice (Figure 4A). A few other cytokines (for example, BLC,

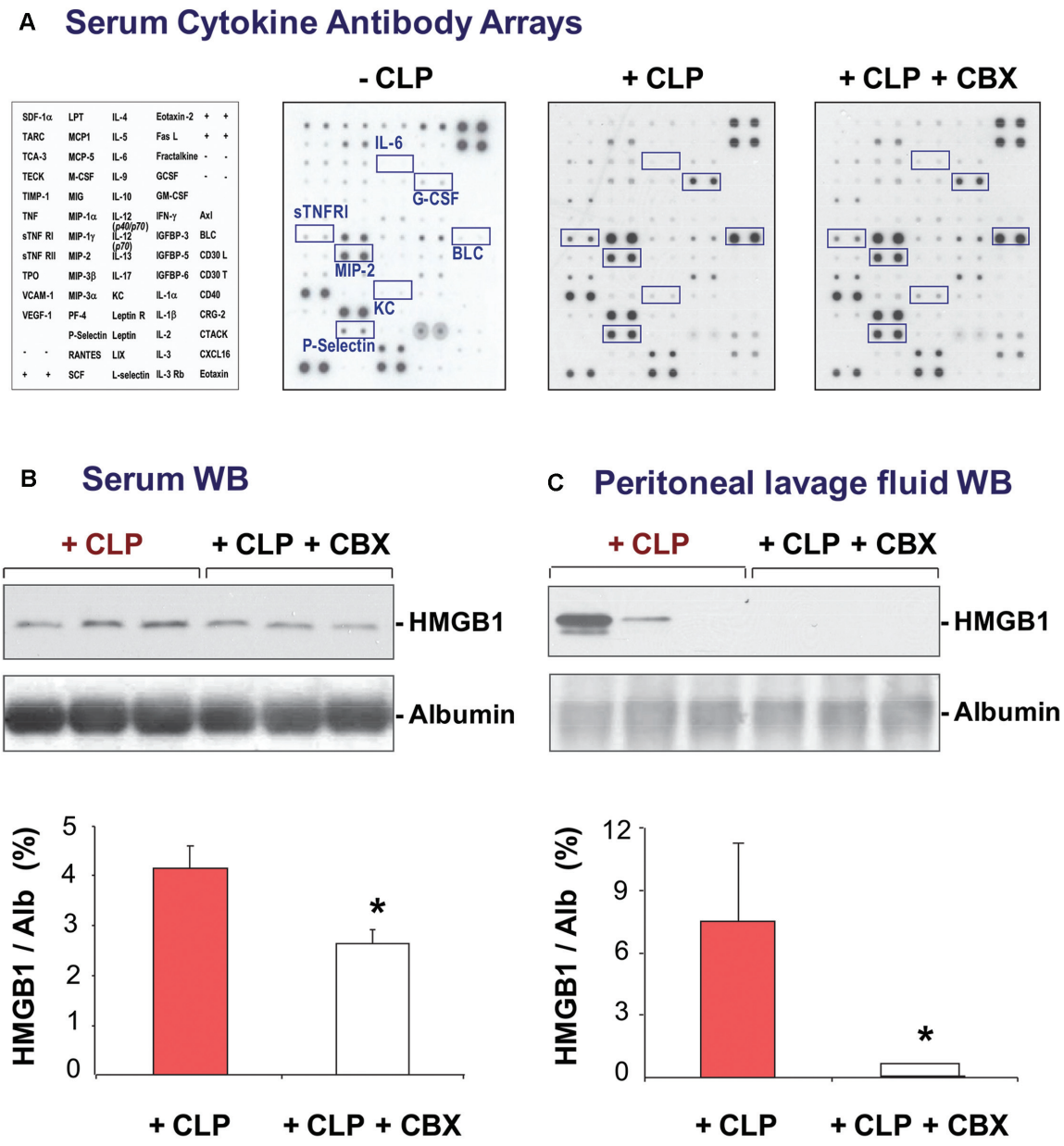

Figure 4. CBX attenuated sepsis-induced HMGB1 accumulation. BALB/c mice were subjected to sepsis by CLP, and administered with control saline (0.2 mL/mouse) or CBX (6.0 mg/kg) at 24 and 48 h CLP. At 50 h after CLP, the levels of HMGB1 and various cytokines in the serum (Panel A,B) and peritoneal lavage fluid (Panel C) were determined by cytokine antibody arrays (Panel A) and Western blotting analysis (Panel B, C), respectively. The relative HMGB1 levels were expressed as mean ± SD of two independent experiments (n = 2) in arbitrary units. *$P < 0.05$ versus saline group (+ CLP).

G-CSF and P-selectin) were still elevated at a late stage of sepsis, but their levels were not affected by CBX administration (see Figure 4A). In a sharp contrast, CBX significantly reduced HMGB1 levels not only systemically in the circulation (Figure 4B), but also locally in the peritoneal lavage fluid (Figure 4C), suggesting that CBX confers protection against lethal sepsis possibly by attenuating local and systemic HMGB1 accumulation.

CBX Effectively Inhibited $P2X_7R$-Gated Channel Activities

To elucidate the mechanisms underlying the CBX-mediated HMGB1 inhibition, we determined whether CBX affects ATP-gated channel activities in macrophage cultures. The ATP-gated channel activities were judged by the cellular uptake of an anionic dye, Lucifer Yellow (LY, MW = 444 Da), which spontaneously fluoresces even after being covalently linked to surrounding biomolecules by formaldehyde

fixation. In quiescent macrophages, approximately 2% cells displayed diffuse fluorescent signal after LY incubation (Figure 5A, left panels). Consistent with a previous report that LPS upregulated $P2X_7R$ expression (21), we found that prolonged LPS stimulation resulted in an elevation in the number of LY-positive cells (Figure 5A, middle panels), suggesting that LPS elevated $P2X_7R$-gated channel activities. However, CBX significantly impaired the LPS-induced elevation of LY uptake (Figure 5A, right panels), suggesting that CBX effectively inhibits LPS-induced HMGB1 release, possibly by blocking $P2X_7R$-gated channel activities.

To test this possibility, we determined whether specific $P2X_7R$ antagonists similarly inhibit LPS-induced HMGB1 release. As predicted, two selective $P2X_7R$ antagonists, oATP and BBG, significantly inhibited LPS-induced LY-uptake in macrophage cultures (Figure 5B). Consistently, both $P2X_7R$ antagonists effectively inhibited LPS-induced HMGB1 release (Figure 5C). Surprisingly, another selective $P2X_7R$ antagonist, KN62, was ineffective in suppressing LPS-induced LY uptake (see Figure 5B), and similarly failed to inhibit LPS-induced HMGB1 release (see Figure 5C). Taken together, these observations demonstrate a strong correlation between $P2X_7R$-gated channel activity and HMGB1 release in macrophages.

CBX Prevented LPS-Induced PKR Upregulation and Phosphorylation

In light of the roles of $P2X_7R$ and PKR in LPS/ATP-induced inflammasome activation (7,20), we tested the effects of CBX and $P2X_7R$ antagonists (for example, oATP) on LPS-induced PKR activation in primary macrophage cultures. Interestingly, prolonged stimulation with crude LPS (containing trace amounts of bacterial proteins and nucleic acids) resulted in a > two-fold increase in total cellular PKR protein levels (Figure 6A), but a more robust (> eight-fold) elevation in phospho-PKR levels (see Figure 6A). Furthermore, this LPS-induced elevation of PKR expression and phosphorylation was both significantly attenuated by CBX (see

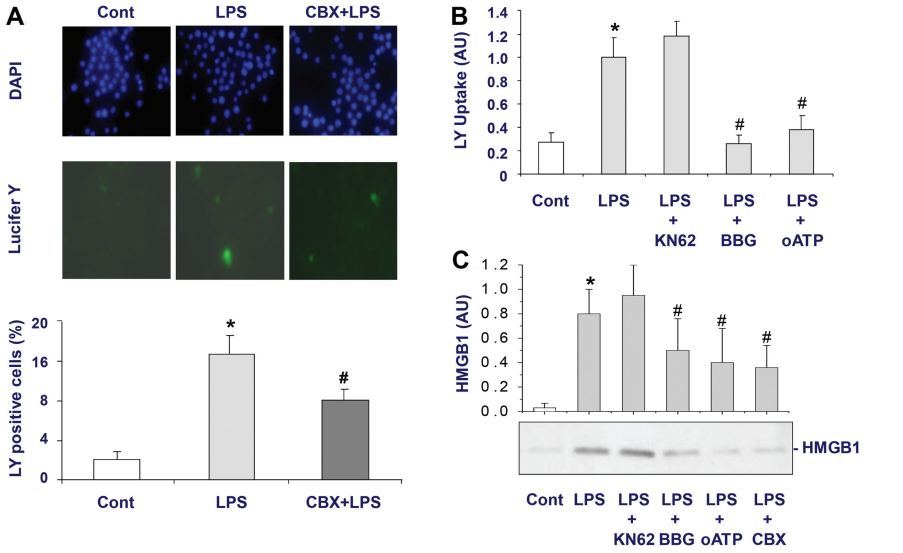

Figure 5. CBX and $P2X_7R$ antagonists inhibited LPS-induced dye uptake and HMGB1 release. A), CBX attenuated LPS-induced elevation of dye uptake. Murine macrophage cultures were stimulated with LPS for 16 h, and subsequently incubated with the LY dye for 15 min. The number of LY dye-positive cells were counted and expressed as a percentage of total number of cells in multiple fields. $^*P < 0.05$ versus negative control; $^{\#}P < 0.05$ versus positive control (+ LPS alone). B, C), $P2X_7R$ antagonists attenuated LPS-induced elevation of dye uptake and HMGB1 release. Macrophage cultures were stimulated with LPS (0.5 μg/mL) in the absence or presence of several $P2X_7R$ antagonists, KN62 (0.5 μmol/L), BBG (0.5 μmol/L), and oATP (50.0 μmol/L). At 16 h after LPS stimulation, the $P2X_7R$ activities were determined by the LY dye uptake assays, and the levels of HMGB1 in the culture medium were determined by Western blotting analysis. $^*P < 0.05$ versus negative control (– LPS); $^{\#}P < 0.05$ versus positive control (+ LPS alone).

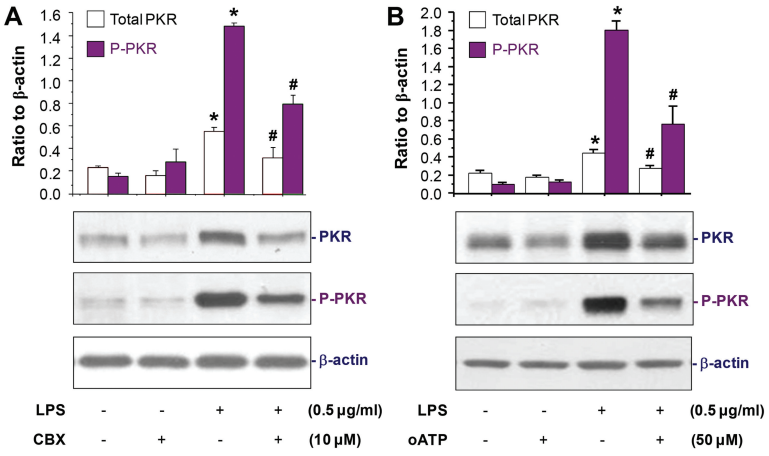

Figure 6. CBX attenuated LPS-induced PKR upregulation and phosphorylation in macrophage cultures. Primary peritoneal macrophages were stimulated with crude LPS in the absence or presence of CBX (panel A) or $P2X_7R$ antagonists (oATP, panel B) for 16 h, and cellular levels of total or phosphorylated PKR were determined by Western blotting analysis with reference to a housekeeping protein, β-actin. $^*P < 0.05$ versus negative control (– LPS and – CBX). $^{\#}P < 0.05$ versus positive controls (+ LPS alone).

Figure 6A) and oATP (Figure 6B), suggesting important roles for $P2X_7R$-gated channels in the regulation of PKR-mediated inflammatory responses.

DISCUSSION

Many medicinal herbs have been developed into effective therapies for various inflammatory ailments. In this study, we demonstrated that CBX, a derivative of gancao component GZA, effectively inhibited endotoxin-induced HMGB1 release, likely through blocking $P2X_7R$-gated panx-1 channels, and rescued mice from lethal sepsis even when given at relative lower doses (1.2–6 mg/kg) in a delayed regimen (24 h after CLP), possibly through reducing both local and systemic HMGB1 levels.

Since its inception, CBX has been shown to dose dependently inhibit a variety of biological activities including the gap junctions (50–100 μmol/L), panx-1 channels (EC_{50} = 1–4 μmol/L) (51,52), and 11β-hydroxysteroid dehydrogenase (1–10 μmol/L, 11β-HSD) (53). However, it is unlikely that CBX inhibits LPS-induced HMGB1 release through impairing the gap junctions. First, macrophages did not form gap junctions even after prolonged LPS stimulation, although the cellular panx-1 levels might be elevated (data not shown). Second, the concentrations of CBX used to block gap junctions (for example, 50–100 μmol/L) are much higher than those (for example, 5–10 μmol/L) used to abrogate LPS-induced HMGB1 release. In fact, at relative high concentrations (50–100 μmol/L), CBX exhibited cytotoxicity to RAW 264.7 cells, thereby enhancing (rather than reducing) passive HMGB1 leakage. Thus, it appears that CBX inhibits HMGB1 release through gap junction-independent mechanisms. Similarly, it is unlikely that CBX exerts its inhibition on LPS-induced HMGB1 release through inhibiting 11β-HSD, an enzyme involved in the production of antiinflammatory glucocorticoids. Our previous study indicated that glucocorticoids such as cortisone and dexamethasone were ineffective in inhibiting LPS-induced HMGB1 release in macrophage cultures

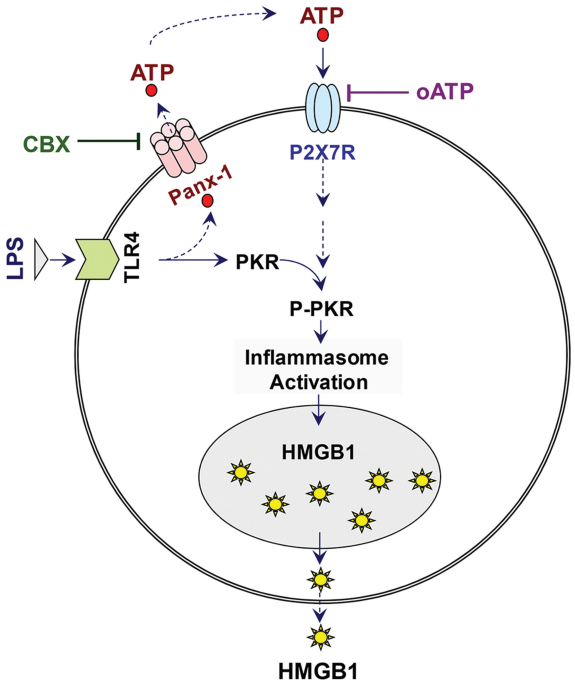

Figure 7. Proposed model for CBX-mediated inhibition of HMGB1 release. Prolonged stimulation with crude LPS may lead to panx-1 hemichannel–mediated ATP efflux, and upregulation of PKR expression. Extracellular ATP then binds to $P2X_7R$ and activates the ATP-gated $P2X_7R$ and panx-1 channels, leading to PKR phosphorylation and subsequent inflammasome-dependent HMGB1 release. As a panx-1 inhibitor, CBX may block LPS-induced ATP efflux, thereby impairing ATP/$P2X_7R$-mediated PKR activation, and subsequent inflammasome-dependent HMGB1 release.

(40), arguing against the possibility that CBX abrogated HMGB1 release by inhibiting 11β-HSD1.

The possibility for the involvement of panx-1 in CBX-mediated inhibition of HMGB1 release is supported by several lines of evidence. First, unlike other pannexin family members, panx-1 is expressed abundantly on macrophage cytoplasmic membranes. Even though panx-1 may not form gap junctions, it can integrate into hemichannels in macrophages or astrocytes. These panx-1 hemichannels are gated by ATP receptors (for example, $P2X_7R$) and responsible for the increased dye-uptake upon ATP stimulation. Second, as an effective panx-1 channel inhibitor (IC_{50} < 5 μmol/L), CBX simultaneously inhibited LPS-induced $P2X_7R$/panx-1 channel activation and HMGB1 release, suggesting that the CBX-mediated inhibition of HMGB1 release is likely associated with the blockade of $P2X_7R$-gated panx-1 channel activities. Consistent with this notion, CBX and other panx-1 channel blockers have recently been shown to block HMGB1 release by neurons during cortical spreading depression (54).

Additionally, we discovered that CBX and $P2X_7R$ antagonists similarly inhibit LPS-induced phosphorylation of PKR, a newly identified regulator of inflammasome activation and HMGB1 release (7). Therefore, we propose that LPS may prime macrophages by upregulating PKR expression and possibly eliciting panx-1-mediated ATP release. Extracellular ATP activates the $P2X_7R$ and causes further elevation of panx-1 channel activity, leading to massive PKR activation and subsequent HMGB1 release (Figure 7). This possibility is consistent with previous findings that panx-1 physically interacts with both $P2X_7R$ and components of the NLRP3 inflammasome (17,55). It also is supported by our observations that both

$P2X_7R$ antagonists (for example, oATP) and panx-1 inhibitors (for example, CBX) effectively inhibited LPS-induced dye uptake, PKR activation, and HMGB1 release. In light of previous reports that CBX inhibited panx-1 hemichannel–mediated ATP release (19,29–31) and passage of anionic dyes (17,51,52), it is tempting to propose that CBX attenuates LPS-induced HMGB1 release by limiting the availability of extracellular ATP to the $P2X_7R$, thereby impairing activation of ATP-gated channels and PKR. This possibility is in agreement with the findings that ATP serves as the second stimuli for the release of other inflammasome-dependent cytokines (for example, IL-1β or IL-18) in LPS-primed macrophages (14–16).

Previously, we, and others, have demonstrated that extracellular accumulation of HMGB1 can be attenuated by HMGB1 inhibitors through distinct mechanisms. For instance, mung bean extracts and green tea components (for example, EGCG) prevent extracellular HMGB1 release through stimulating HMGB1 aggregation and autophagic degradation (38,43). Danshen components (for example, tanshinone IIA sodium sulphonate) effectively stimulate endocytic HMGB1 uptake, thereby recycling extracellular HMGB1 back to cytoplasmic vesicles for eventual degradation (36). In a sharp contrast, CBX could neither induce autophagic HMGB1 degradation, nor stimulate endocytic HMGB1 uptake in macrophage cultures (data not shown). Instead, it blocks the LPS-induced activation of $P2X_7R$/panx-1 channels, indicating the selective targeting of LPS-triggered inflammatory signaling cascade in macrophages (see Figure 7). This is noteworthy especially in light of observations that CBX appears to be more effective in blocking the release of HMGB1 than NO and TNF *in vitro* and in selectively attenuating circulating HMGB1 in septic mice. It is plausible that, as a molecular target of CBX, the $P2X_7R$-gated channels occupy a more important role in the regulation of late (rather than early) mediator of lethal sepsis.

CONCLUSION

In summary, we have validated the therapeutic potential of CBX in an animal model of polymicrobial sepsis by administering it in a delayed regimen. Furthermore, we have uncovered a novel mechanism by which CBX effectively inhibits HMGB1 release possibly by blocking $P2X_7R$-gated channels and preventing endotoxin-induced PKR activation. Given the central role of PKR in the regulation of inflammasome activation and HMGB1 release, it is important to further delineate the role of $P2X_7R$-gated channels in inflammatory diseases and explore novel PKR-targeting therapeutic strategies.

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DISCLOSURES

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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