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Renal protective effects of zingerone in a mouse model of sepsis

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Abstract

Zingerone (ZGR), a phenolic alkanone isolated from ginger, has been reported to possess pharmacological activities such as anti-inflammatory and anti-apoptotic effects. This study was initiated to determine whether ZGR could modulate renal functional damage in a mouse model of sepsis and to elucidate the underlying mechanisms. The potential of ZGR treatment to reduce renal damage induced by cecal ligation and puncture (CLP) surgery in mice was measured by assessment of serum creatinine, blood urea nitrogen (BUN), lipid peroxidation, total glutathione, glutathione peroxidase activity, catalase activity, and superoxide dismutase activity. Treatment with ZGR resulted in elevated plasma levels of BUN and creatinine, and of protein in urine in mice with CLP-induced renal damage. Moreover, ZGR inhibited nuclear factor-κB activation and reduced the induction of nitric oxide synthase and excessive production of nitric acid. ZGR treatment also reduced the plasma levels of interleukin-6 and tumor necrosis factor-α, reduced lethality due to CLP-induced sepsis, increased lipid peroxidation, and markedly enhanced the antioxidant defense system by restoring the levels of superoxide dismutase, glutathione peroxidase, and catalase in kidney tissues. Our study showed renal suppressive effects of zingerone in a mouse model of sepsis, suggesting that ZGR protects mice against sepsis-triggered renal injury.

Key words; zingerone, sepsis, antioxidant, renal injury, renal toxicity

Introduction

Sepsis is defined as a systemic inflammatory response syndrome caused by infection and is a common cause of morbidity and mortality, despite recent advances in antibiotic therapy and intensive care (1). In the past 30 years, sepsis has become one of the major causes of hospital admission and medical expenses (2). Although activation of cytokines is part of the host defense response to infection, excessive production and secretion of cytokines can cause widespread tissue injury and organ failure (3). Septic conditions activate inducible nitric oxide synthase (iNOS) and increase the plasma concentration of nitric oxide (NO), which ultimately leads to cytotoxicity (4-5). Sepsis is also known to enhance the synthesis of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide (4-5). The excessive production of ROS can cause significant oxidative stress as indicated by a decrease in endogenous antioxidant defenses and lipid peroxidation. The rate of organ failure due to sepsis can be diminished by reduction of inflammatory cytokines and inhibition of iNOS activity (4-6). Since interventions that reduce the production or the effect of ROS have been shown to have beneficial effects on sepsis (7-8), agents that can decrease cytokine production and ROS might also prevent or lessen the pathological cascade of inflammation caused by sepsis.

The herbal plant *Zingiber officinale* is a natural dietary spice with potent anti-inflammatory, antioxidative, and anticancer properties (9). Zingerone (ZGR) [4-(4-hydroxy-3-methoxyphenyl) butan-2-one] is a stable active component of dry ginger rhizome (10) and has been reported to exhibit pharmacological activities such as anti-inflammatory and anti-apoptotic effects and to confer protection against myocardial infarction and irritable bowel disorder (11-15). However, to our knowledge, the possible protective effects ZGR against renal damage have not been studied. In the current study, we seek to remedy this deficit by investigating the renal

protective effects of ZGR in an animal sepsis model.

Results

Effects of ZGR on CLP-induced renal tissue injury

Previous reports showed that treatment of cells or mice with ZGR inhibited LPS-induced expression and activity of sPLA2-IIA which contributes to vascular inflammation (16) and ZGR has anti-platelet aggregation activity (17). In those studies, ZGR was used from at 10 μM to 50 μM in human endothelial cells and from at 0.14, 0.36, or 0.72 mg/kg in mice (16-17). Therefore, in this study, ZGR was also used from 0.07 to 0.72 mg/kg.

The effects of CLP surgery on nephrotoxic markers are shown in Table 1: plasma levels of BUN and creatinine, and protein levels in urine were significantly higher on the fourth day after CLP surgery than in the sham-operated group. Sham operation or administration of ZGR only to mice did not result in any obvious changes in plasma levels of BUN and creatinine, or of protein in urine. The increased levels of BUN and creatinine, and of protein in urine after surgery were blocked by a single administration of ZGR (0.07, 0.14, 0.29 or 0.72 mg/kg, 12 h after CLP, data not shown). Thus, we administered two equal doses of ZGR, one at 12 h after CLP and the other at 50 h after CLP. We found that ZGR decreased BUN, creatinine, and protein in urine levels (Table 1). Another important marker of tissue injury, LDH, was also reduced by ZGR in CLP-operated mice (Table 1).

Effects of ZGR on plasma nitrite and nitrate production after CLP surgery

The effects of ZGR treatment on inflammatory response in kidney tissue were investigated *in vivo* by measuring plasma nitrite and nitrate levels (stable end products of NO). In sham-operated and ZGR-only mice, the levels of plasma NO did not significantly change (Table 2). However, CLP surgery caused an approximately 7-fold increase in mouse plasma NO production with respect to that in control mice (Table 2). Post-surgery treatment with ZGR resulted in NO levels being up to 39% lower than those in the CLP group (Table 2).

Effects of ZGR on plasma TNF-α, IL-6, and MPO levels

The effects of ZGR on CLP-induced inflammatory responses were investigated through measurement of plasma levels of TNF-α and IL-6. CLP surgery significantly increased plasma TNF-α and IL-6 levels; post-surgery treatment with ZGR inhibited these increases (Table 2). Plasma concentrations of TNF-α and IL-6 were lower by 67 and 71%, respectively, in CLP + ZGR (0.72 mg/kg) group than in the CLP group. Next, we determined the effects of ZGR treatment on infiltration of neutrophils after CLP surgery. Kidney tissues were collected, homogenized, and centrifuged, and supernatants were assayed for MPO level by ELISA. MPO activity can act as an indicator of renal infiltration by neutrophils. We identified a marked increase in MPO level after CLP surgery (Table 2), which was associated with nephritis. Treatment with ZGR post-surgery resulted in a significantly lower MPO concentration in renal tissues than that in the CLP-operated mice. Moreover, during inflammatory response, inflammatory mediator proteins expression and MAPKs signaling pathways are closely involved in the regulation of inflammatory response. Therefore, we determined the effects of ZGR on the transcriptional regulation of cyclooxygenase (COX)-2 and mitogen-activated protein kinase (MAPK) such as p38 and janus kinase (JNK). Data showed that ZGR inhibited CLP-mediated expressions of COX-2, p38, and

JNK (Supp. Table 1).

Effect of ZGR on kidney tissue MDA

MDA concentration is an indicator of lipid peroxidation levels. In kidney tissues of CLP-operated mice, a significant increase in MDA levels was present (Table 3). Treatment with ZGR post-surgery led to significantly lower MDA levels than those in the control group.

Effect of ZGR on total GSH and activities of antioxidant enzymes in renal tissues

To test the effect of ZGR on CLP-induced oxidative stress, we analyzed the activities of the antioxidant GSH and the oxidative stress associated enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT). Total GSH levels and the activities of SOD, GSH-Px, and CAT were similar in the ZGR-only and sham-operated groups. In contrast, total GSH levels and renal activities of all three enzymes were reduced in the CLP mice. However, post-surgery treatment with *ZGR* increased total GSH and renal enzyme activities (Table 3). Moreover, ZGR suppressed the transcriptional expression levels of SOD, GSH-Px, and CAT by CLP (Supp. Table 1). Noting that nuclear factor-erythroid 2-related factor 2 (Nrf2) is a potential pathway to protective responses against oxidative stress, and heme oxygenase-1 (HO-1) is one of the most important enzymes for antioxidant pathway (18-19). Therefore, we determined the effects of ZGR on the induction of HO-1 and on the nuclear accumulation of Nrf2. Data showed that HO-1 protein expression was induced by ZGR (Figure 1A) and ZGR mediated the translocation of Nrf2 from the cytosol to nucleus in a concentration-dependent manner (S. Figure 1). Therefore, these results indicate that the Nrf2/HO-1 signaling axis plays an

important role in the anti-oxidant effects of ZGR.

Effect of ZGR on the levels of the renal proteins iNOS, IκB and NF-κB and on cellular toxicity

To explore the mechanisms responsible for mediating the anti-inflammatory effects of ZGR, we measured the levels of iNOS, IκB, and NF-κB proteins in kidney tissues of the mice. iNOS protein levels were low in kidney homogenates obtained from the control groups, but were significantly elevated in CLP-operated mice. Post-surgery treatment with ZGR significantly reduced this increase in iNOS (Figure 1B). Next, we investigated whether ZGR could inhibit the CLP-induced degradation of IκB and prevent the translocation of the subunit of NF-κB p65 from the cytosol to the nucleus. As shown in figure 1B, the translocation of nuclear factor (NF)-κB p65 to nucleus and activation of NF-κB p65 in nucleus and I inhibitory kappa B (IκB) phosphorylation in kidney significantly increased after CLP. Moreover, ZGR administration after CLP decreased NF-κB p65 activation and IκB phosphorylation compared with the CLP group. In addition, degradation of IκB was lower in the ZGR + CLP group than in the CLP-only group (Figure 1B).

Effect of ZGR in CLP-induced septic lethality

To evaluate whether the renal protective responses identified after ZGR treatment influenced the survival rate of mice with CLP-induced sepsis, we administered two equal doses of ZGR (0.72 mg/kg), one at 12 h after CLP and the other at 50 h after CLP. The ZGR treatment increased the rate of survival of mice with sepsis (55%), according to a Kaplan-Meier survival analysis ($p < 0.0001$, Figure 1C). The marked improvement in survival rate achieved by the treatment suggested that ZGR might be of value in therapies for severe vascular

inflammatory diseases, such as sepsis and septic shock.

Discussion

The aim of the present study was to evaluate the potential effects of ZGR, an active compound isolated from *C. tricuspidata*, on renal damage in mice with acute CLP-induced sepsis. Our data demonstrated that post-surgical treatment with ZGR significantly ameliorated CLP-induced deterioration in renal function. Furthermore, ZGR reduced the levels of TNF-α, IL-6, NO, and MPO that were elevated after CLP; additionally, ZGR treatment reduced elevated iNOS levels after CLP surgery. These ameliorative effects were accompanied by an increase in the activities of antioxidant enzymes and a reduction in the levels of lipid peroxidation products in renal tissues. The underlying molecular mechanism of ZGRs renal protective effects appeared to involve the suppression of NF-κB activation. Therefore, the results of the present study suggested that ZGR might potentially have beneficial effects in therapies to prevent acute renal injury due to sepsis.

We found that excretion of urinary proteins and the levels of plasma BUN and creatinine were increased after CLP surgery; these findings are consistent with previous reports (20-22). Our study also found that changes in kidney function after CLP surgery could be ameliorated by ZGR treatment, since significant reductions in BUN, creatinine, and urine protein levels were identified.

NO is an important proinflammatory molecule that is released during inflammatory responses. In pathological conditions, iNOS is induced and then NO is synthesized, and can affect many parts of the inflammatory cascade (8). There is substantial evidence that CLP-mediated renal inflammatory damage may be

due to increased iNOS activity and consequent abnormal NO levels (4-6). Our results showed that CLP-operated mice had enhanced NO production in blood and iNOS expression in kidney tissue; these changes were significantly reduced by treatment with ZGR.

TNF-α and IL-6 are involved in CLP-induced tissue damage and are regarded as major regulators of severe inflammatory diseases such as sepsis or septic shock (3, 23). In this study, we found that ZGR treatment reduced the levels of TNF-α and IL-6. Since the increased release of cytokines, particularly TNF-α and IL-6, appears to be an essential aspect of pathogenesis in the inflammation process, the inhibitory effects of ZGR on CLP-induced TNF-α and IL-6 production might be a crucial step in the anti-inflammatory action of ZGR. The nuclear transcription factor NF-κB amplifies and regulates many genes, including multiple cytokines and iNOS in response to inflammatory stimuli. When activated by such stimuli, NF-κB dissociates from IκB and translocates to the nucleus, leading to gene transcription (24). NF-κB is a promising target for treating a variety of diseases because it plays a diverse role in the expression of inflammatory genes. In this study, ZGR blocked the CLP-induced activation of NF-κB by inhibiting the degradation of IκB. These observations indicate that interference with NF-κB might explain, as least in part, the inhibitory effects of ZGR on iNOS, TNF-α, and IL-6 levels. Recently, we reported the anti-septic effects of ZGR on high mobility group box 1 (HMGB1), a late mediator of sepsis, -mediated severe vascular inflammatory responses (11). We showed that ZGR reduced HMGB1 release in bacterial lipopolysaccharide-activated HUVECs, suppressed CLP-mediated release of HMGB1, expression of HMGB1 receptors, and HMGB1-mediated barrier disruption by increasing barrier integrity (11). Therefore, the renal protective activities of ZGR in this study were further confirmed by the anti-inflammatory activities of ZGR on the HMGB1-mediated severe vascular inflammatory responses (11).

The antioxidant enzymes SOD, CAT, and GSH-Px are considered to be the primary defenses against oxidative damage in tissues (25). Septic conditions have been found to impair the balance between free radical scavenging and production by cellular antioxidant systems (26-27). Our data showed decreased levels of the three enzymes in kidney tissue in CLP-operated mice; the activities of these enzymes were significantly raised by ZGR treatment. These results indicate that may have potential therapeutic value in oxidative stress-associated kidney diseases. The major lipid peroxidation product, MDA, is a good indicator of oxidative stress; a negative correlation has been reported between the MDA level and the activities of endogenous antioxidant enzymes (28-30). Here, our data showed that renal MDA levels were increased in CLP-operated mice and that ZGR treatment significantly reduced this increase. As described above, ZGR may promote the activities and levels of SOD, CAT, and GSH-Px in kidney tissues of CLP mice. Therefore, our data indicate that ZGR could provide renal protection against CLP-induced oxidative injury via inhibiting lipid peroxidation as well as promoting the activities and expression of endogenous antioxidant enzymes.

In conclusion, this study has demonstrated a renal protective effect by ZGR against CLP-induced kidney injury and septic lethality. The ameliorative effects of ZGR were associated with down-regulation of TNF-α and IL-6 production reduction of iNOS expression, and lowering of NO production by blocking the NF-κB pathway. These effects were accompanied by enhanced antioxidant defense and decreased lipid peroxidation in the kidney and plasma *in vivo*. Overall, our results suggest that ZGR has the potential to be considered for therapeutic use in the treatment of renal inflammatory damage and sepsis-induced oxidative stress.

Materials and Methods

Reagents

ZGR was purchased from Sigma (St. Louis, MO). Nuclear and cytoplasmic protein extraction reagent kits were obtained from Thermo Scientific Company (Rockford, lL). Antibodies against iNOS, IκB, phospho-IκB, NF-κB, phosphor-NF-κB, Lamin-B, and β-actin were purchased from Cell Signaling Technology (Danvers, MA).

Animals and cecal ligation and puncture

Male C57BL/6 mice (6-7 weeks old) were obtained from Orient Bio Co. (Sungnam, Republic of Korea) and were given a 12 d acclimatization period. The mice were housed under controlled temperature (20–25°C) and humidity conditions (40–45% RH), with a 12 h light:12 h dark cycle. They were fed a normal rodent pellet diet and had *ad libitum* access to water during acclimatization. To induce sepsis, the mice were first anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and Rompum (xylazine, 10 mg/kg). Sepsis was induced using cecal ligation and puncture (CLP) as previously described (31-32). As controls, sham-operated animals were used: in these mice, the cecum was exposed, but not ligated or punctured, and then returned to the abdominal cavity. Animals were randomly divided into 7 treatment groups (n = 10 each): sham-operated control; ZGR-only (0.72 mg/kg body weight) in 0.5% DMSO; CLP surgery only; and CLP + ZGR (0.07, 0.14, 0.29, or 0.72 mg/kg body weight). ZGR was intravenously injected at 12 h after CLP and again at 50 h after CLP. Blood and organ samples were collected 4 days after ZGR injection for functional assays. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2017-102).

Sample preparation

Four days after ZGR injection, the mice were anesthetized as described above and sacrificed. Blood samples were collected from the posterior vena cava and allowed to clot. Serum was separated by centrifugation at 4,000 rpm for 10 min, stored at -80°C until analyzed and was used for the assessment of plasma BUN and creatinine levels. Kidney samples were immediately removed and weighed. The kidneys were then minced with scissors and homogenized in 0.1 M phosphate buffer saline (pH 7.4); the tissue was fractionated under refrigeration by centrifugation at 10,000 x g for 10 min. The homogenate was stored at -80°C until analyzed in the various biochemical assays. Protein concentrations were determined using the Bradford assay.

Evaluation of nephrotoxicity and lactate dehydrogenase

Renal dysfunction was assessed by measuring the changes in levels of BUN and creatinine, and of protein in urine. BUN, creatinine, and lactate dehydrogenase (LDH), another important marker of tissue injury, were measured using commercial assay kits (Pointe Scientific, Linclon Park, MI). Urine samples were collected from each animal using a metabolic cage at 12 h after CLP surgery and the supernatant was obtained. Urinary protein concentrations were determined by the Bradford assay, using BSA as the protein standard.

Plasma nitrite/nitrate determination

Nitrite and nitrate concentrations in the plasma were determined using Griess reagents and vanadium solution (VCl_3) as previously described (33). Briefly, 100 μL of VCl_3 were added to 100 μL of sample,

immediately followed by Griess reagents (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). After 30 min of color development, absorbance was determined by measuring optical density (OD) at 540 nm using a microplate reader (Tecan Austria GmbH, Austria). Concentrations were determined by comparing absorptions with those of a standard curve of sodium nitrite.

ELISA for TNF-α, IL-6, and HO-1

The plasma concentrations of IL-6, TNF-α, HO-1 were determined using ELISA kits (R&D Systems, Minneapolis, MN). Values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

Renal myeloperoxidase activity

Renal myeloperoxidase (MPO) activity was used as a quantitative indicator for neutrophil influx into the kidney; MPO activity was measured using ELISA kits (Abcam, UK).

Evaluation of oxidative stress markers

Lipid peroxidation was determined using a method to measure the formation of thiobarbituric acid reactive substances (TBARSs). The level of malondialdehyde (MDA) in kidney tissue was measured spectrophotometrically using an OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA). MDA values were expressed as nM/mg protein. Total glutathione (GSH) contents of kidney tissue were measured as described previously (34). A tissue homogenate was prepared, and then samples were added to metaphosphoric acid and allowed to stand for 5 min to precipitate proteins. Phosphate buffer and 5,5′-dithiobis-2-nitro-benzoic acid were

added for color development. GSH was determined by measuring absorbance at 415 nm and absolute concentrations were calculated using a GSH standard (Sigma Aldrich, St. Louis, MO). Values of total GSH were expressed as nM/mg protein. Superoxide dismutase (SOD) activity was measured using a SOD assay kit (Fluka). Values of SOD were expressed as U/mg protein. Glutathione peroxidase (GSH-Px) activity was determined using the cellular activity assay kit CGP-1 (Sigma Aldrich). Values of GSH-Px were expressed as U/mg protein. Catalase activity (CAT) was determined by a CAT assay kit (Sigma Aldrich) using the decomposition rate of the substrate H_2O_2 as determined at 240 nm. Total CAT values were expressed as U/mg protein.

Western blots from renal tissue

Kidney samples were homogenized with radioimmunoprecipitation (RIPA) buffer containing protease inhibitors; equal amounts of protein were separated by SDS-PAGE (10%) and were electroblotted overnight onto an Immobilon membrane (Millipore, Billerica, MA). Or, nuclear and cytoplasmic protein was prepared using extraction kit (Thermo Scientific Company, Rockford, lL). The membranes were blocked for 1 h with 5% low-fat milk-powder TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and were then incubated with primary antibodies for iNOS, IκB, phospho-IκB, phospho-NF-κB, NF-κB, Nrf2, Lamin-B, and β-actin, at 4°C overnight. Subsequently, the membranes were incubated with horseradish-peroxidase-conjugated secondary antibody, and enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained as previously described (31, 35). HUVECs at passages 3-5 were used.

Statistical Analysis

All experiments were performed independently at least three times. Values are expressed as means ± standard deviation (SD). The statistical significance of differences between test groups was evaluated using SPSS for Windows, version 16.0 (SPSS, Chicago, IL). Statistical relevance was determined by one-way analysis of variance (ANOVA) and Tukey's post-hoc test. *P* values less than 0.05 were considered to indicate significance.

References

1. Russell JA (2006) Management of sepsis. N Engl J Med 355, 1699-1713
2. Singer M, Deutschman CS, Seymour CW et al. (2016) The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA 315, 801-810
3. Chaudhry H, Zhou J, Zhong Y et al. (2013) Role of cytokines as a double-edged sword in sepsis. In Vivo 27, 669-684
4. Parratt JR (1998) Nitric oxide in sepsis and endotoxaemia. J Antimicrob Chemother 41 Suppl A, 31-39
5. Symeonides S and Balk RA (1999) Nitric oxide in the pathogenesis of sepsis. Infect Dis Clin North Am 13, 449-463, x
6. Draisma A, Dorresteijn MJ, Bouw MP, van der Hoeven JG and Pickkers P (2010) The role of cytokines and inducible nitric oxide synthase in endotoxemia-induced endothelial dysfunction. J Cardiovasc Pharmacol 55, 595-600
7. Vincent JL, Zhang H, Szabo C and Preiser JC (2000) Effects of nitric oxide in septic shock. Am J Respir Crit Care Med 161, 1781-1785
8. Cadenas S and Cadenas AM (2002) Fighting the stranger-antioxidant protection against endotoxin toxicity. Toxicology 180, 45-63

9. Park KK, Chun KS, Lee JM, Lee SS and Surh YJ (1998) Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice. Cancer Lett 129, 139-144
10. Sies H and Masumoto H (1997) Ebselen as a glutathione peroxidase mimic and as a scavenger of peroxynitrite. Adv Pharmacol 38, 229-246
11. Lee W, Ku SK and Bae JS (2017) Zingerone reduces HMGB1-mediated septic responses and improves survival in septic mice. Toxicol Appl Pharmacol 329, 202-211
12. Kim MK, Chung SW, Kim DH et al. (2010) Modulation of age-related NF-kappaB activation by dietary zingerone via MAPK pathway. Exp Gerontol 45, 419-426
13. Rao BN, Archana PR, Aithal BK and Rao BS (2011) Protective effect of zingerone, a dietary compound against radiation induced genetic damage and apoptosis in human lymphocytes. Eur J Pharmacol 657, 59-66
14. Hemalatha KL and Prince PS (2015) Preventive effects of zingerone on altered lipid peroxides and nonenzymatic antioxidants in the circulation of isoproterenol-induced myocardial infarcted rats. J Biochem Mol Toxicol 29, 63-69
15. Banji D, Banji OJ, Pavani B, Kranthi Kumar C and Annamalai AR (2014) Zingerone regulates intestinal transit, attenuates behavioral and oxidative perturbations in irritable bowel disorder in rats. Phytomedicine 21, 423-429
16. Lee IC, Kim DY and Bae JS (2017) Inhibitory Effect of Zingerone on Secretory Group IIA Phospholipase A2. Natural Product Communications 12, 929-932
17. Lee W, Ku SK, Kim MA and Bae JS (2017) Anti-factor Xa activities of zingerone with anti-platelet aggregation activity. Food Chem Toxicol 105, 186-193
18. Agca CA, Tuzcu M, Hayirli A and Sahin K (2014) Taurine ameliorates neuropathy via regulating NF-kappaB and Nrf2/HO-1 signaling cascades in diabetic rats. Food Chem Toxicol 71, 116-121
19. Yu M, Xu M, Liu Y et al. (2013) Nrf2/ARE is the potential pathway to protect Sprague-Dawley rats against oxidative stress induced by quinocetone. Regul Toxicol Pharmacol 66, 279-285
20. Huber-Lang M, Sarma VJ, Lu KT et al. (2001) Role of C5a in multiorgan failure during sepsis. J Immunol 166, 1193-1199
21. Bhargava R, Altmann CJ, Andres-Hernando A et al. (2013) Acute lung injury and acute kidney injury are established by four hours in experimental sepsis and are improved with pre, but not post, sepsis administration of TNF-alpha antibodies. PLoS One 8, e79037
22. Guo RF and Ward PA (2006) C5a, a therapeutic target in sepsis. Recent Pat Antiinfect Drug Discov 1, 57-65
23. Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S and Remick DG (2011) The pathogenesis of sepsis. Annu Rev Pathol 6, 19-48
24. Oeckinghaus A and Ghosh S (2009) The NF-kappaB family of transcription factors and its regulation. Cold Spring Harb Perspect Biol 1, a000034
25. Birben E, Sahiner UM, Sackesen C, Erzurum S and Kalayci O (2012) Oxidative stress and antioxidant defense. World Allergy Organ J 5, 9-19
26. Horton JW (2003) Free radicals and lipid peroxidation mediated injury in burn trauma: the role

of antioxidant therapy. Toxicology 189, 75-88
27. Hatwalne MS (2012) Free radical scavengers in anaesthesiology and critical care. Indian J Anaesth 56, 227-233
28. Qiao Y, Bai XF and Du YG (2011) Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. Int Immunopharmacol 11, 121-127
29. Zhang J, Yu Y, Zhang Z, Ding Y, Dai X and Li Y (2011) Effect of polysaccharide from cultured Cordyceps sinensis on immune function and anti-oxidation activity of mice exposed to 60Co. Int Immunopharmacol 11, 2251-2257
30. Xiao JH, Xiao DM, Chen DX, Xiao Y, Liang ZQ and Zhong JJ (2012) Polysaccharides from the Medicinal Mushroom Cordyceps taii Show Antioxidant and Immunoenhancing Activities in a D-Galactose-Induced Aging Mouse Model. Evidence-Based Complementary and Alternative Medicine
31. Jung B, Kang H, Lee W et al. (2016) Anti-septic effects of dabrafenib on HMGB1-mediated inflammatory responses. BMB Rep 49, 214-219
32. Wang H, Liao H, Ochani M et al. (2004) Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 10, 1216-1221
33. Miranda KM, Espey MG and Wink DA (2001) A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5, 62-71
34. Beutler E, Duron O and Kelly BM (1963) Improved method for the determination of blood glutathione. J Lab Clin Med 61, 882-888
35. Kim J and Bae JS (2016) ROS homeostasis and metabolism: a critical liaison for cancer therapy. Exp Mol Med 48, e269

Additional Information

Competing financial interests: The authors declare no competing financial interests.

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Figure Legend

Figure 1. Effects of ZGR treatment on the expression levels of HO-1 in HUVECs, renal iNOS, IκB and NF-κB expression in CLP-operated mice and on CLP-induced septic lethality. (A) HUVECs were harvested after treatment with aloin (0–50 μM) for 16 h. The extracted proteins were subjected to ELISA for HO-1 expression. (B) Sham, sham-operated mice; Sham + ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h after sham operation. CLP, CLP-operated mice; CLP + ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h after CLP surgery (from left line). Western blots of iNOS, phosphor-IκB, IκB, phosphor-NF-κB, NF-κB, Lamin-B, and β-actin. The image is representative of results obtained from three different experiments. (C) Male C57BL/6 mice (n = 20) were administered ZGR at 0.72mg/kg (i.v. □) at 12 h and 50 h after CLP. Animal survival was monitored every 12 h for 132 h after CLP. Control CLP mice (●) and sham-operated mice (○) were administered sterile saline (n = 20). Kaplan-Meier survival analysis was used to determine the overall survival rates versus CLP treated mice. D = 0.2% DMSO is the vehicle control. *$p < 0.05$ versus DMSO (A).

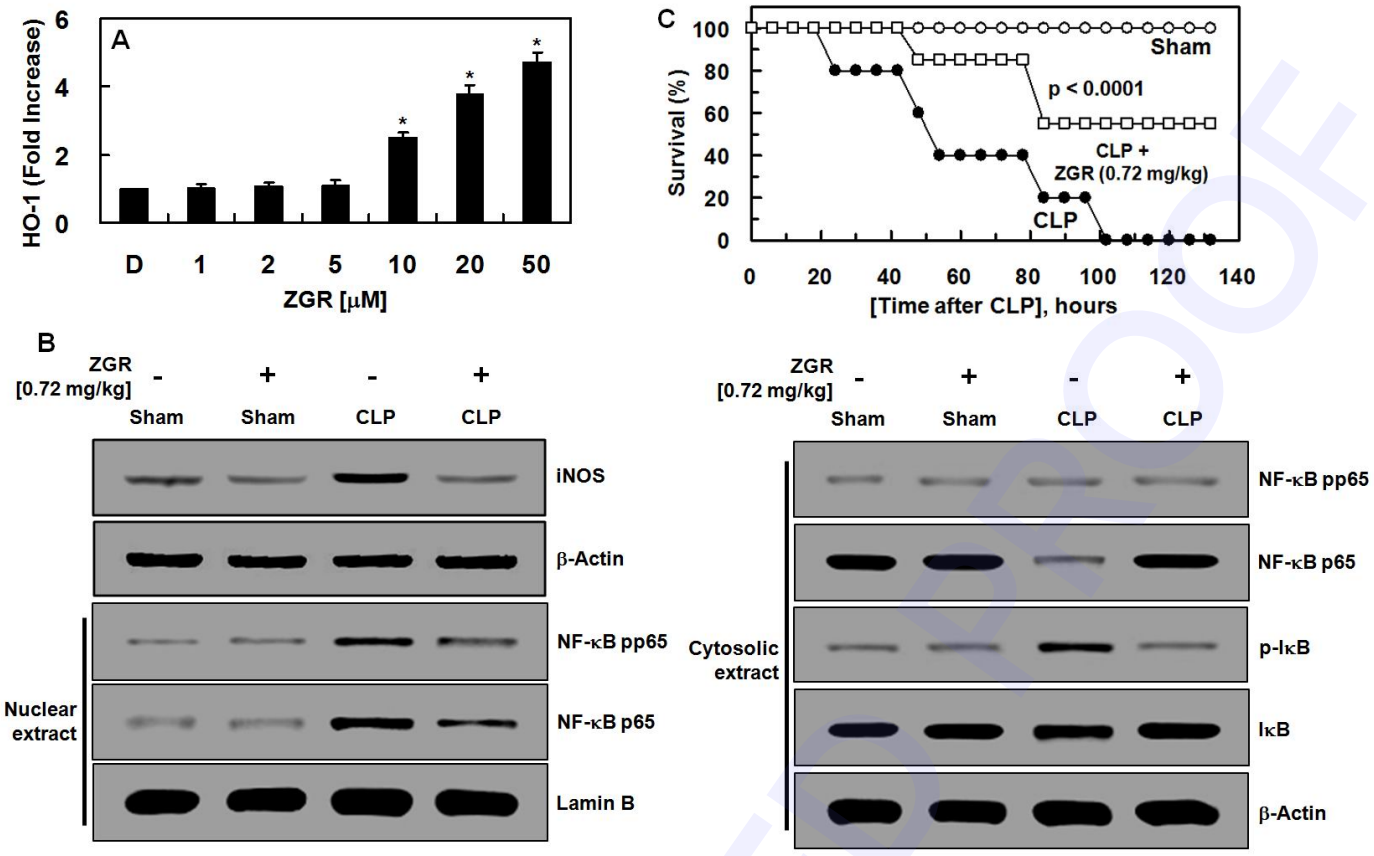


Fig. 1. Figure 1

Table 1. Effects of ZGR treatment on plasma levels of BUN and creatinine and urine level of protein in CLP-operated mice[a].

	BUN (mg/dL)	Creatinine (mg/dL)	Urine protein (mg/12 hour)	LDH (U/dL)
Sham	17.2 ± 1.1	0.118 ± 0.011	2.2 ± 0.15	291 ± 23.3
ZGR (0.72 mg/kg)	18.1 ± 0.9	0.121 ± 0.017	2.3 ± 0.28	285 ± 23.6
CLP	78.4 ± 6.3	0.489 ± 0.035	14.1 ± 0.89	3520 ± 255.2
CLP + ZGR (0.07 mg/kg)	77.5 ± 5.1	0.492 ± 0.031	13.8 ± 0.55	3495 ± 205.6
CLP + ZGR (0.14 mg/kg)	65.2 ± 5.2*	0.325 ± 0.023*	8.5 ± 0.57*	2584 ± 189.5*
CLP + ZGR (0.29 mg/kg)	47.1 ± 3.5*	0.288 ± 0.023*	5.3 ± 0.41*	1875 ± 156.7*
CLP + ZGR (0.72 mg/kg)	29.7 ± 2.2*	0.231 ± 0.022*	3.5 ± 0.22*	1285 ± 105.4*

[a]Each value represents the mean ± SD (n = 10).

Sham, sham-operated mice; ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; ZGR + CLP, mice treated with ZGR at 12 and 50 h after CLP surgery.

* $p < 0.05$ as compared to CLP.

Table 2. Effects of ZGR treatment on NO, TNF-α, IL-6 levels and renal MPO activity in CLP-operated mice[a].

	NO (μM)	TNF-α (pg/mL)	IL-6 (pg/mL)	MPO (U/g tissue)
Sham	32.52 ± 2.35	125.69 ± 10.21	0.85 ± 0.05	0.58 ± 0.06
ZGR (0.72 mg/kg)	34.26 ± 3.11	135.69 ± 12.38	0.67 ± 0.04	0.68 ± 0.07
CLP	231.62 ± 15.98	582.35 ± 29.65	85.61 ± 4.95	3.89 ± 0.52
CLP + ZGR (0.07 mg/kg)	225.57 ± 20.31	548.25 ± 32.65	86.39 ± 5.15	3.89 ± 0.47
CLP + ZGR (0.14 mg/kg)	174.36 ± 15.37*	358.24 ± 25.96*	62.27 ± 2.58*	2.69 ± 0.25*
CLP + ZGR (0.29 mg/kg)	135.29 ± 12.17*	304.58 ± 25.65*	35.21 ± 3.58*	2.03 ± 0.15*
CLP + ZGR (0.72 mg/kg)	92.28 ± 8.57*	189.51 ± 12.38*	25.14 ± 2.39*	1.25 ± 0.11*

[a]Each value represents the mean ± SD (n = 10).

Sham, sham-operated mice; ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; ZGR + CLP, mice treated with ZGR at 12 and 50 h after CLP surgery.

* $p < 0.05$ as compared to CLP.

Table 3. Effects of ZGR treatment on MDA level and the activities of renal antioxidant enzymes in CLP-operated mice[a].

	MDA (nM/mg protein)	GSH (nM/mg protein)	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)
Sham	189.31 ± 11.65	26.37 ± 2.02	1.11 ± 0.03	2.38 ± 0.19	4.31 ± 0.25
ZGR (0.72 mg/kg)	191.57 ± 15.68	28.05 ± 1.89	1.09 ± 0.05	2.41 ± 0.24	4.52 ± 0.33
CLP	325.69 ± 30.25	16.89 ± 1.41	0.72 ± 0.05	1.35 ± 0.12	2.95 ± 0.29
CLP + ZGR (0.07 mg/kg)	318.72 ± 27.68	17.05 ± 1.68	0.74 ± 0.06	1.37 ± 0.11	2.89 ± 0.22
CLP + ZGR (0.14 mg/kg)	260.17 ± 18.98*	21.08 ± 1.85*	0.82 ± 0.07*	1.78 ± 0.11*	3.51 ± 0.31*
CLP + ZGR (0.29 mg/kg)	224.58 ± 18.68*	23.58 ± 2.32*	0.89 ± 0.05*	1.92 ± 0.14*	3.95 ± 0.25*
CLP + ZGR (0.72 mg/kg)	201.15 ± 17.52*	24.85 ± 2.62*	0.94 ± 0.08*	2.14 ± 0.15*	4.15 ± 0.28*

[a]Each value represents the mean ± SD (n = 10).

Sham, sham-operated mice; ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; ZGR + CLP, mice treated with ZGR at 12 and 50 h after CLP surgery.

* $p < 0.05$ as compared to CLP.

[Supplementary Information]

Renal protective effects of zingerone in a mouse model of sepsis

Bong-Seon Lee[1†], Changhun Lee[1†], Sumin Yang[1], Sae-Kwang Ku[2]*, and Jong-Sup Bae[1]*

Materials and Methods

RNA isolation and real-time quantitative RT-PCR

Total RNA was isolated using RNeasy (Qiagen, Valencia, CA). An aliquot (5 μg) of extracted RNA was reverse transcribed into first-strand cDNA with a PX2 Thermal Cycler (Thermo Scientific), using 200 U/μL M-MLV reverse-transcriptase (Invitrogen, Grand Island, NY) and 0.5 mg/μL of oligo (dT)-adapter primer (Invitrogen, Grand Island, NY) in a 20-μL reaction mixture. Real-time PCR for COX-2, p38, JNK, and GAPDH was performed with a Mini Opticon Real-Time PCR System (Bio-Rad, Hercules, CA), using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The sequences of the primers were as follows: for COX-2, sense 5′-GCAAATCCTTGCTGTTCCAATC-3′ and antisense 5′-GGAGAAGGCTTCCCAGCTTTTG-3′; for p38 sense 5′-GGA GAA GAT GCT CGT TTT GGA-3′ and antisense 5′-TTG GTC AAG GGG TGG TGG-3; for JNK sense 5′-CGT CTG GTG GAA GGA GAG AG-3′ and antisense 5′-TAA TAA CGG GGG TGG AGG AT-3′; for SOD1 sense 5′-CCA GTG CAG GAC CTC ATT TT-3′ and antisense 5′-CAC CTT TGC CCA AGT CAT CT-3′; for GSH-Px1 sense 5′-GGT TCG AGC CCA ATT TTA CA-3′ and antisense 5′-CCC ACC AGG AAC TTC TCA AA-3′; for CAT sense 5′-GCT GAG AAG CCT AAG AAC GCA AT-3′ and antisense 5′-CCC TTC GCA GCC ATG TG-3′; for GAPDH, sense 5′-CGG AGT CAA CGG ATT TGG TCG TAT -3′ and antisense 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′. The PCR settings were as follows: initial denaturation at 95°C was followed by 35 cycles of amplification for 15 s at 95°C and 20 s at 60°C, with subsequent melting curve analysis, increasing the temperature from 72 to 98°C. Quantification of gene expression was calculated relative to GAPDH.

Figure Legend

Figure 1. Effects of ZGR on the nuclear accumulation of Nrf2. HUVECs were harvested after treatment with aloin (5–50 μM) for 16 h, and their cytosolic and nuclear fractions were extracted using a separation kit. The extracted proteins were subjected to western blotting for Nrf2. The image is representative of results obtained from three different experiments.

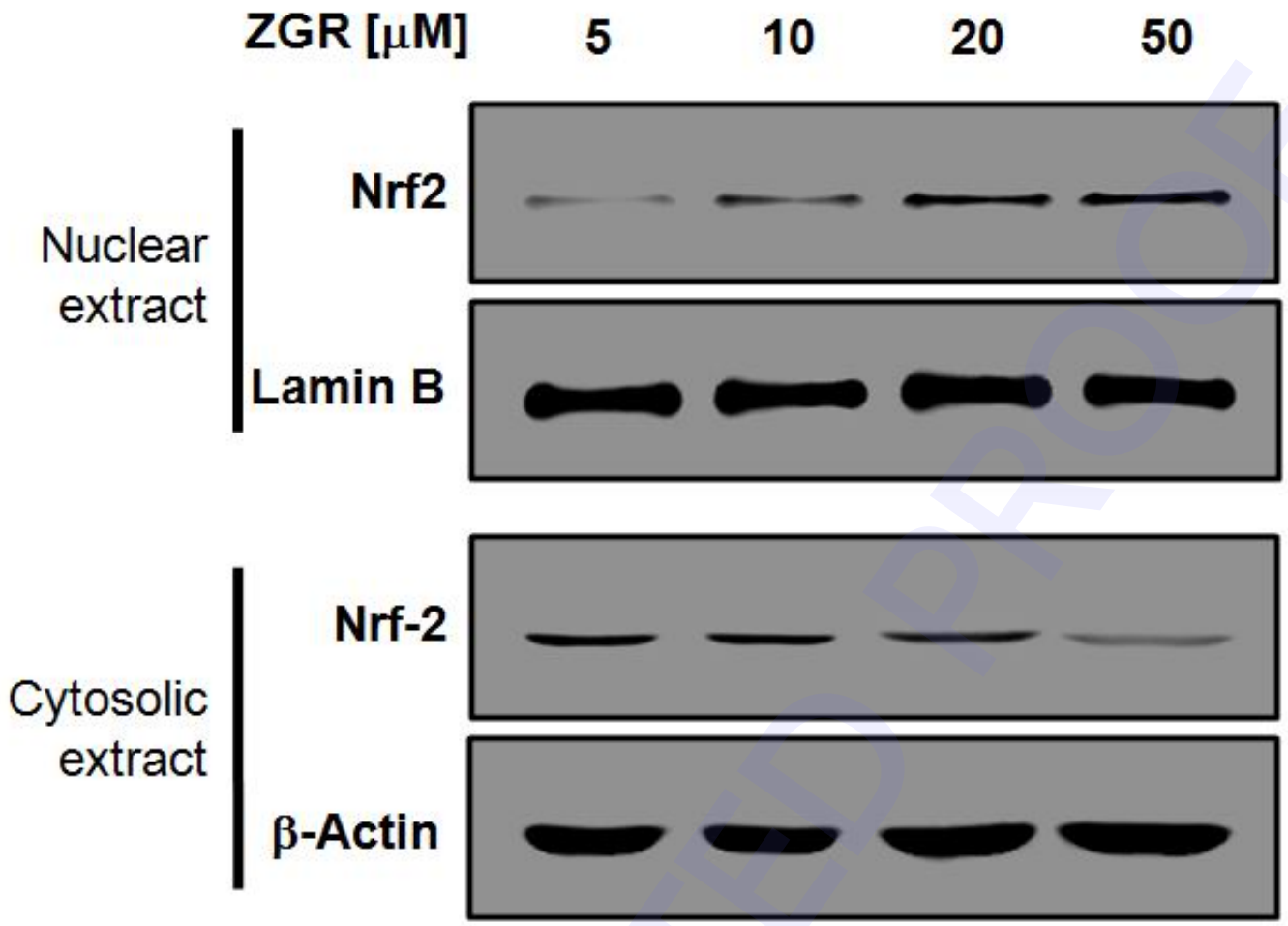
Supplementary Table 1. Effect of ZGR on the expressions of COX-2, p38, JNK MAPK, SOD, GSH-Px, and CAT mRNA[a].

	COX-2 mRNA (fold change)	p38 mRNA (fold change)	JNK mRNA (fold change)	SOD 1 mRNA (fold change)	GSH-Px mRNA (fold change)	CAT mRNA (fold change)
Sham	1	1	1	1	1	1
ZGR (0.72 mg/kg)	1.11 ± 0.07	1.18 ± 0.05	1.15 ± 0.05	1.05 ± 0.03	1.06 ± 0.06	1.11 ± 0.04
CLP	11.28 ± 1.23	7.72 ± 0.62	8.35 ± 0.52	6.95 ± 0.47	4.01 ± 0.32	6.59 ± 0.42
CLP + ZGR (0.07 mg/kg)	11.38 ± 1.05	7.82 ± 0.59	8.29 ± 0.65	7.02 ± 0.56	4.32 ± 0.38	6.39 ± 0.53
CLP + ZGR (0.14 mg/kg)	7.36 ± 0.52*	5.82 ± 0.43*	5.32 ± 0.41*	5.15 ± 0.38*	3.65 ± 0.25*	4.35 ± 0.37*
CLP + ZGR (0.29 mg/kg)	5.72 ± 0.41*	4.35 ± 0.35*	3.95 ± 0.37*	4.06 ± 0.39*	2.85 ± 0.23*	3.52 ± 0.31*
CLP + ZGR (0.72 mg/kg)	3.17 ± 0.21*	3.08 ± 0.29*	2.68 ± 0.19*	3.25 ± 0.21*	2.07 ± 0.21*	2.68 ± 0.22*

[a]Each value represents the mean ± SD (n = 10).

Sham, sham-operated mice; ZGR, mice treated with ZGR (0.72 mg/kg body weight) at 12 and 50 h; CLP, CLP-operated mice; ZGR + CLP, mice treated with ZGR at 12 and 50 h after CLP surgery.

* $p < 0.05$ as compared to CLP.



Supp. Fig. 1

Sup. 3.