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Journal of Cellular Biochemistry 114:336–345 (2013)

Journal of Cellular Biochemistry

Anti-Inflammatory Activities of Isorhamnetin-3-*O*-Galactoside Against HMGB1-Induced Inflammatory Responses in Both HUVECs and CLP-Induced Septic Mice

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ABSTRACT

High mobility group box 1 (HMGB1) protein is a crucial nuclear cytokine that elicits severe vascular inflammatory diseases. *Oenanthe javanica* (water dropwort) extract has anti-arrhythmic, neuroprotective and anti-diabetic activity. However, isorhamnetin-3-*O*-galactoside (I3G), an active compound from *O. javanica*, is not researched well for its biological activity. Here, we investigated the anti-inflammatory activities of I3G by monitoring the effects of I3G on the lipopolysaccharide (LPS) or cecal ligation and puncture (CLP)-mediated release of HMGB1 and HMGB1 or CLP-mediated modulation of inflammatory responses. I3G potently inhibited the release of HMGB1 and down-regulated HMGB1-dependent inflammatory responses in human endothelial cells. I3G also inhibited HMGB1-mediated hyperpermeability and leukocyte migration in mice. Further studies revealed that I3G suppressed the production of tumor necrosis factor-α and activation of nuclear factor-κB by HMGB1. In addition, I3G reduced CLP-induced HMGB1 release and sepsis-related mortality. Given these results, I3G should be viewed as a candidate therapeutic agent for the treatment of severe vascular inflammatory diseases such as sepsis or septic shock via inhibition of the HMGB1 signaling pathway. J. Cell. Biochem. 114: 336–345, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: ISORHAMNETIN-3-*O*-GALACTOSIDE; HMGB1; SEPSIS; INFLAMMATION; BARRIER INTEGRITY

High mobility group box 1 (HMGB1) was originally described as a nuclear protein that binds DNA, and functions as a co-factor that is required for proper transcriptional regulation and gene expression [Wang et al., 1999; Ulloa et al., 2002; Yang et al., 2004]. Studies show that extracellular HMGB1 contributes to a variety of pathophysiological process, including diabetes, antibacterial activity, smooth-muscle cell chemotaxis, cell differentiation, myocardial regeneration, angiogenesis, tissue repair, and cancer [Wang et al., 1999; Yang et al., 2004]. In addition, HMGB1 is a potent proinflammatory cytokine and associates with a variety of severe vascular inflammatory diseases such as sepsis and septic shock, especially and has been categorized as a danger signal [Wang et al., 1999; Yang et al., 2004]. Sepsis, which is a systemic inflammatory response disease, is the most severe complication of infection and is a deadly disease, and HMGB1 acting as a potent proinflammatory cytokine involve in the delayed endotoxin lethality and sepsis [Huang et al., 2010]. Therefore, targeting HMGB1 with specific antagonists is protective in established preclinical inflammatory disease models including lethal endotoxemia or sepsis [Huang et al., 2010].

The search for anti-inflammatory agents from natural herbal medicines represents an area of great interest worldwide [Aggarwal

Tae Hoon Kim and Sae-Kwang Ku have contributed equally to this work.

Conflict of interest statement: The authors declare no conflicts of interest.

Grant sponsor: Korea Government (MEST); Grant number: 2011-0030124; Grant sponsor: Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea; Grant number: A111305.

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Manuscript Received: 1 June 2012; Manuscript Accepted: 15 August 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 August 2012

DOI 10.1002/jcb.24361 • © 2012 Wiley Periodicals, Inc.

et al., 2006]. *Oenanthe javanica* (water dropwort), cultivated in Australia and East Asian countries such as Korea, China, and Japan, is a perennial herb and consumes as a spicy vegetable especially in early spring because of its distinctive aroma and taste [Ma et al., 2010]. The fresh stems and leaves are used as a salad or as a seasoning in soups and stews in Korea [Seo and Baek, 2005]. According to the traditional Chinese medicine, it has well known used in the treatment of jaundice, hypertension, fever, abdominal pain, leucorrhea, mumps, and difficult urination [Park et al., 1993]. Indeed, Chinese researchers have already shown that *O. javanica* extract has anti-arrhythmic activity [Ji et al., 1990] and anti-diabetic activity by promoting insulin release from Langerhan's β-cells [Yang et al., 2000]. The total phenolics of water dropwort are known to have an anti-hepatitis B virus activity [Han et al., 2008]. Although isolation and determination of structure of isorhamnetin-3-*O*-galactoside (I3G, synonym is Quercetin 3-methoxy-3-*O*-galactoside) is well studied in previous reports [Radwan et al., 2007; Materska, 2008], the biological activities of I3G rarely studied. And to our knowledge to date, any studies related to the anti-inflammatory activity of I3G have not been reported. Therefore, the anti-inflammatory role of I3G and underlying mechanism in HMGB1-mediated inflammatory responses remains to be elucidated. The purpose of this study is to investigate the anti-inflammatory effect of I3G against HMGB1-induced inflammatory responses in human endothelial cells and cecal ligation and puncture (CLP)-induced septic mice. To do this end, we monitored the effects of I3G on LPS or CLP-induced HMGB1 release, LPS or HMGB1-mediated hyperpermeability in HUVEC, HMGB1-mediated barrier disruption in mice, expression of cell adhesion molecules, and adhesion/trans-endothelial migration of leukocytes. Our results indicate I3G is able to reduce LPS-induced HMGB1 release and HMGB1-mediated inflammatory responses, thereby reducing the mortality in CLP-induced septic mice.

Because the inhibition of the vascular endothelial inflammatory responses is considered a promising target for the treatment of many vascular diseases such as atherosclerosis, shock, heart attack, and sepsis, the significance of this study is that I3G can affect barrier functions and some of the factors that can influence barrier functions and provide novel insights into the anti-inflammatory activity of I3G and its potential applications as a therapeutic tool to treat severe vascular disease such as sepsis and septic shock.

MATERIALS AND METHODS

REGENTS

HMGB1 was purchased from Abnova (Taipei City, Taiwan). Bacterial lipopolysaccharide (serotype: 0111:B4, L5293, used at 100 ng/ml), Evans blue, crystal violet, MTT (3-[4,5-dimethyl-2-yl]-2,5-diphenyltetrazolium bromide), quercetin and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Vybrant DiD (used at 5 μM) was obtained from Invitrogen (Carlsbad, CA). Kaempferol-3-*O*-sophoroside (KP, Fig. 1A) was prepared as described before [Kim et al., 2012b].

PLANT MATERIALS, EXTRACTION AND ISOLATION OF HYPEROSIDE AND I3G

The aerial parts of *O. javanica* was collected from agricultural farms in Pyeongyang-ri, Cheongdo-gun, Gyoungsanbuk-do, Korea in April 2011, and identified by Dr. Tae Hoon Kim. A voucher specimen (KAJ-0075) was deposited at the Natural Product Chemistry Laboratory of Daegu Hanny University. Fresh milled *O. javanica* plant material (1.4 kg) was extracted with 70% EtOH (10 l × 3) at room temperature, and the solvent was evaporated in vacuo. The combined crude EtOH extract (81.5 g) was suspended in 20% MeOH (3 L), and then partitioned in turn with n-hexane (3 l × 3), EtOAc (3 l × 3) and n-BuOH (3 l × 3) to yield dried n-hexane-(5.5 g), EtOAc-(1.8 g), n-BuOH (10.6 g), and H_2O-soluble (53.6 g) residues. A portion (1.7 g) of the EtOAc extract was chromatographed on a Toyopearl HW-40 column (coarse grade; 3.0 cm i.d. × 48 cm) with H_2O containing increasing amounts of MeOH in a stepwise gradient mode. The 40% MeOH eluate was subjected to column chromatography over a YMC GEL ODS AQ 120–50S column (1.6 cm i.d. × 38 cm) with aqueous MeOH, to yield pure compounds **1** (7.7 mg; $t_R = 8.8$ min) and **2** (7.0 mg; $t_R = 9.1$ min) as shown in Figure 1. HPLC analysis was carried out on a YMC-Pack ODS A-302 column (4.6 mm i.d. × 150 mm; YMC Co., Kyoto, Japan) and the solvent system consisted of a linear gradient that started with 10% (v/v) MeCN in 0.1% HCOOH/H_2O (detection: UV 280 nm; flow rate: 1.0 ml/min; at 40°C), increased to 90% MeCN over 30 min, and

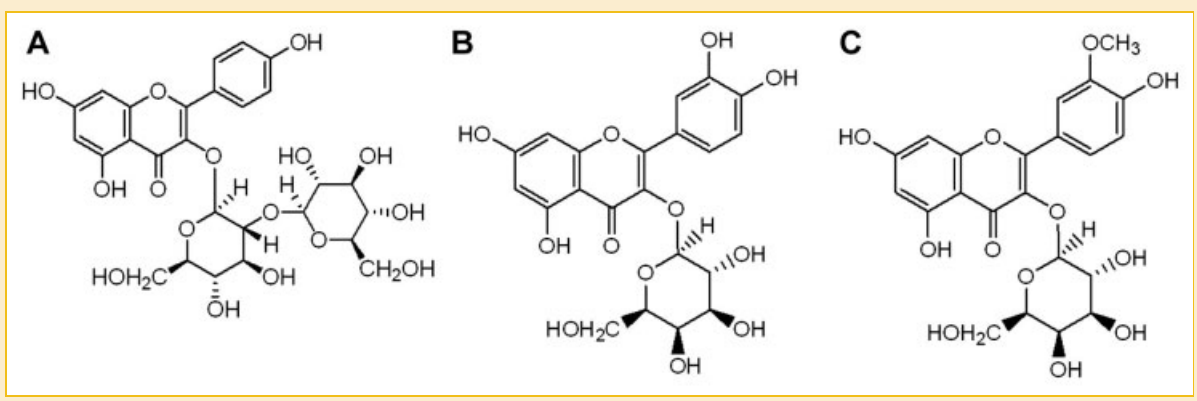

Fig. 1. Chemical structure of Kaempferol-3-*O*-sophoroside (KP, A), hyperoside (B), and isorhamnetin-3-*O*-galactoside (I3G, C).

then increased to 100% MeCN over 5 min. The isolated compounds 1 and 2 were identified as hyperoside (1) and I3G (2) from comparisons of their physicochemical and spectroscopic data (^{1}H, ^{13}C NMR, 2D NMR, and MS) with those of reference data [Liu et al., 2010; Sikorska and Matlawska, 2001].

Hyperoside (1): ^{1}H NMR (600 MHz, CD_3OD) δ 7.83 (1H, d, J = 2.4 Hz, H-2′), 7.57 (1H, d, J = 8.4, 2.4 Hz, H-6′), 6.85 (1H, d, J = 8.4 Hz, H-5′), 6.39 (1H, d, J = 1.8 Hz, H-8), 6.19 (1H, d, J = 1.8 Hz, H-6), 5.15 (1H, d, J = 7.8 Hz, H-1″), 3.90∼3.10 (6H, m, H-2″–H-6″).

I3G (2): ^{1}H NMR (600 MHz, CD_3OD) ^{1}H-NMR (600 MHz, CD_3OD) δ 8.02 (1H, d, J = 1.8 Hz, H-2′), 7.56 (1H, d, J = 8.4, 1.8 Hz, H-6′), 6.89 (1H, d, J = 8.4 Hz, H-5′), 6.38 (1H, s, H-8), 6.19 (1H, s, H-6), 5.32 (1H, d, J = 7.8 Hz, H-1″), 3.95 (3H, s, MeO-3′), 3.97∼3.30 (6H, m, H-2″–H-6″).

ANIMALS AND HUSBANDRY

Male C57BL/6 mice (6–7-week old, weighting 18–20 g) were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea), and used after a 12-day acclimatization period. Animals were housed five per polycarbonate cage under controlled temperature (20–25°C) and humidity (40–45%) under a 12:12 hour light/dark cycle, and fed normal rodent pellet diet and supplied with water ad libitum. All animals were treated in accordance with the Guidelines for the Care and Use of Laboratory Animals issued by Kyungpook National University.

CELL CULTURE

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described [Bae and Rezaie, 2011]. HUVECs of passage numbers 3 or 4 were used in the experiments. Human leukocytes were freshly isolated from whole blood (15 ml) obtained by viniculture from five healthy volunteers, and maintained as previously described [Hofbauer et al., 1998].

COMPETITIVE ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY) FOR HMGB1

Measurement of concentration of HMGB1 was quantitated as previously described [Lee et al., 2012]. Briefly, lyophilized culture media were pre-incubated with anti-HMGB1 antibodies (Abnova, diluted 1:1,000 in PBS-T) for 90 min at 37 °C. Pre-incubated samples were transferred to HMGB1 protein pre-coated plate, and incubated for 30 min at room temperature. The plates incubated for 90 min at RT with the peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2,000 in PBS-T, Amersham Pharmacia Biotech). The plates were rinsed and incubated for 60 min at RT in dark space with 200 μl substrate solution (100 μg/ml *O*-phenylenediamine and 0.003% H_2O_2). After stopping the reaction with 50 μl of 8N H_2SO_4, the absorbance was read at 490 nm.

CELL VIABILITY ASSAY

MTT was used as an indicator of cell viability as previously described [Lee et al., 2012]. The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and then treated with I3G. After 48 h incubation, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and the OD was measured at 540 nm using a microplate reader (Tecan Austria GmbH, Austria).

CECAL LIGATION AND PUNCTURE

To induce sepsis, male mice were anesthetized with zoletil 50 and rompun. The CLP-induced sepsis model was prepared as described previously [Wang et al., 2004a]. In brief, a 2-cm midline incision was placed to allow exposure of the cecum with adjoining intestine. The cecum was then 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 perforation sites and returned to the peritoneal cavity. The laparotomy site was then stitched with 4.0-silk. In sham control animals, the cecum was exposed but not ligated or punctured and then returned to the abdominal cavity. This protocol was approved in advance by the Animal Care Committee at the Kyungpook National University.

PERMEABILITY ASSAY

In vitro: permeability was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional HUVECs monolayers as previously described [Bae and Rezaie, 2008]. The confluent monolayers were incubated with each compound for 6 h followed by HMGB1 (1 μg/ml) for 16 h or LPS (100 ng/ml) for 4 h.

In vivo: mice were pretreated with each compound (KP; 6 μg/mouse, hyperoside; 4.6 μg/mouse, I3G; 4.8 μg/mouse) intravenously and after 6 h, 1% Evans blue dye solution in normal saline was injected intravenously in each mouse immediately followed by an intravenous injection of HMGB1 (2 μg/mouse). Measurement of in vivo permeability was quantitated as previously described [Lee et al., 2012].

IMMUNOFLUORESCENCE STAINING

HUVECs were grown to confluence on glass cover slips coated with 0.05% poly-L-lysine in complete media containing 10% fetal bovine serum (FBS) and maintained for 48 h. Cells were then stimulated with HMGB1 (1 μg/ml) for 1 h with or without each compound. For cytoskeletal staining, cells were fixed in 4% formaldehyde in PBS (v/v) for 15 min at room temperature, and for immunostaining, cells were permeabilized in 0.05% Triton X-100 in PBS for 15 min, and blocked in blocking buffer (5% BSA in PBS) overnight at 4°C. Cells were then incubated with primary mouse monoclonal antibody (1:400, diluted in 5% BSA in PBS). F-actin labeled fluorescein phalloidin (F 432; Molecular Probes, Invitrogen) overnight at 4°C. Cells were analyzed by fluorescence microscopy at ×400 (Carl Zeiss, AG, Germany).

EXPRESSION OF VCAM-1

The expression of vascular cell adhesion molecule-1 (VCAM-1) on HUVECs was determined by a whole-cell ELISA as described [Che et al., 2002; Bae and Bae, 2011]. Briefly, confluent monolayers of HUVECs were treated with each compound for 6 h followed by HMGB1 (1 μg/ml) for 16 h. The medium was removed, cells were washed with PBS and fixed by adding 50 μl of 1% paraformaldehyde for 15 min at room temperature. After washing, 100 μl of

mouse anti-human monoclonal antibodies (VCAM-1, CA, 1:50 each) were added. After 1 h (37°C, 5% CO_2), the cells were washed three times and then 100 μl of 1:2,000 peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO) was added for 1 h. The cells were washed again three times and developed using *O*-phenylenediamine substrate (Sigma, St. Louis, MO). Colorimetric analysis was performed by measuring absorbance at 490 nm. All measurements were performed in triplicate wells.

CELL–CELL ADHESION ASSAY

Adherence of leukocytes to endothelial cells was evaluated by fluorescent labeling of monocytes as described [Akeson and Woods, 1993; Kim et al., 2001; Bae and Bae, 2011]. Briefly, leukocytes were labeled with 5 μM Vybrant DiD for 20 min at 37°C in phenol red-free RPMI containing 5% FBS. Following two washings, cells (1.5×10^6/ml, 200 μl/well) were resuspended in adhesion medium (RPMI containing 2% FBS and 20 mM HEPES) and added to confluent monolayers of HUVECs in 96-well plates which were treated for 6 h with each compound followed by HMGB1 (1 μg/ml) for 16 h. The fluorescence of labeled cells was measured (total signal) using a fluorescence microplate reader (Tecan Austria GmbH, Austria). After incubation for 1 h at 37°C, non-adherent cells were removed by washing four times with pre-warmed RPMI and the fluorescent signals of adherent cells were measured. The percentage of adherent monocytes was calculated by the formula: % adherence = (adherent signal/total signal) × 100 as described [Akeson and Woods, 1993; Kim et al., 2001]. The data are expressed as means ± SD from at least three independent experiments.

MIGRATION ASSAY

In vitro: measurement of migration was quantitated as previously described [Lee et al., 2012]. Briefly, confluent HUVECs monolayers which were treated for 6 h with each compound followed by HMGB1 (1 μg/ml) for 16 h were washed three times with PBS and leukocytes (1.5×10^6/0.2 ml) were added to the upper compartment. Leukocytes on the lower side of the filter were fixed and stained. Nine randomly selected high power microscopic fields (HPF, 200×) were counted and expressed as a migration index.

In vivo: measurement of migration was quantitated as previously described [Lee et al., 2012]. Briefly, animals received an invenous injection of HMGB1 (2 μg/mouse) in normal saline 1 h after pretreated with each compound (KP, 6 μg/mouse; hyperoside, 4.6 μg/mouse; and I3G, 4.8 μg/mouse) 6 h later, the mice were sacrificed; 20 μl of peritoneal fluid was mixed with 0.38 ml of Turk's solution (0.01% crystal violet in 3% acetic acid) and the number of leukocytes was counted under a light microscope.

ELISA FOR NUCLEAR FACTOR-κB (NF-κB) AND TUMOR NECROSIS FACTOR- (TNF-α)

The activity of Phospho-NF-κB p65 (No. 7173, Cell Signaling Technology, Danvers, MA) in the nuclear lysates and concentrations of TNF-α (R&D Systems, Minneapolis, MN) in cell culture supernatants were determined by ELISA according to the manufacturer's protocol. The sensitivity of the TNF-α ELISA was 0.152–0.736 pg/ml. TNF-α was determined by comparison to standard curves.

STATISTICAL ANALYSIS

Results are expressed as means ± SD of at least three independent experiments. Statistical significance was determined using analysis of variance (ANOVA; SPSS, version 14.0, SPSS Science, Chicago, IL) and *P*-values less than 0.05 ($P < 0.05$) were considered significant.

RESULTS AND DISCUSSION

In this study, we used one flavonoid, kaempferol-3-*O*-sophoroside (KP, Fig. 1A), as a positive control because we recently reported that KP showed anti-inflammatory responses against HMGB1-mediated inflammatory responses [Kim et al., 2012b]. Hyperoside (Fig. 1B), another compound from *O. javanica*, was used as a negative control because the structure of quercetin is similar to I3G and it has no barrier protective effects in this study.

EFFECT OF I3G ON LPS OR CLP-MEDIATED HMGB1 RELEASE

Previous studies have demonstrated that LPS stimulates HMGB1 release in murine macrophages and human endothelial cells [Mullins et al., 2004; El Gazzar, 2007; Bae and Rezaie, 2011]. In agreement with previous results, LPS (100 ng/ml) stimulated HMGB1 release by HUVECs (Fig. 2A). To investigate the effect of I3G on LPS-mediated HMGB1 release, endothelial cells were pretreated with increasing concentrations of I3G for 6 h before stimulation of cells with 100 ng/ml LPS for 16 h. The results shown in Figure 2A indicate that I3G inhibits HMGB1 release by LPS in endothelial cells, with the optimal effect occurring at a concentration above 1–5 μM I3G. However, I3G alone did not affect HMGB1 release (Fig 2A). To confirm this effect in vivo, we next subjected mice to severe sepsis in a standardized model of CLP since the CLP model more closely resembles human sepsis than LPS-induced endotoxemia [Yang et al., 2004; Buras et al., 2005]. As shown in Figure 2B, I3G markedly inhibited the CLP-induced HMGB1 release in mice. Assuming that the average weight of a mouse is 20 g and the average blood volume is 2 ml, the injected each compound (KP, 6 μg; hyperoside, 4.6 μg; and I3G, 4.8 μg per mouse) concentration is approximately 5 μM in peripheral blood. To exclude the possibility that the inhibition of HMGB1 release was due to cytotoxicity caused by I3G, cellular viability assays were performed in HUVECs treated with I3G for 24 h. At the concentrations used (up to 10 μM), I3G did not affect cell viability (Fig. 2C). The plasma level of HMGB1 is elevated in patients with severe inflammatory diseases and animal models of endotoxemia [Yang and Tracey, 2010]. However, hyperoside did not show any inhibitory effect on HMGB1 release in both cellular and mice model. This result suggests that methoxy group in I3G positively regulate the inhibitory effect of HMGB1 release.

A higher plasma level of HMGB1 is associated with poor prognosis and higher mortality rate in severe inflammatory diseases [Yang and Tracey, 2010]. Because of its delayed release kinetics, HMGB1 is known as the late mediator of endotoxin lethality in mice [Wang et al., 2001]. Previous results, together with our own in this study, suggest that vascular endothelial cells are a rich source of HMGB1, which can be released in response to bacterial endotoxin and proinflammatory cytokines, thereby contributing to the

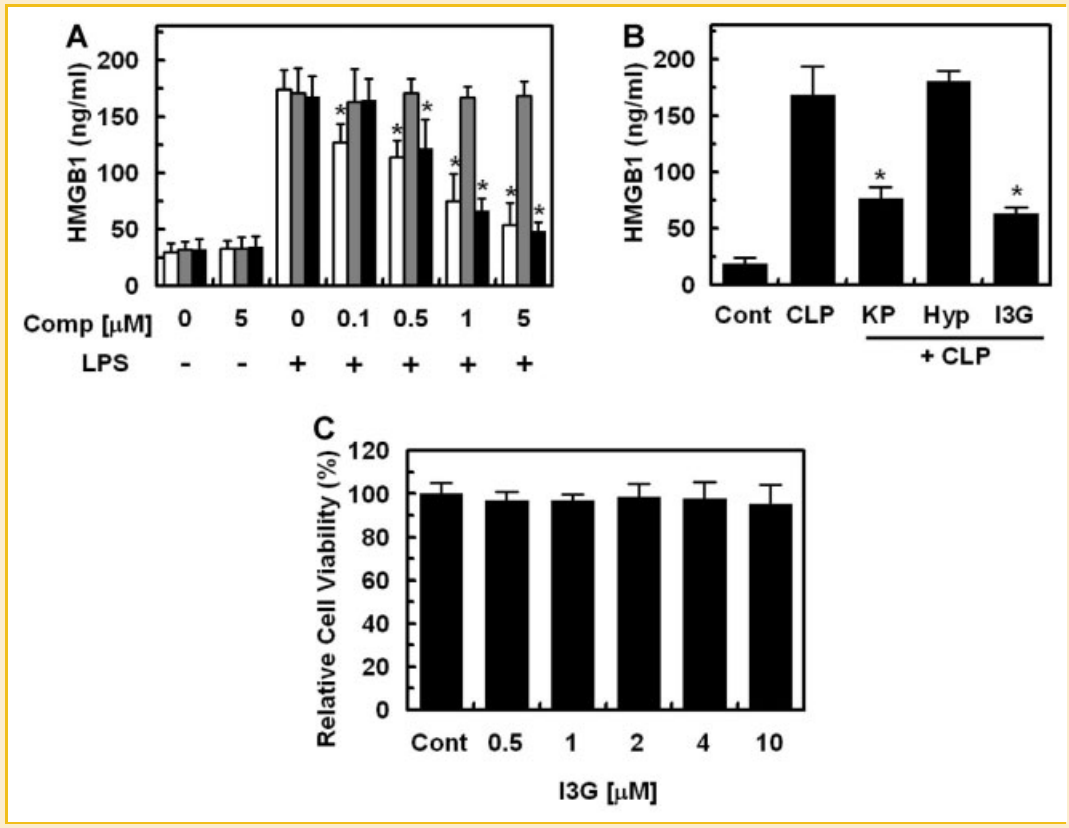

Fig. 2. Effect of I3G on HMGB1 release. A: HUVECs were stimulated with LPS 100 ng/ml for 16 h after treating HUVEC monolayer with the indicated concentrations of KP (black box), hyperoside (gray box), and I3G (white box) for 6 h and the release of HMGB1 was measured by ELISA. B: Male C57BL/6 mice underwent CLP and were administered KP (6 μg/mouse), hyperoside (4.6 μg/mouse), and I3G (4.8 μg/mouse) intravenously at 12 h after CLP ($n = 5$). Mice were euthanized at 24 h after CLP. Serum HMGB1 levels were measured by ELISA. C: Effect of I3G on cellular viability was measured by MTT assay. All results are shown as means ± SD of three different experiments. $^{*}P < 0.01$ compared with LPS only (A) or CLP (B).

pathology of inflammatory diseases and the preventions of LPS or CLP-induced HMGB1 release by I3G suggest that I3G might be used for the treatment of vascular inflammatory diseases.

EFFECT OF I3G ON LPS OR HMGB1-MEDIATED BARRIER DISRUPTION

The vascular endothelium acts as a dynamic barrier that selectively restricts the passage of plasma and cells from the blood into adjacent tissues [Fisher, 2008]. During inflammatory responses, over-production of inflammatory mediators may irreversibly damage vascular integrity and cause excessive loss of fluid from the circulation. This hazardous event may lead to prolonged tissue hypoperfusion, organ dysfunction and death [Fisher, 2008]. Therefore, prevention of vascular damage and maintenance of barrier integrity could improve survival of patients suffering from inflammatory diseases. The exogenous administration of HMGB1 to animals leads to disruption of the intestinal barrier function, tissue injury and death [Wang et al., 2001; Yang et al., 2005; Wolfson et al., 2011]. To determine the effects of I3G on barrier protection in LPS or HMGB1-stimulated primary HUVECs, the flux of albumin in a dual chamber system was monitored. LPS and HMGB1 are known to cleave and disrupt barrier integrity of human endothelial cells [Bae and Rezaie, 2008, 2011; Kim and Bae, 2010]. HUVECs were treated with various concentrations of I3G for 6 h before addition of LPS (100 ng/ml for 4 h) or HMGB1 (1 μg/ml for 16 h). I3G decreased LPS (Fig. 3A) or HMGB1 (Fig. 3B)-induced barrier disruption in dose-dependent manner in vitro. However, I3G alone could not alter barrier integrity (Fig. 3A and B). To confirm this effect in vivo, HMGB1-induced vascular permeability in mice was assessed. As shown in Figure 3C, I3G markedly inhibited the leakage of dye into the peritoneum in mice. These results suggest that I3G could be used to maintain vascular barrier integrity. Based on the results that hyperoside did not affect barrier protective effects, we conclude that methoxy group in I3G positively regulate the barrier protective effects.

It has been reported that stimulation of endothelial cells is associated with reductions and redistributions of extra- and intracellular proteins [Goldblum et al., 1993; Schnittler et al., 2001]. Furthermore, these proteins, like actin, are important for maintaining cell integrity and shape [Schnittler et al., 2001]. Redistribution of the actin cytoskeleton, detachment of cells, and loss of cell–cell contact due to cytokine stimulation are all associated with increased endothelial monolayer permeability [Friedl et al., 2002; Petrache et al., 2003]. Therefore, we next examined the effects of I3G on

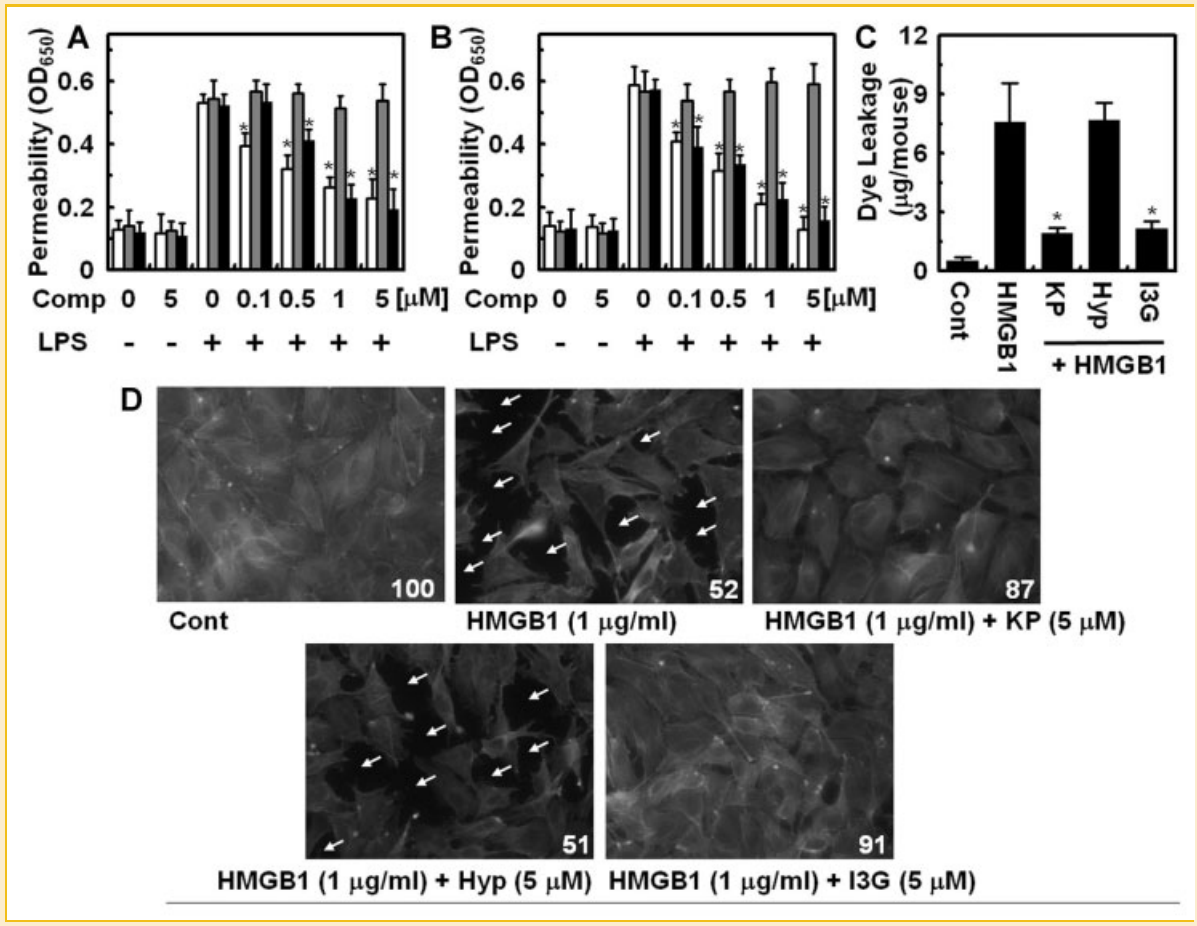

Fig. 3. Effects of I3G on vascular barrier permeability in vitro and in vivo. Effect of various concentrations of KP (black box), hyperoside (gray box), and I3G (white box) on LPS (A, 100 ng/ml) or HMGB1 (B, 1 μg/ml)-mediated barrier disruption was monitored by the flux of Evans blue bound albumin across HUVECs. C: Effect of KP (6 μg/mouse, i.v.), hyperoside (4.6 μg/mouse, i.v.), and I3G (4.8 μg/mouse, i.v.) on HMGB1-induced (2 μg/mouse, i.v.) vascular permeability in mice was examined by the flux of Evans blue in mice (expressed μg/mouse, $n = 5$). Results are expressed as means ± SD of different three experiments. $^{*}P < 0.01$ as compared with LPS alone (A), HMGB1 alone (B and C). D: Staining for F-actin. HUVECs monolayers grown on glass coverslips were stimulated with HMGB1 for 1 h and then immunofluorescence stained for F-actin. Arrows depict intercellular gaps. The results shown are representative of three independent experiments. F-actin content was quantified using Image J software. Quantification data (inserted in the pictures) are represented as F-actin content expressed as relative arbitrary units.

HUVEC actin cytoskeletal arrangement by immunofluorescence staining HUVEC monolayers with F-actin labeled fluorescein phalloidin. Control HUVECs displayed a random F-actin distribution throughout cells with some localization of actin filament bundles at cell boundaries (Fig. 3D). Barrier disruption by HMGB1 (1 μg/ml) was manifested by formation of paracellular gaps (shown by arrows) in HUVECs. A similar cytoskeletal arrangement was induced by LPS (100 ng/ml) (data not shown). Furthermore, pretreatment with I3G (5 μM) inhibited the formation of HMGB1-induced paracellular gaps with formation of the dense F-actin rings (Fig 3D). These results suggested that I3G maintains HMGB1-mediated morphological changes and gap formation of endothelial cells associated with F-actin redistribution, and thereby, increase vascular barrier integrity.

I3G INHIBITS THE EXPRESSION OF CAMS AND PROINFLAMMATORY RESPONSES

It is well known that human endothelial cells treated with HMGB1 are associated with upregulation of several CAMs such as VCAM-1, ICAM-1, and E-selectin [Andersson et al., 2000; Park et al., 2003; Treutiger et al., 2003]. To determine the effect of I3G on the expressions of CAMs in HMGB1-stimulated endothelial cells, we monitored the expression of VCAM-1, ICAM-1, and E-selectin in HMGB1-stimulated HUVECs which were pretreated with I3G. As demonstrated in Figure 4, I3G suppressed the expression of VCAM-1 (Fig. 4A), ICAM-1, and E-selectin (data not shown). The adhesion of leukocytes to endothelial cells and transendothelial migration (TEM) of monocytes are important steps in the proinflammatory response [Bae et al., 2007; Hansson and Libby, 2006]. We conducted studies to determine whether the expression of CAMs correlated with enhanced binding of leukocytes and whether I3G could block the adhesion of monocytes to HMGB1-stimulated HUVECs. The results shown in Figure 4B demonstrated that I3G effectively inhibited the binding of leukocytes to HMGB1-stimulated endothelial cells. Further studies revealed that the adhesion of leukocytes to endothelial cells was associated with their subsequent TEM and that I3G also effectively inhibited this step (Fig. 4C). To confirm this

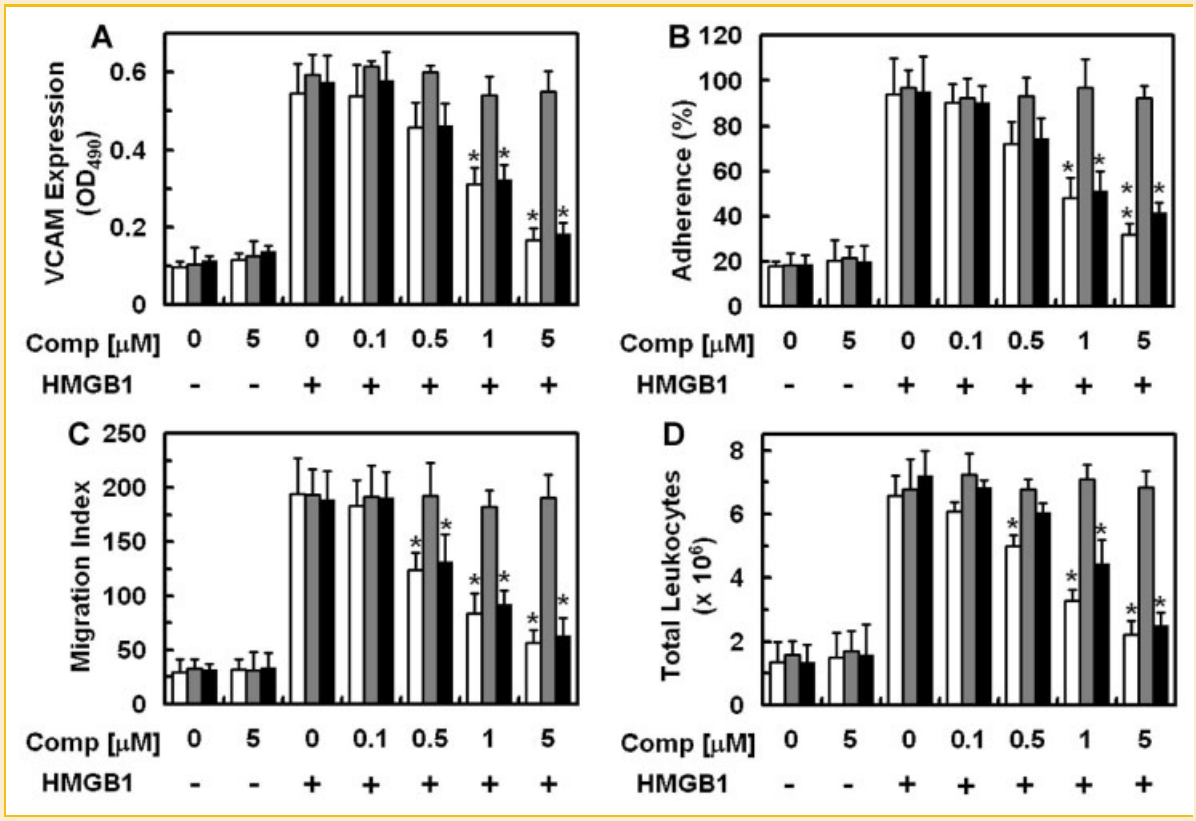

Fig. 4. Effects of I3G on HMGB1-induced CAM expressions, adhesion, and migration. A: HMGB1 (1 μg/ml)-mediated expression of VCAM-1 in HUVECs was analyzed after treating monolayers with the indicated concentrations of KP (black box), hyperoside (gray box), and I3G (white box) for 6 h. B: HMGB1 (1 μg/ml)-mediated adherence of leukocytes to HUVEC monolayers was analyzed after treating cells with the indicated concentrations of KP (black box), hyperoside (gray box), and I3G (white box) for 6 h. C: HMGB1 (1 μg/ml for 16 h)-mediated migration of leukocytes through HUVEC monolayers were analyzed after treating cells with the indicated concentrations of KP (black box), hyperoside (gray box), and I3G (white box) for 6 h. D: HMGB1 (2 μg/mouse, i.v.) mediated migration of leukocytes into the peritoneal cavity of mice was analyzed after treating mice with KP (6 μg/mouse, i.v., black box), hyperoside (4.6 μg/mouse, i.v., gray box), and I3G (4.8 μg/mouse, i.v., white box; expressed $\times 10^6$, n = 5). All results are expressed as means ± SD of three experiments. $^*P < 0.01$ as compared with HMGB1 alone.

effect in vivo, HMGB1-induced leukocyte migration in mice was examined. HMGB1 significantly stimulated leukocyte migration into the peritoneal cavity of mice and I3G at doses of 1–5 μM significantly decreased leukocytes counts (Fig. 4D). These results suggest I3G not only inhibits the endotoxin-mediated release of HMGB1 by endothelial cells but that it also down regulates the pro-inflammatory signaling effect of released HMGB1, and thereby inhibits the amplification of inflammatory pathways by nuclear cytokines.

I3G INHIBITS HMGB1-STIMULATED NF-κB ACTIVATION AND PRODUCTION OF TNF-α

Activation of NF-κB is necessary for proinflammatory responses [Marui et al., 1993; Lockyer et al., 1998]. Thus, we next examined the effect of I3G on the activation of Phospho-NF-κB p65 in HMGB1-stimulated HUVECs. HMGB1 treatment caused a significant increase the activation of Phospho-NF-κB p65 as measured by ELISA (Fig. 5A). In contrast, pretreatment of I3G in HUVECs significantly suppressed the activation of Phospho-NF-κB p65 by HMGB1 (Fig. 5A). We next attempted to evaluate the potential effect of I3G on the production of the proinflammatory cytokine, TNF-α. HUVECs were preincubated with I3G for 6 h and TNF-α levels were measured in the culture media using ELISA. The level of TNF-α was increased in HMGB1-stimulated endothelial cells and this increase was significantly decreased by I3G (Fig. 5B). Therefore, these results indicate that the anti-inflammatory effects of I3G were mediated through the inhibition of HMGB1-mediated activation of the NF-κB pathway as well as suppression of the induction of TNF-α in endothelial cells treated with HMGB1.

PROTECTIVE EFFECT OF I3G IN THE CLP MODEL

We hypothesized that treatment with I3G would reduce mortality in our CLP-induced sepsis mouse model. A mouse CLP model of sepsis was used to determine the effect of I3G on sepsis lethality. Twenty-four hours after operation, animals manifested signs of sepsis, such as, shivering, bristled hair, and weakness. To investigate whether I3G protects mice from CLP-induced sepsis lethality, I3G were administered to mice after CLP. The administration of I3G at a single dose (4 μg/mouse, 12 h after CLP) did not prevent CLP-induced death (data not shown). Therefore, we administered I3G twice (4.8 μg/mouse, once 12 h after CLP and once 50 h after CLP) and this

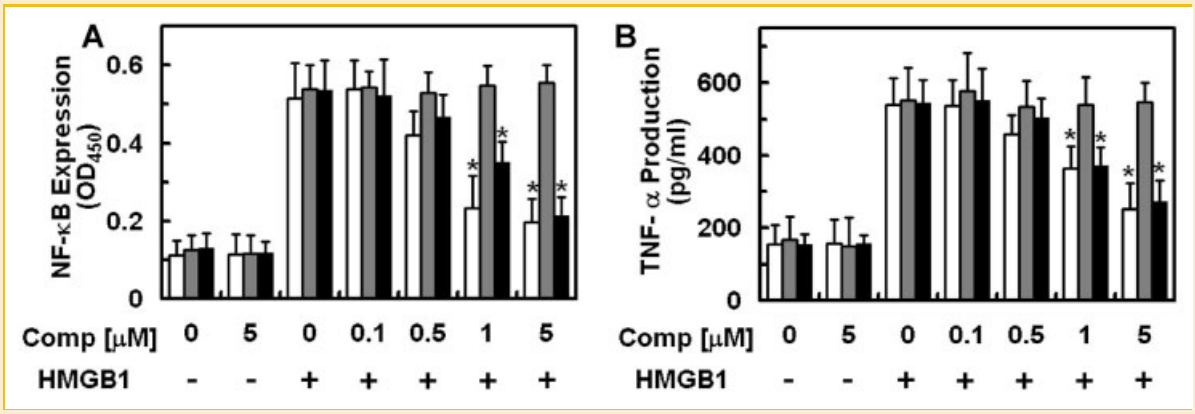

Fig. 5. Effects of I3G on HMGB1-induced NF-κB activation and production of TNF-α in HUVECs. A: HMGB1 (1 μg/ml)-mediated activation of Phospho-NF-κB p65 in HUVECs was analyzed after treating cells with KP (black box), hyperoside (gray box), and I3G (white box). B: HMGB1 (1 μg/ml)-mediated production of TNF-α was analyzed after treating cells with KP (black box), hyperoside (gray box), and I3G (white box). Results are expressed as means ± SD of different three experiments. $^{*}P < 0.01$ as compared with HMGB1 alone.

increased the survival rate to 40% (Fig. 6B). However, no benefit effect was observed for a lower I3G dose (1 μg/mouse, data not shown). This marked survival benefit achieved by administering I3G suggests that the suppression of HMGB1 release and of HMGB1-mediated inflammatory responses provides a therapeutic strategy for the management of sepsis and septic shock.

The molecular mechanisms of I3G for its anti-inflammatory responses are due to its inhibitory effects on the receptors of HMGB1. Recently, we reported that KP downregulated the cell surface expression of the two receptors, TLR2 and TLR4 [Kim et al., 2012a], which are known to bind HMGB1 to initiate pro-inflammatory responses in endothelial cells [Park et al., 2004]. In this study, we also showed that I3G inhibited LPS or HMGB1-mediated TLR2/4 expressions (data not shown). Although all three receptors for HMGB1 such as TLR2, TLR4, and RAGE (receptor for advanced glycation end products) were involved in the HMGB1-mediated signaling pathway [Wang et al., 2004b], the anti-inflammatory effects of I3G on HMGB1-mediated inflammatory responses were regulated by blocking the expression of TLR2 and TLR4. Therefore, there are two potential molecular mechanisms by which I3G could inhibit HMGB1-mediated inflammatory responses: (1) although I3G blocked HMGB1-mediated expression of TLR2 and TLR4 but not RAGE, I3G might block downstream signaling pathway of RAGE; (2) I3G could inhibit common downstream signaling molecules of all three receptors such as the activation of NF-κB and production TNF-α. HMGB1 and LPS both significantly increase the nuclear translocation of NF-κB and the phosphorylation of Akt and p38 MAPK [Silva et al., 2007]. Collectively, the finding that I3G inhibited the release of HMGB1 by LPS, the expressions of HMGB1 receptors and HMGB1-mediated activation of NF-κB and production of TNF-α strongly suggest that I3G could inhibit HMGB1 signaling pathway from the beginning (inhibition of expression of receptors) and to the following next step (inhibition of common inflammatory pathway such as inhibition of activation of NF-κB and production of TNF-α).

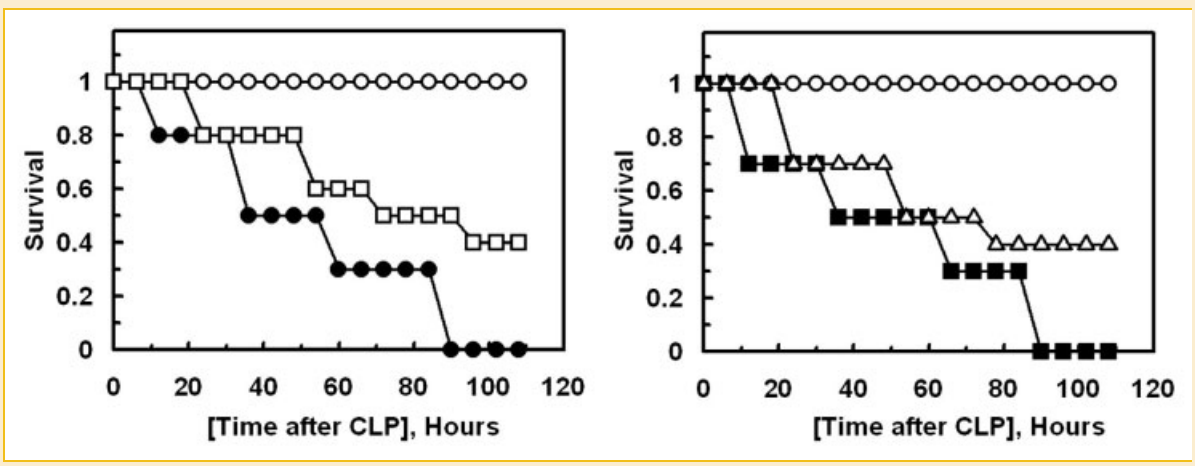

Fig. 6. Effects of I3G on lethality after CLP. Male C57BL/6 mice (n = 10) were administered KP (6 μg/mouse, i.v., □), hyperoside (4.6 μg/mouse, i.v., ■), and I3G (4.8 μg/mouse, i.v., △) at 12 and 50 h after CLP. Animal survival was monitored at 108 h after CLP. Survival was monitored six hourly. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline (n = 10).

This study shows that I3G inhibits LPS or CLP-mediated HMGB1 release and HMGB1-mediated barrier disruption by increasing barrier integrity and inhibiting the expressions of CAMs, and that these effects reduce leukocytes adhesion and migration in HUVECs. We also found the I3G inhibited phenomena associated with HMGB1-mediated cytoskeletal rearrangement. Moreover, these barrier protective effects of I3G were confirmed in a mouse model and I3G reduced mortality in CLP-induced septic mice. The methoxyl group at C-3′ in I3G positively regulate the anti-inflammatory activities because hyperoside in which a methoxyl group is changed to a hydroxyl group has no anti-inflammatory activities. Our findings indicate that I3G is a potential candidate for the treatment of severe vascular inflammatory diseases, such as, sepsis and septic shock.

ACKNOWLEDGMENTS

This study was supported by the National Research Foundation of Korea (NRF) funded by the Korea Government (MEST) (Grant No. 2011-0030124) and by the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No. A111305).

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