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Full Length Article

# Cilostazol inhibits HMGB1 release in LPS-activated RAW 264.7 cells and increases the survival of septic mice

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ABSTRACT

*Introduction:* Inflammation and coagulation play important roles in the pathogenesis of sepsis. Anticoagulants with anti-inflammatory action draw attention as therapeutic agent in sepsis.
*Objective:* Whether cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2-(1H)-quinolinone), anticoagulant, protects mice against sepsis and underlying mechanism(s) were investigated.
*Methods:* Induction of heme oxygenase (HO)-1 protein, phosphorylation of 5' adenosine monophosphate-activated protein kinase (AMPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) luciferase activity, and release of high mobility group box 1 (HMGB1) were analyzed using signal inhibitors and transfection techniques. Survival and organ damage were compared in septic mice with and without cilostazol.
*Results:* In RAW264.7 cells, cilostazol increased phosphorylation of AMPK which was followed by HO-1 induction. Lipopolysaccharide (LPS)-activated HMGB1 release was reduced by cilostazol which was reversed by both SB203580 and silencing of HO-1 or AMPK RNA. Interestingly, silencing AMPK reduced HO-1 expression, whereas silencing HO-1 did not affect p-AMPK by cilostazol. Both compound C and zinc protoporphyrin IX (ZnPPIX) antagonized inhibitory effect of HMGB1 by cilostazol. Cilostazol inhibited NF-κB luciferase activity which was antagonized by SB203580. Finally, the administration of cilostazol increased the survival of endotoxemic mice but failed to do so when co-treated with rHMGB1. Cilostazol reduced circulating HMGB1, plasminogen activator inhibitor-1 (PAI-1) levels, organ damages and protein expression of PAI-1 in lung tissues of CLP-septic mice, which were antagonized by ZnPPIX.
*Conclusion:* These findings suggest that HMGB1 can be a target molecule of cilostazol by 1) AMPK activation, and 2) induction of HO-1 by p38 MAPK and AMPK. Therefore, cilostazol may be useful for treatment of sepsis.


## 1. Introduction

Sepsis is defined as a systemic inflammatory response syndrome resulting from a microbial infection. Despite recent advances in antibiotic therapy and intensive care, sepsis is still the most common cause of death in intensive care units [1,2]. The pathogenesis of sepsis is attributable, at least in part, to dysregulated systemic inflammatory responses characterized by an excessive accumulation of proinflammatory mediators such as tumor necrosis factor and interleukin-1 [3], interferon-γ [4], and nitric oxide [5]. During sepsis, the release of endotoxin (lipopolysaccharide, or LPS) and proinflammatory cytokines initiate an activation cascade in endothelial cells (ECs), leading to increased coagulation and inflammatory response [6]. Recently, high-mobility group box 1 (HMGB1), a highly conserved nuclear DNA-binding protein, has been identified as a novel inflammatory cytokine and a late mediator of endotoxin lethality in mice [7]. In addition, Wolfson et al. [8] reported that HMGB1 plays a critical role in EC barrier disruption and that this process is mediated by the rearrangement of the actin cytoskeleton into a contractile phenotype. In sepsis conditions, the elevated HMGB1 and its induced inflammatory factors activate platelets and exacerbate the endothelial injury and EC dysfunction, causing the loss of physiologic anticoagulant property of normal endothelium. Indeed, microvessel thrombosis occurs in septic patients [9] Moreover, HMGB1 has been reported to play a role in the pathogenesis of disseminated intravascular coagulation (DIC) because plasma HMGB1 levels correlate with DIC scores [10]. In sepsis-related DIC, anticoagulant therapy draws attention due to the mounting evidence for the anti-inflammatory effects which these agents possess and can prove beneficial in septic situations [9]. Given important role of HMGB1 between coagulation and inflammation in patients with sepsis, we hypothesize that cilostazol [(6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2-(1H)-quinolinone)], anti-platelet agent,

*Abbreviations:* AMPK, 5' adenosine monophosphate-activated protein kinase; ATCC, American Type Culture Collection; CO, Carbon monoxide; CLP, Cecal ligation and puncture; Cilostazol, (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2-(1H)-quinolinone); DMEM, Dulbecco's modified Eagle's medium; ECs, Endothelial cells; ECL, Enhanced chemiluminescence; FBS, Fetal bovine serum; HO-1, Heme oxygenase-1; HMGB1, High-mobility group box 1; IFN-γ, Interferon-gamma; IL-1, Interleukin-1; LPS, Lipopolysaccharide; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; rHMGB1, Recombinant human HMGB1; ZnPPIX, Zinc protoporphyrin IX.

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might be beneficial by reducing HMGB1 in septic conditions. Thus, the aim of the present study was to test hypothesis that heme oxygenase-1 (HO-1) induction by cilostazol is responsible for inhibition of HMGB1 release in LPS-activated RAW 264.7 cells and increase survival in endotoxaemic mice and cecal ligation and puncture (CLP) induced septic mice. We and others demonstrated that HO-1 and the carbon monoxide (CO) system can play very important roles in sepsis through the negative regulation of HMGB1 [7,11–13].

## 2. Materials & Methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. Anti-HMGB1 antibody was purchased from Abcam (Cambridge, MA, USA), and antibodies against AMPK, p-AMPK, p38, and p-p38 were purchased from Cell Signaling Technology (Beverly, MA, USA). Scrambled siRNAs were purchased from Invitrogen (Carlsbad, CA, USA). Anti-β-actin, anti-heme oxygenase-1, and anti-PAI-1 antibodies, siHO-1, and siAMPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence (ECL) western blotting detection reagent was purchased from Amersham (Buckinghamshire, UK). Compound C was obtained from Calbiochem (San Diego, CA, USA). All other chemicals, including LPS (*Escherichia coli* 0111:B4) and recombinant human HMGB1 (rHMGB1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cilostazol was kindly supplied by Otsuka Pharmaceutical Co., Ltd. (Seoul). A p38 MAPK inhibitor (SB203580) was purchased from Calbiochem (San Diego, CA). An inhibitor of HO enzyme (ZnPPIX) and an AMPK inhibitor (compound C) were obtained from from Sigma-Aldrich (St. Louis, MO).

### 2.2. Cell Culture and Stimulation

RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were grown in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (4.5 mg/ml), and glucose (4.5 mg/ml). RAW 264.7 cells were plated at a density of $1 \times 10^7$ cells per 100-mm dish. The cells were rinsed with fresh medium and stimulated with LPS (1 μg/ml) in the presence or absence of different concentrations of cilostazol (1-100 μM).

### 2.3. Cell Viability

Cell viability was determined colorimetrically using the MTT assay. Cells in the exponential phase were seeded at $1 \times 10^4$ cells per well in 24-well plates. After different treatments, 20 μl of 5 mg/ml MTT solution was added to each well (0.1 mg per well), and the wells were incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 200 μl of dimethyl sulfoxide for 30 min at 37 °C. The optical density was read at 570 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

### 2.4. Western Blot Analysis

Total protein was obtained using lysis buffer containing 0.5% SDS, 1% Nonidet P-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and protease inhibitors. The protein concentration was determined using the Bradford assay. The absorbance of the mixture at 595 nm was determined using an ELISA plate reader. An equal amount of protein from each sample was electrophoresed on 8% and 10% polyacrylamide gels, followed by electrophoretic transfer onto PVDF western blotting membranes (Roche, Germany).

Each membrane was blocked with 5% skim milk and sequentially incubated with a primary antibody and a horseradish peroxidase-conjugated secondary antibody, followed by ECL detection (Animal Genetics).

### 2.5. Silencing RNA and Vector Transfection Technique

RAW 264.7 cells were seeded into 60-mm cell culture dishes at $3 \times 10^5$ cells per dish 18-24 h prior to transfection. Depending on the purpose of the experiment, the cells were transfected with 2 μg of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) luciferase vector, 100 nM AMPK siRNA, 100 nM HO-1 siRNA, or 100 nM scrambled siRNA using SuperFect (Qiagen, Valencia, CA, USA). After 4 h of incubation, the medium was replaced with fresh medium.

### 2.6. NF-κB Luciferase Activity

After the scheduled treatment, the cells were washed twice with cold PBS, lysed in the lysis buffer provided in the Dual-Luciferase kit (Promega, Madison, WI, USA), and assayed for luciferase activity using a TD-20/20 Luminometer (Tumer Designs, Sunnyvale, CA, USA) according to the manufacturer's protocol. All transfections were performed in triplicate. The data are presented as the ratio between firefly and Renilla luciferase activities.

### 2.7. Survival Experiments

To examine the survival on endotoxaemic mice, mice BALB/c mice (male, 7-8 wk, 20-25 g) were randomly divided into 3 groups. 1) LPS group (15 mg/kg, i.p., n = 20). 2) LPS + cilostazol group (10 mg/kg, i.p., $n = 20$), and 3) LPS + cilostazol + rHMGB1 group (100 μg per mouse, n = 20). Endotoxemia was induced by i.p. injection of LPS. Cilostazol group mice were treated with cilostazol 2 h prior to the injection of LPS, and boost injection at 12 h after the onset of endotoxemia. Recombinant HMGB1 group mice were co-treated with cilostazol (10 mg/kg) and rHMGB1 (100 mg per mouse) 2 h prior to and 12 h post LPS injection. Survival was monitored every 12 h for up to 3 days. For the CLP-induced sepsis experiment, BALB/c mice (male, 7-8 wk, 20-25 g) were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). Next, a 2-cm midline incision was performed to allow exposure of the cecum and the adjoining intestines. The cecum was tightly ligated with a 3.0 silk suture at 5.0 mm from the cecal tip and punctured once with a 22-gauge needle. The cecum was then gently squeezed to extrude a small amount of feces from the perforation sites and returned to the peritoneal cavity. For sham animals (n = 8) the cecum was exposed but not ligated or punctured and returned to the abdominal cavity. The laparotomy site was then stitched with 4.0 silk. To evaluate the effect of cilostazol on the survival of CLP mice, mice were treated with vehicle (DMSO, 0.1 ml per mice, n = 8) or cilostazol (10 mg/kg, i.p., $n = 20$) or cilostazol (10 mg/kg) with ZnPPIX (10 mg/kg, i.p., $n = 20$) 2 h prior to and at 12 h after the operation. Survival was monitored every 12 h for up to 3 days. The mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996) and were treated ethically. The protocol was approved by the Animal Research Committee of Gyeongsang National University.

### 2.8. Measurement of Serum ALT, AST, HMGB1 and PAI-1 Levels

To measure circulating HMGB1 and PAI-1 levels, BALB/c mice (male, 7-8 wk, 20-25 g) were anesthetized with ketamine (30 mg/kg) and xylazine (6 mg/kg). And CLP operation was done as described above. Animals were randomly divided into 4 groups. 1) Sham (n = 6), 2) CLP (n = 10), 3) CLP + Cilostazol (1 mg/kg and 10 mg/kg, i.p., n = 10, respectively) and 4) CLP + Cilostazol (10 mg/kg) + ZnPPIX (10 mg/kg, i.p, n = 10). Cilostazol was treated 2 h prior to and 12 h post CLP operation. ZnPPIX was treated 2 h prior to CLP operation. Twenty-four hours after CLP, all animals were sacrificed under