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OMEPRAZOLE INCREASES SURVIVAL THROUGH THE INHIBITION OF INFLAMMATORY MEDIATERS IN TWO RAT SEPSIS MODELS

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ABSTRACT—**Background:** Omeprazole (OMZ) is a proton pump inhibitor that is used to reduce gastric acid secretion, but little is known about its possible liver protective effects. This study investigated whether OMZ has beneficial effects in rat septic models of LPS-induced liver injury after D-galactosamine (GalN) treatment and 70% hepatectomy (PH), and to determine the mechanisms of OMZ in an *in vitro* model of liver injury. **Methods:** In the *in vivo* models, the effects of OMZ were examined 1 h before treatments in both models on survival, nuclear factor (NF)-κB activation, histopathological analysis, and proinflammatory mediator expression in the liver and serum. In the *in vitro* model, primary cultured rat hepatocytes were treated with IL-1β in the presence or absence of OMZ. The influence of OMZ on nitric oxide (NO) product and inducible NO synthase (iNOS) induction and on the associated signaling pathway was analyzed. **Results:** OMZ increased survival and decreased tumor necrosis factor-alpha, iNOS, cytokine-induced neutrophil chemoattractant 1, IL-6, and IL-1β mRNA expression, and increased IL-10 mRNA expression in the livers of both GalN/LPS- and PH/LPS-treated rats. Necrosis and apoptosis were inhibited by OMZ in GalN/LPS rats, but OMZ had no effects on necrosis in PH/LPS rats. OMZ inhibited iNOS induction partially through suppression of NF-κB signaling in hepatocytes. **Conclusions:** OMZ inhibited the induction of several inflammatory mediators, resulting in the prevention of LPS-induced liver injury after GalN liver failure and PH, although OMZ showed different doses and mechanisms in the two models.

KEYWORDS—D-galactosamine with lipopolysaccharide, inducible nitric oxide synthase, nuclear factor-kappa B, omeprazole, partial hepatectomy with lipopolysaccharide, primary cultured hepatocytes, septic acute liver injury

ABBREVIATIONS—CINC-1/CXCL-1—cytokine-induced neutrophil chemoattractant 1/chemokine (C-X-C motif) ligand 1; GalN—D-galactosamine hydrochloride; IL—interleukin; iNOS—inducible nitric oxide synthase; LPS—lipopolysaccharide; OMZ—omeprazole; PH—70% hepatectomy; PPIs—proton pump inhibitors; TNF-α—tumor necrosis factor-alpha

INTRODUCTION

The incidence sepsis has gradually increased (1, 2). Over the last decade, the number of reported sepsis diagnoses in the emergency department has tripled, exceeding the number of diagnoses of myocardial infarction. The reason for this increase is thought to be aging and the advancement of medical care. Many older people suffer from chronic disease and are susceptible to infectious diseases that are likely to become severe. In addition, with the progress of medical treatment, the number of cases in which treatments that suppress immunity are applied is increasing with the rise in transplant surgery and chemotherapy for cancer. Furthermore, patients treated for immunosuppression are more susceptible to infections. Most importantly for patient outcome, early diagnosis of sepsis and source control are essential.

There are several experimental animal models of endotoxemia and sepsis with liver failure, including two that we reported previously: simultaneous administration of D-galactosamine and LPS (GalN/LPS) and a partial (70%) hepatectomy followed by LPS administration (PH/LPS) (3–5). In our previous studies, high doses of LPS, such as ≥50 μg/kg and ≥250 μg/kg, resulted in poor survival (less than 10%) in two models of GalN/LPS and PH/LPS, respectively. In this study, we decreased the levels of LPS injected after GalN and PH treatment to closely reflect the conditions in human cases.

In both sepsis models, nitric oxide (NO)/inducible nitric oxide synthase (iNOS) and inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and cytokine-induced neutrophil chemoattractant 1/chemokine ligand 1 (CINC-1) (IL-8 rat analogue) are excessively induced in hepatocytes within 3 h of LPS injection (3–5). Excessive NO production by iNOS contributes to liver injury (6, 7).

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MKo performed the experiments and acquired and analyzed the data. YH and RN helped to generate the GalN/LPS and PH/LPS models under the supervision of MKa and MS. MKo, RN, and TaO wrote the manuscript. YH, MH, and TY assisted in the primary culture of isolated rat hepatocytes and their analyses. MN and TeO helped to the transfection and electrophoretic mobility shift assay experiment (NF-κB activation). TaO was a mentor of this study and attended all experiments. All authors approved the final version of the manuscript for submission.

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Next, to determine the mechanisms of these hepatoprotective effects, we examined if omeprazole (OMZ) inhibits iNOS induction and NO production in primary cultured rat hepatocytes (8). In the liver during inflammation, in addition to the production of various inflammatory cytokines, the induction of iNOS gene expression is enhanced. Overproduction of NO by iNOS is considered a hepatic disorder, and suppression of iNOS induction is important for the alleviation of hepatic injuries. We analyzed the organ-protective effects of various clinical drugs, conventional therapeutic drugs, herbal medicines, and functional drugs. We demonstrated that NO produced by iNOS was an index of liver damage.

OMZ, the first clinically approved proton pump inhibitor (PPI), is used for the treatment of gastric acid-related disorders including gastroduodenal ulcers, reflux esophagitis, and nonsteroidal anti-inflammatory drug -induced gastric lesions. OMZ is a substituted benzimidazole that interacts with the gastric proton pump (H+, K+-ATPase) in the secretory membrane, resulting in potent long-acting inhibition of gastric acid secretion (9, 10), and it is extensively metabolized by the liver (11). PPIs inhibit hydrogen potassium adenosine triphosphatase, which in turn leads to reduced gastric acid secretion from parietal cells (12). Many studies have proposed other mechanisms by which PPIs exert their anti-inflammatory effects (13–16). PPIs were demonstrated to be a revolutionary treatment for acid-related diseases, and they minimized the need for elective surgery for ulcers or reflux when introduced to clinical practice (17, 18).

Previously, we reported the liver-protective effects of lansoprazole (LPZ) (4), a PPI. However, few studies have examined whether other PPIs influence survival and the expression of proinflammatory mediators in animal models of liver injury or septic shock. Current study will prove the universality of hepatoprotective and anti-inflammatory effects of PPIs, as those effects can be found by another PPI, OMZ. This may suggest that in the future, prophylactic administration of PPIs after clinically invasive liver surgery or in situations where sepsis needs to be treated may avert the risk of liver failure and septic shock. We first used two rat models of liver injury induced with GaIN/LPS or PH/LPS and examined if OMZ influences survival and various inflammatory mediators. Next, to determine the mechanisms of these hepatoprotective effects, we examined if OMZ inhibits iNOS induction and NO production in primary cultured rat hepatocytes (8).

MATERIALS AND METHODS

Ethics statement

Animal care and experiments were performed in accordance with the standards in the ARRIVE and PREPARE guidelines (19, 20). In addition to these, our study was in accordance with the relevant guidelines and regulations, which was approved by the Animal Care Committee of Kansai Medical University (19-009 and 20-059). All methods proposed in these studies were also carried out according to the standards of relevant institutional guidelines and regulations.

Drugs

OMZ (20 mg) and recombinant human IL-1β (2×10^7 U/mg protein) were purchased from Nichi-iko Co, Ltd. (Toyama, Japan) and MyBioSource (San Diego, Calif). Isoflurane, pentobarbital sodium, collagenase, Transaminase CII-test kit, GalN, 10% formalin, and PicaGene Luminescence kit were from Wako Pure Chemical Industries (Osaka, Japan). LPS (*Escherichia coli*; O111:B4) and mouse anti-β-tubulin were from Sigma-Aldrich Japan (Tokyo, Japan). Enzyme-linked immunosorbent assay (kits were from Life Technologies Japan (Tokyo, Japan). TRIzol Reagent was from Thermo Scientific (Waltham, Mass). T4 polynucleotide kinase, Oligo (dT) Primer (25 ng), dNTPs Mixture, RNase Inhibitor, and Rever Tra Ace were from Toyobo (Osaka, Japan). Beta-Glo kits and mouse immunoglobulin κ light chain were from Promega (Fitchburg, Wis).

Animals

Male Wistar and Sprague-Dawley (male) strain rats were purchased from Charles River Laboratories Japan (Yokohama, Japan), maintained at 22°C under a 12-h light/dark cycle, and fed γ-irradiated CRF-1 (Oriental Bioservices, Kyoto, Japan) and water ad libitum.

Rat GalN/LPS and PH/LPS models

To examine effect of LPS on survival, acute liver injury was induced in the *in vivo* model. Male Sprague–Dawley rats (8 weeks old, 300 g ± 20) were anesthetized with isoflurane (Abbott Laboratories, Abbott Park, Ill) before receiving an i.v. injection of GalN/LPS (500 mg/kg GalN and 0.5–50 μg/kg LPS) via the penile vein (3). Survival was monitored for 3 days after GalN/LPS injection. To examine the effects of OMZ on survival in the liver injury model with GalN/LPS (2.5 μg/kg LPS), rats that were randomly assigned to receive OMZ were injected (i.p.) with various doses of OMZ (40 mg/kg –240 mg/kg) 1 h before GalN/LPS treatment. Survival was monitored for 5 days.

To investigate the effects of LPS on survival, the PH/LPS model was induced. Rats were anesthetized with pentobarbital and isoflurane prior to undergoing 70% hepatectomy, as reported previously (21). Forty-eight hours after surgery, LPS (6.25–250 μg/kg) was injected into the penile vein. Survival was monitored for 3 days after LPS injection. To determine the effects of OMZ levels on survival in the liver injury model with PH/LPS (25 μg/kg), rats were randomly divided into control, OMZ, PH/LPS, and PH/LPS + OMZ groups. Forty-eight hours after surgery, LPS (25 μg/kg) was injected into the penile vein. The rats that were randomly assigned to receive OMZ were injected (i.p.) with various doses of OMZ (40 mg/kg–100 mg/kg) 1 h before LPS treatment. Survival was monitored for 5 days. The rats were killed when they appeared weak and moribund because of the progression of liver failure, congestion, and multi-organ failure. We used the NIH Office of Animal Care and Use score and severity assessment to assess the animals following liver resection (22). Liver and blood samples were collected from the rats 1 and 6 h after GaIN/LPS treatment, and 1 and 4 h after LPS treatment in PH/LPS.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously with a minor modification, as described elsewhere (5). Nuclear extracts were prepared from frozen liver at −80°C or cultured hepatocytes. Binding reactions were undertaken by incubating the nuclear extracts in reaction buffer (20 mM HEPES-KOH, pH 7.9, containing 1 mM EDTA, 60 mM KCl, 10% glycerol, and 1 μg poly[dI-dC]) with a probe (40,000 dpm) for 20 min at room temperature. Products were electrophoresed on a 4.8% polyacrylamide gel in high-ionic-strength buffer, and dried gels were analyzed by autoradiography. An nuclear factor (NF)-κB consensus oligonucleotide (5′-AGTTGAG GGGA-CTTTCCCAGGC) from the mouse immunoglobulin κ light chain was purchased and labeled with [γ-^{32}P]-ATP (PerkinElmer, Tokyo, Japan) and T4 polynucleotide kinase. Protein was measured using the Bradford method. Bands corresponding to NF-κB were quantified by densitometry using ImageJ.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the frozen liver samples or cultured hepatocytes using TRIzol reagent (the guanidinium thiocyanate-phenol-chloroform mixture) (3). cDNA was synthesized from 1 μg total RNA from each sample with Oligo(dT)20 Primer (25 ng/μL), 5 × RT Buffer (5 μL), 10 mM dNTPs Mixture (2.5 μL), RNase Inhibitor (20 units/0.5 μL), Rever Tra Ace (100 units/μL), and UltraPure DNase/RNase-free distilled water (total volume, 25 μL). The conditions of thermal cycling using iCycler (Bio-Rad Laboratories, Hercules, Calif) were 42°C for 60 min and 95°C for 5 min. Real-time PCR was performed using THUNDERBIRDTM SYBR™ qPCR Mix (TOYOBO, Osaka, Japan) Green and primers for each gene. Primer sequences were synthesized by Eurofins Genomics (Tokyo, Japan) (Table 1). The conditions of thermal cycling using a Rotor-Gene Q (Qiagen, Stanford, Va) were 95°C for 5 min followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. Collection and analyses of data were undertaken using the system software. mRNA expression levels of each gene were measured as computed tomography threshold levels and normalized to those of eukaryotic

TABLE 1. **Primer sets for RT-PCR**

Gene	RT primer	PCR forward primer	PCR reverse primer	Amplification (bp)
EF-1α	oligo (dT)20	5'-TCTGGTTGGAATGGTGACAACATGC-3'	5'-CCAGGAAGAGCTTCACTCAAAGCTT-3'	332
iNOS	oligo (dT)20	5'-CCAACCTGCAGGTCTTCGATG-3'	5'-GTCGATGCACAACTGGGTGAAC-3'	257
TNF-α	oligo (dT)20	5'-TCCCAACAAGGAGGAGAAGTTCC-3'	5'-GGCAGCCTTGTCCCTTGAAGAGA-3'	275
IL-1β	oligo (dT)20	5'-TCTTTGAAGAAGAGCCCGTCCTC-3'	5'-GGATCCACACTCTCCAGCTGCA-3'	321
IL-6	oligo (dT)20	5'-GAGAAAAGAGTTGTGCAATGGCA-3'	5'-TGAGTCTTTTATCTCTTGTTTGAAG-3'	286
CINC-1	oligo (dT)20	5'-GCCAAGCCACAGGGGCGCCCGT-3'	5'-ACTTGGGGACACCCTTTAGCATC-3'	231
IL-10	oligo (dT)20	5'-GCAGGACTTTAAGGGTTACTTGG-3'	5'-CCTTTGTCTTGGAGCTTATTAAA-3'	245

CINC-1 (CXCL-1), cytokine-induced neutrophil chemoattractant 1 (chemokine (C-X-C motif) ligand 1); EF-1α, elongation factor-1-alpha; IL-1β, interleukin-1 beta; IL-10, interleukin-10; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; TNF-α, tumor necrosis factor-alpha.

elongation factor-1α. The cDNA sequence for rat *NOS2* mRNA was deposited in the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank under accession number AB250951.

Serum biochemical analyses

Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels were quantified using commercial kits. The serum levels of nitrite and nitrate (stable metabolites of NO) were measured using a commercial kit (Roche, Mannheim, Germany) according to the Griess method (23).

Histopathological analyses

Excised liver specimens from the Sprague–Dawley rats were collected and fixed in 10% formalin and embedded in paraffin. Sections of 3 μm to 5 μm in size were cut and stained with hematoxylin-eosin. Neutrophil infiltration was evaluated by staining with myeloperoxidase (MPO) using anti- MPO antibodies (A0398; DAKO, Glostrup, Denmark) before hematoxylin-eosin staining. Apoptotic bodies in the hepatocyte nuclei were detected by triphosphate-digoxigenin nick-end labeling (TUNEL) staining using an *in-situ* Apoptosis Detection Kit (MK500; Takara Bio Inc, Kusatsu, Shiga, Japan). The number of MPO- and TUNEL-positive cells per square millimeter was counted by analysts who were blinded to the treatment arm.

Preparation of rat primary cultured hepatocytes

Collagenase perfusion was used to isolate hepatocytes from male Wistar rats (200 g–250 g, 6 weeks–7 weeks old) (8). The isolated hepatocytes were cultured with Williams' medium E (supplemented with 10% fetal calf serum, HEPES (5 mmol/L), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), aprotinin (0.1 μg/mL; Roche, Basel, Switzerland), dexamethasone (10 nmol/L), and insulin (10 nmol/L). After 7 h, the medium was changed with fresh hormone-free medium and the cells were cultured overnight. The number of cells attached to the dishes was estimated by counting the number of nuclei and applying a ratio of 1.37 ± 0.04 nuclei/cell (mean ± standard error; n = 7 experiments) (24).

Treatment of the cultured hepatocytes with OMZ

OMZ was dissolved in Williams' medium E under sterile conditions. On day 1 after cell culture, the hepatocytes were washed with fresh serum- and hormone-free Williams' medium E and incubated with IL-1β (1 nmol/L) in the same medium, either in the presence or absence of OMZ (dose range, 0.1 mmol/L –0.5 mmol/L).

Determination of NO production and lactate dehydrogenase (LDH) activity in the cultured hepatocytes

The amount of nitrite (a stable metabolite of NO) in the cell culture medium of the hepatocytes was measured using the Griess method (23). Cell viability was measured on the basis of LDH activity using a commercial kit (Cytotoxicity LDH Assay Kit-WST; Dojindo Inc, Tokyo, Japan).

Western blotting in the cultured hepatocytes

Total cell lysates were obtained from the cultured hepatocytes using a previously described method with minor modifications (3, 25). Immunostaining was performed with primary antibodies against mouse iNOS (Affinity BioReagents, Golden, Colo), human inhibitor of κB alpha (IκBα; Santa Cruz Biotechnology, Santa Cruz, Calif), and rat β-tubulin. Immunoreactive proteins were visualized by an enhanced chemiluminescence detection kit (GE Healthcare Biosciences, Piscataway, NJ).

Transfection and luciferase assay in the cultured hepatocytes

Transfection of the cultured hepatocytes was performed using a previously described method (26). Hepatocytes were cultured at 3×10^5 cells/dish (35×10 mm) in Williams' medium E with serum, dexamethasone, and insulin for 7 h before undergoing magnet-assisted transfection. Reporter constructs pRiNOS-Luc-SVpA (for detecting the transactivation of the *NOS2* promoter) or pRiNOS-Luc-3′UTR (for detecting the stability of mRNA) (1 μg) and the cytomegalovirus promoter-driven β-galactosidase plasmid pCMV-LacZ (1 ng; internal control) were mixed with a magnet-assisted transfection reagent (1 μL; IBA Lifesciences, Göttingen, Germany) in fresh serum- and hormone-free Williams' medium E (1.5 mL), followed by incubation with cultured cells. After a 15-min incubation period on a magnetic plate at room temperature, the medium was replaced with fresh Williams' medium E with serum. The cells were then cultured overnight and treated with IL-1β in the presence or absence of OMZ.

Statistical analyses

Quantitative results were obtained from three to four independent experiments for each of the various analyses, and the mean values and their standard deviations were calculated. Differences between groups and survival rates were identified using the Student *t* test, log-rank test and one-way ANOVA, followed by the Tukey-Kramer method, respectively (JMP 14, SAS Institute Inc, Cary, NC). $P < 0.05$ was considered significant.

RESULTS

Effects of LPS on survival in rat GalN/LPS and PH/LPS models

Previously, we applied GalN (500 mg/kg)/LPS (50 μg/kg) and PH (70% hepatectomy)/LPS (250 μg/kg) in two rat liver injury (septic) models (4, 21). More than 90% of rats had died 72 h after GalN/LPS or LPS injection in these models. In the current study, we examined the effects of lower doses of LPS on survival without changing other conditions to obtain milder survival curves.

In the GalN/LPS model, 0.5 μg/kg to 50 μg/kg LPS was injected with GalN (500 mg/kg). Cumulative survival was 0 with 2.5 μg/kg to 50 μg/kg LPS (Fig. 1A), whereas it varied with 0.5 μg/kg LPS. Therefore, we used 2.5 μg/kg LPS in the GalN/LPS model as the positive control (PC). Similarly, we examined cumulative survival with 6.25 μg/kg to 250 μg/kg LPS (Fig. 1B) in the PH/LPS model. We determined that 25 μg/kg LPS was the most appropriate dose for further experiments in the PH/LPS model, but it varied with 6.25 μg/kg LPS.

Effects of omeprazole on increased survival in rat GalN/LPS and PH/LPS models

In the GalN/LPS (2.5 μg/kg) model, rats were treated with 40 mg/kg, 80 mg/kg, and 120 mg/kg OMZ (intraperitoneally,

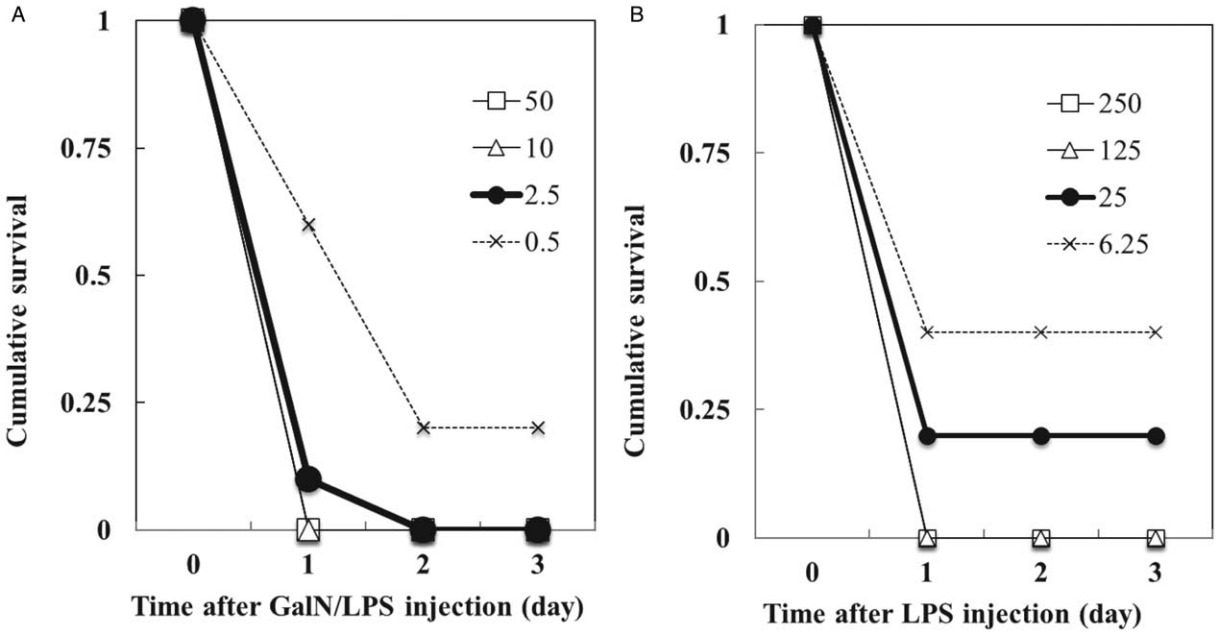

FIG. 1. **Survival rates in rat GalN/LPS and PH/LPS models with various concentrations of LPS.** Survival rates in rat GalN/LPS (A) and PH/LPS (B) models were examined following treatment with various concentrations of LPS (injected into the penile vein, i.v.). A, 0.5 (×, n = 13), 2.5 (●, n = 13), 10 (Δ, n = 13), and 50 (□, n = 13) μg/kg LPS. B, 6.25 (×, n = 10), 25 (●, n = 10), 125 (Δ, n = 10), and 250 (□, n = 10) μg/kg LPS. GalN, 500 mg D-galactosamine/kg rat; LPS, lipopolysaccharide (μg of LPS/kg rat); PH, 70% hepatectomy.

i.p.) for 1 h before GalN/LPS injection (PC); no significant effects on survival were observed. However, two injections (second injection was administered 3 h after GalN/LPS) of 120 mg/kg OMZ increased survival (Fig. 2A1). One injection of higher-dose OMZ (180 mg/kg and 240 mg/kg) increased survival, although 240 mg/kg OMZ had no effect compared with 180 mg/kg, indicating that adverse effects might occur by overdosing (Fig. 2A2). In the PH/LPS (25 μg/kg) model (PC), 100 mg/kg OMZ (i.p., 1 h before LPS injection) significantly increased survival (Fig. 2B).

In the subsequent biochemical and histopathological analyses, OMZ (180 mg/kg) was used in the GalN (500 mg/kg)/LPS

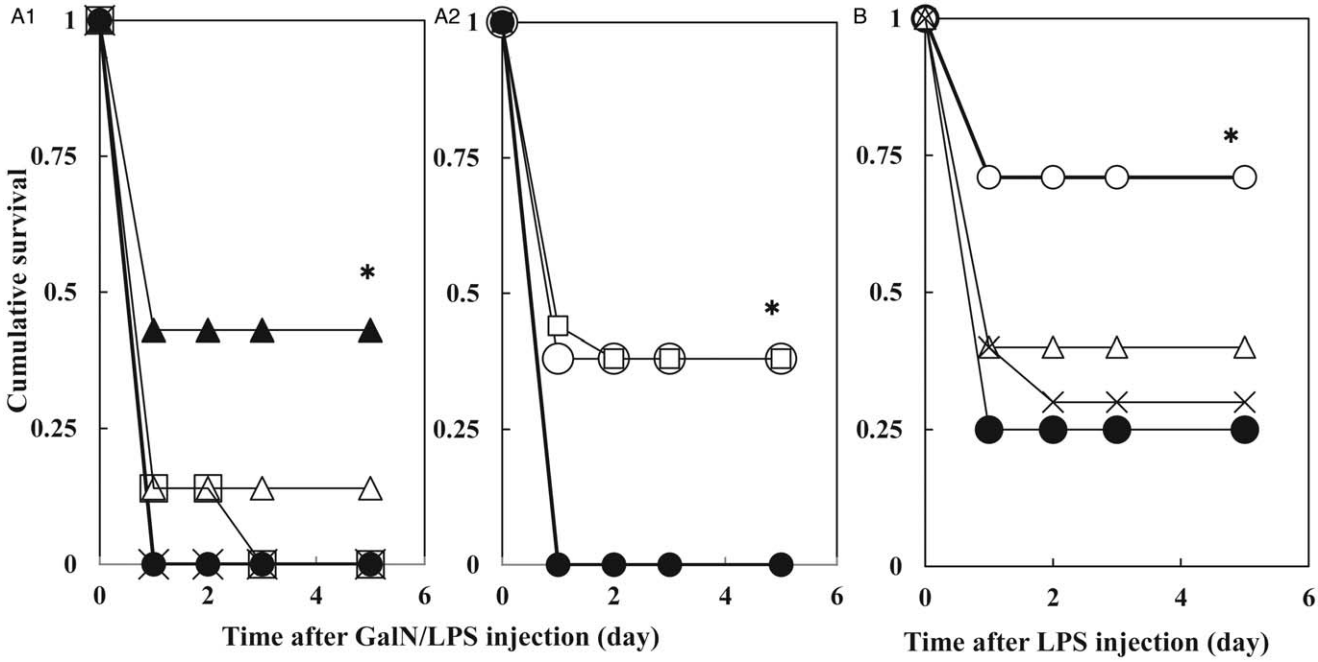

FIG. 2. **Increased survival in rat GalN/LPS and PH/LPS models following omeprazole treatment.** A, Positive control (PC): GalN/LPS (2.5 μg/kg, ●, n = 10, i.v.); (A1) PC + 40 (×, n = 5), 80 (Δ, n = 13), 120 (□, n = 12), 120 (×2) (▲, n = 12), and (A2) PC + 180 (○, n = 10), and 240 mg (□, n = 12) omeprazole (OMZ)/kg (OMZ was administered 1 h before GalN/LPS treatment; intraperitoneally, i.p.). In cases with two injections (120 (×2)), the second injection was administered 3 h after GalN/LPS. B, PC: PH/LPS (25 μg/kg, ●, n = 10, i.v.); PC + 40 (×, n = 10), 80 (Δ, n = 10), and 100 mg (○, n = 7) OMZ/kg (OMZ was administered 1 h before LPS treatment, i.p.). *$P < 0.05$ versus PC.

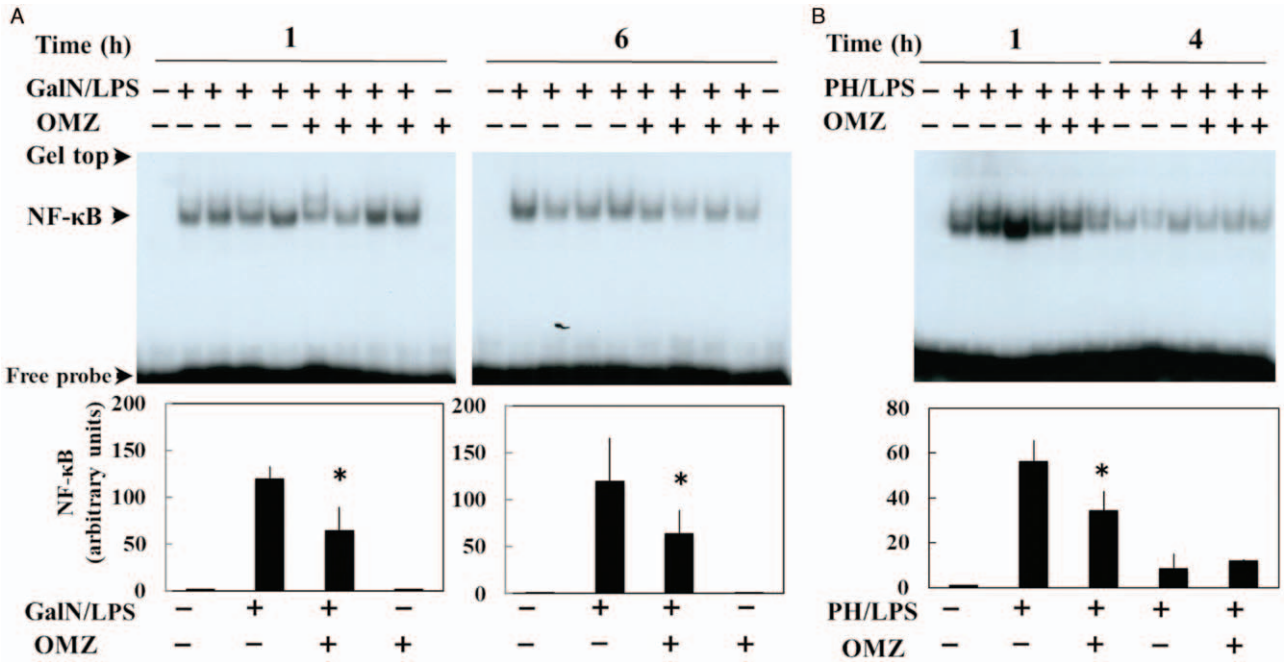

FIG. 3. **Effects of omeprazole on the activation of NF-κB in the livers of rat GalN/LPS and PH/LPS models.** A, GalN (500 mg/kg)/LPS (2.5 μg/kg) ± OMZ (180 mg/kg). B, PH (70% hepatectomy)/LPS (25 μg/kg) ± OMZ (100 mg/kg). $^*P < 0.05$ versus positive control (n = 3–5). OMZ, omeprazole.

(2.5 μg/kg) rat model (Fig. 2A2) and OMZ (100 mg/kg) was used in the PH/LPS (25 μg/kg) rat model (Fig. 2B).

Effects of omeprazole on nuclear factor (NF)-κB activation in the livers of rat liver injury models

In the GalN (500 mg/kg)/LPS (2.5 μg/kg) and PH/LPS (25 μg/kg) models, EMSA experiments revealed that OMZ (180 mg/kg and 100 mg/kg) inhibited the activation of NF-κB at both 1 and 6 h in GalN/LPS rats (Fig. 3A) and at 1 h in PH/LPS rats (Fig. 3B), respectively. However, LPS had less effect on NF-κB activation at 4 h in PH/LPS rats.

Effects of omeprazole on mRNA expression of inflammatory mediators in the livers of rat liver injury models

In the GalN/LPS model, OMZ decreased the mRNA levels of TNF-α (1 h), iNOS (6 h), CINC-1 (1 h), IL-6 (6 h), and IL-1β (6 h), but increased IL-10 (1 and 6 h), compared with the positive control (Fig. 4A). In the PH/LPS model, OMZ also decreased the mRNA levels of TNF-α (1 and 4 h), iNOS (4 h), CINC-1 (1 h and 4 h), IL-6 (1 h), and IL-1β (1 h), but increased IL-10 (4 h), compared with the positive control (Fig. 4B).

Effects of omeprazole on nitric oxide, alanine/aspartate transaminase (ALT/AST), and cytokines in the serum of rat GalN/LPS and PH/LPS models

In the GalN/LPS model, OMZ decreased NO production (6 h), ALT/AST (6 h), TNF-α (1 and 6 h), IL-6 (6 h), and IL-1β (6 h) compared with the positive control (Fig. 5A). In the PH/LPS model, OMZ also decreased NO production (4 h), ALT/AST (4 h), TNF-α (1 and 4 h), IL-6 (4 h), and IL-1β (1 and 4 h) compared with the positive control (Fig. 5B).

Effects of omeprazole on pathological changes in the livers of GalN/LPS and PH/LPS models

In both rat models, the areas of focal necrosis with inflammatory cell infiltration and massive hemorrhage were increased in the positive controls at 1 and 6 h (or 4 h), while it was reduced by OMZ at 1 and 6 h (or 4 h) (Figs. 6 and 7). In MPO staining (necrosis), OMZ decreased MPO-positive cells compared with the positive controls in both models (Fig. 6; B5 and Fig. 7; B5). In terminal deoxynucleotidyl transferase-mediated deoxyuridine TUNEL staining (apoptosis), OMZ also decreased TUNEL-positive cells as compared with the positive controls at 6 h in the GalN/LPS model (Fig. 6; C3), but no differences were observed with OMZ in the PH/LPS models (Fig. 7; C3).

Effects of omeprazole on nitric oxide production, iNOS protein expression, and inflammatory mediator mRNA expression in primary cultured rat hepatocytes

In primary cultured rat hepatocytes, OMZ inhibited the production of NO (Fig. 8A, upper) and the expression of iNOS protein (Fig. 8A, middle) in a dose-dependent manner. OMZ showed no cellular toxicity at the indicated concentrations, as evaluated by LDH release and trypan blue exclusion (data not shown). OMZ also reduced the mRNA expression of iNOS, TNF-α, IL-1β, and CINC-1 (Fig. 8B), indicating that OMZ affects these genes at the transcriptional and/or post-transcriptional levels.

Effects of omeprazole on NF-κB activation and iNOS mRNA levels in primary cultured rat hepatocytes

Although OMZ had no effects on the degradation of IκBα (Fig. 9A), OMZ inhibited NF-κB activation at 2, 3, and 4 h (Fig. 9B1 and B2). iNOS mRNA expression is regulated

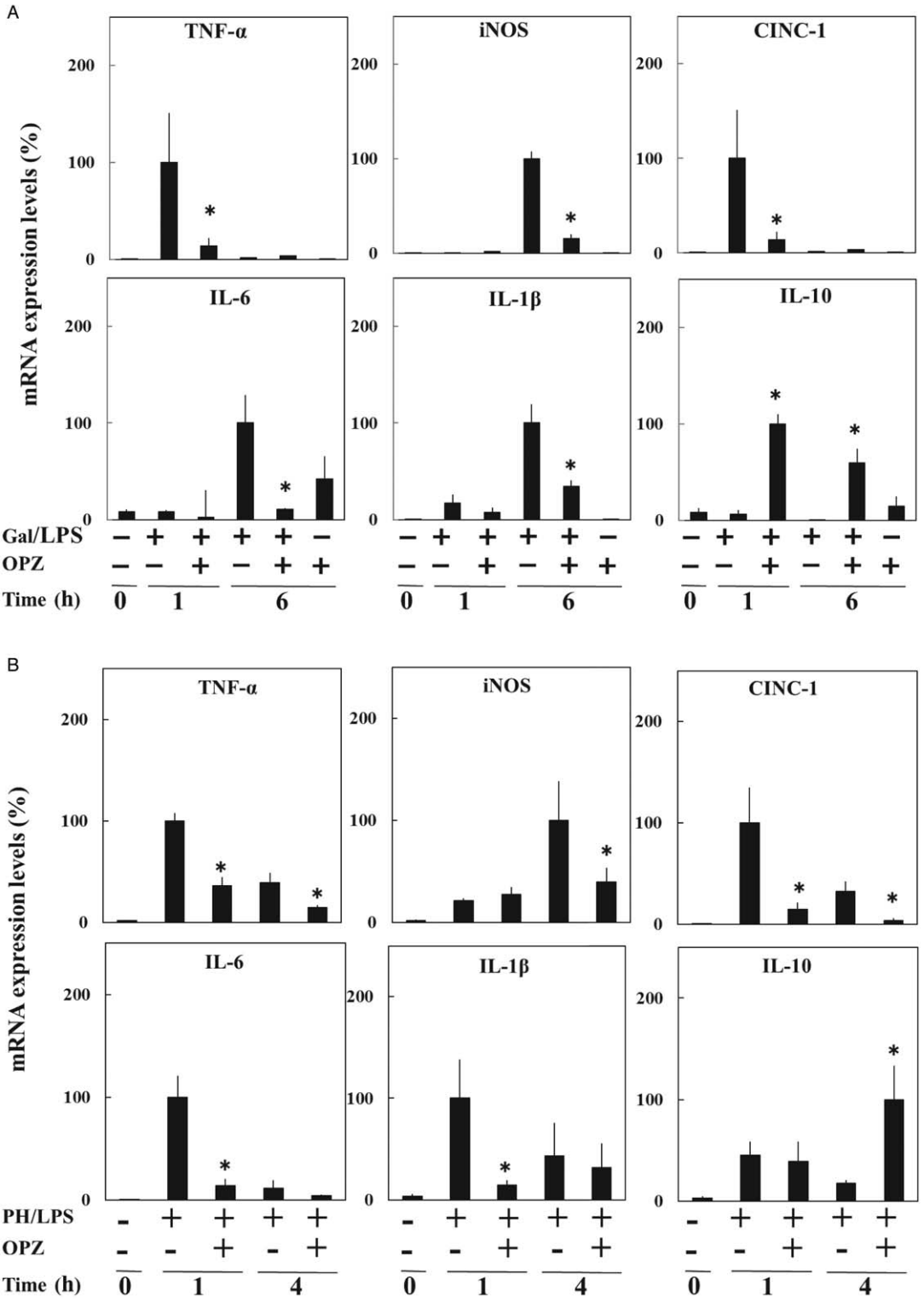

FIG. 4. **Effects of omeprazole on the mRNA expression of inflammatory mediators in the liver of rat GalN/LPS and PH/LPS models.** A, GalN/LPS. B, PH/LPS. $^{*}P < 0.05$ versus positive control (n = 3–5). CINC-1, cytokine-induced neutrophil chemoattractant-1; IL-6, interleukin-6; IL-1β, interleukin-1beta; IL-10, interleukin-10; iNOS, inducible nitric oxide synthase; OMZ, omeprazole; TNF-α, tumor necrosis factor-alpha.

through activation of the iNOS promoter by transcription factors such as NF-κB and through post-transcriptional modifications such as mRNA stabilization (26, 27). Transfection was performed using pRiNOS-Luc-SVpA and pRiNOS-Luc-3′UTR, which detected iNOS promoter activation (i.e., mRNA synthesis) and mRNA stability, respectively (26). IL-1β increased the luciferase activity of these vector constructs, and these effects were inhibited by OMZ (Fig. 9C).

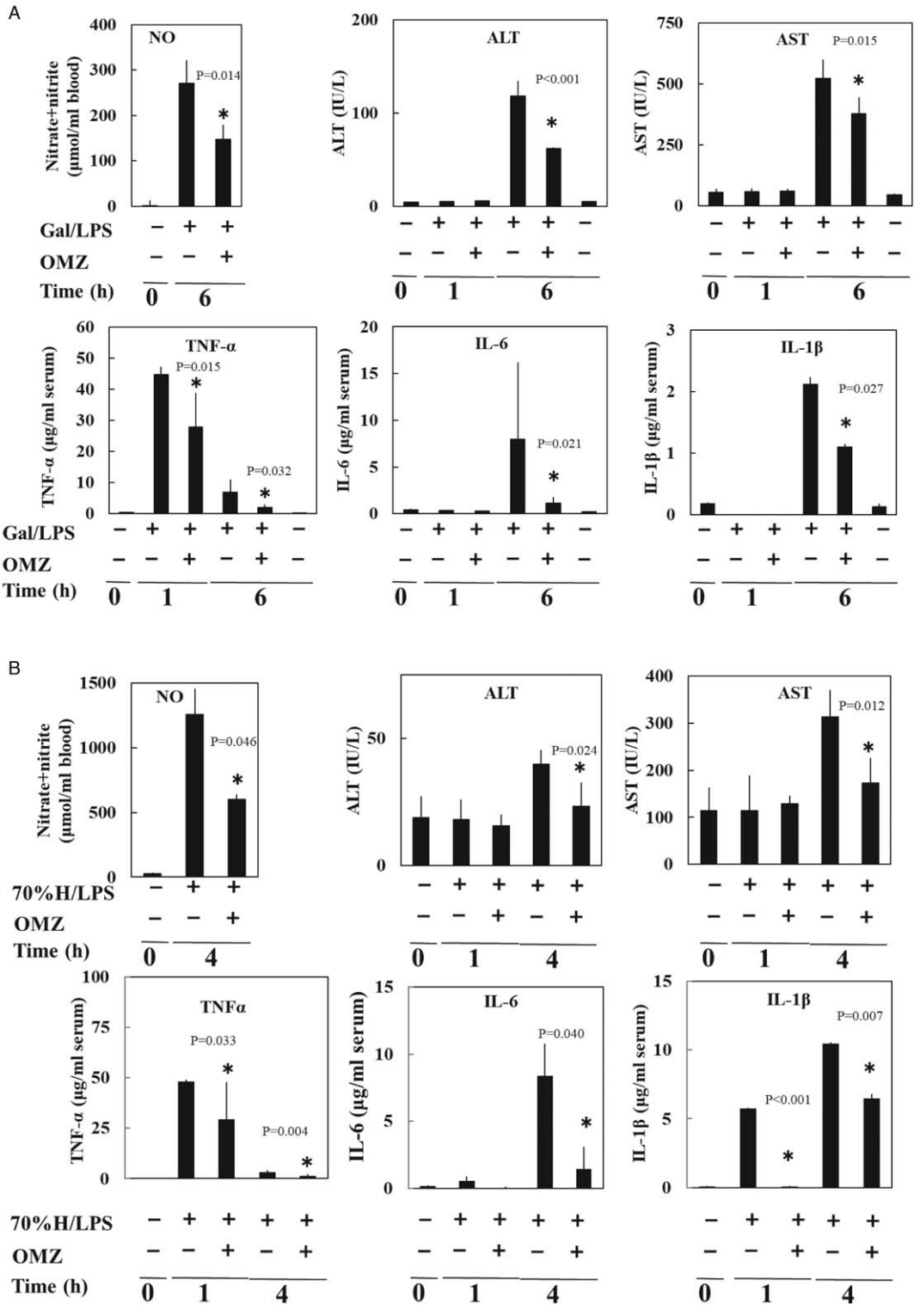

FIG. 5. **Effects of omeprazole on nitric oxide, alanine/aspartate transaminases, and cytokines in the serum of rat GalN/LPS and PH/LPS models.** A, GalN/LPS. B, PH/LPS. $^{*}P < 0.05$ versus positive control (n = 3–5). ALT/AST, alanine/aspartate transaminases; IL-6, interleukin-6; IL-1β, interleukin-1beta; NO, nitric oxide; OMZ, omeprazole; TNF-α, tumor necrosis factor-alpha.

DISCUSSION

In this study, we investigated the liver-protective effects of OMZ using two septic rat models (GaIN/LPS and PH/LPS) as *in vivo* liver injury models. We also attempted to clarify the protective mechanisms of OMZ in IL-1β-stimulated rat hepatocytes in an *in vitro* liver injury model (8). OMZ demonstrated hepatoprotective effects in both *in vivo* models, and our experiments in the *in vitro* liver injury model indicated several possible mechanisms for these effects.

PPIs are effective on elements of the immune system including monocytes, neutrophils, and endothelial cells (28). PPIs suppress neutrophil functions such as chemotaxis, superoxide production,

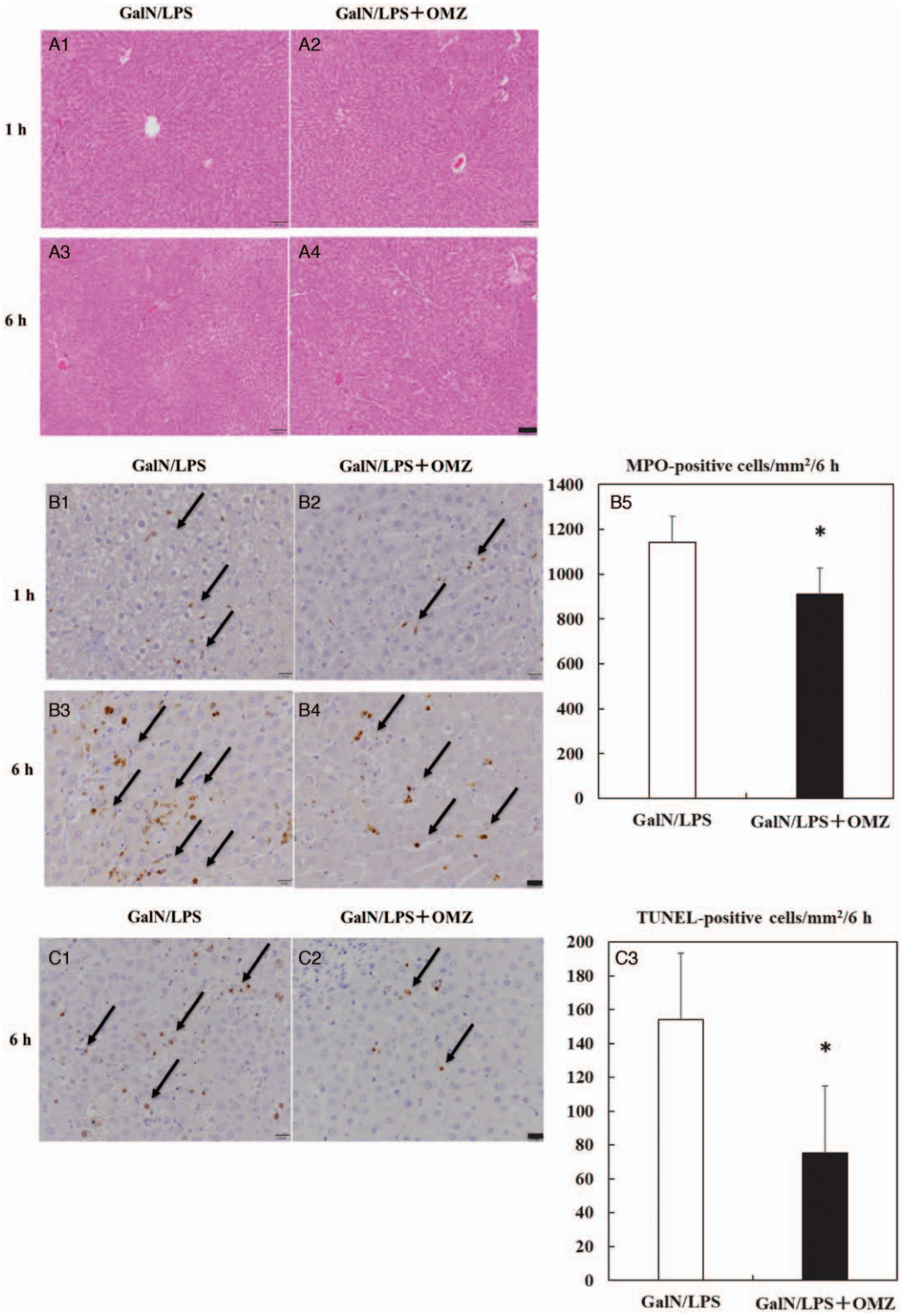

FIG. 6. **Effects of omeprazole on pathological changes in the livers of GalN/LPS model.** Omeprazole (OMZ, 180 mg/kg) was administered (i.p.) 1 h before GalN/LPS treatment, and liver sections were stained in (A–C; 1 and 6 h) rats. H&E; note the areas of focal necrosis with inflammatory cell infiltration and massive hemorrhage in PC rats (A1–2 (1 h) and A3–4 (6 h), bar = 100 microns, magnification × 100). MPO (necrosis); B1-5. TUNEL (apoptosis); C1–3. Note the brown nuclei in the positive cells (arrows: positive cells, bar = 20 microns, magnification × 400). The numbers of MPO- and TUNEL-positive cells per square millimeter were counted at 6 h (B5 and C3). The values in the bar graphs represent the mean ± standard error (n = 4 rats per time-point per group). * $P < 0.05$ versus positive control (GalN/LPS).

and degranulation via IL-8 (29). In addition, P-type proton-ATPase inhibitors have anti-inflammatory effects by reducing neutrophil adhesion molecules and free oxygen radicals (30). They are also known to activate heme oxyegenase-1, an endogenous antioxidant (31). We have previously reported these hepatoprotective effects of LPZ, a PPI, because of its ability to induce an anti-oxidative stress

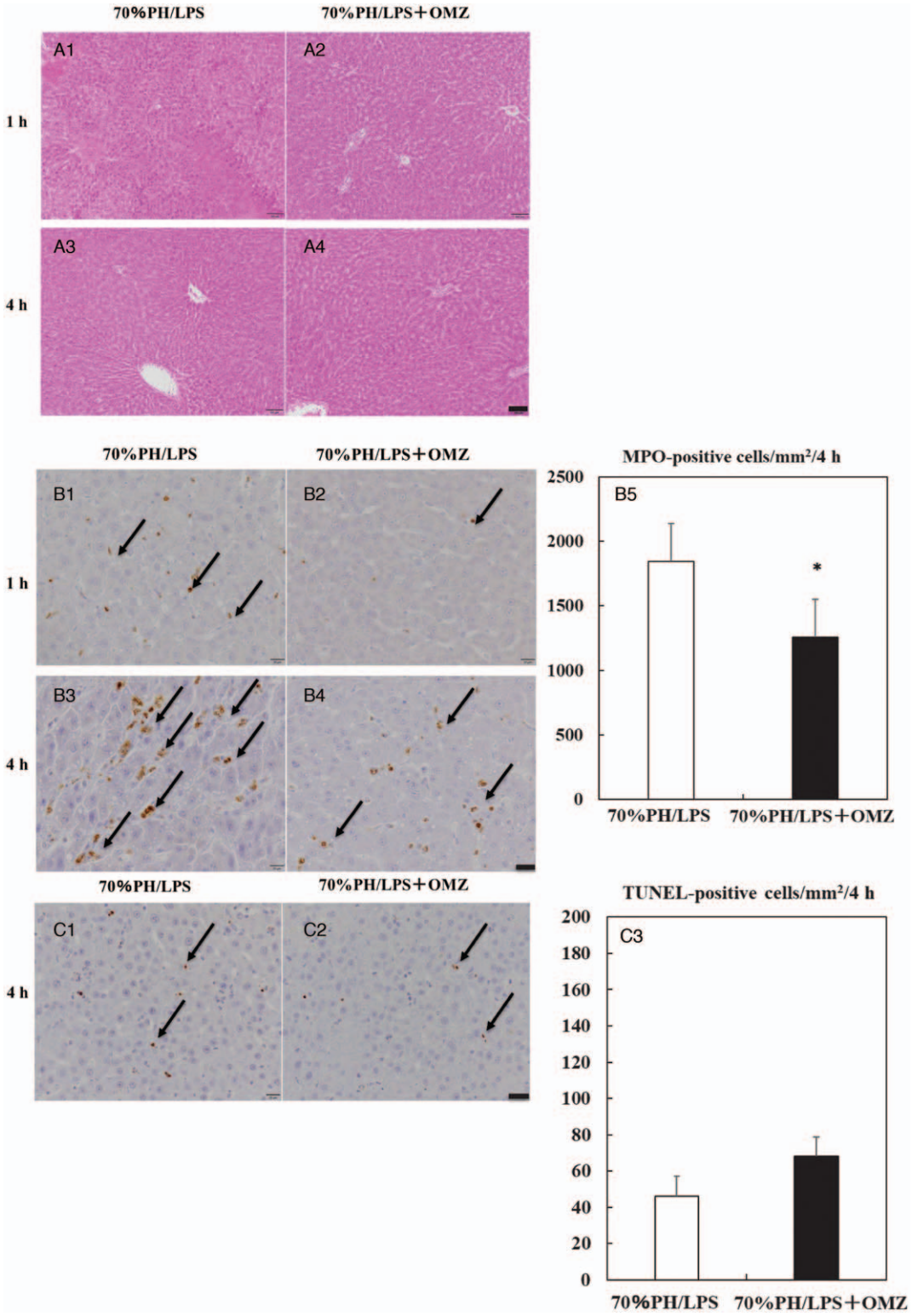

FIG. 7. **Effects of omeprazole on pathological changes in the livers of PH/LPS model.** Omeprazole (OMZ, 100 mg/kg) was administered (i.p.) 1 h before LPS treatment, and liver sections were stained in (A–C; 1 and 4 h) rats. H&E; note the areas of focal necrosis with inflammatory cell infiltration and massive hemorrhage in both rats (A1–2 (1 h) and A3–4 (6 h), bar = 100 microns, magnification × 100). MPO (necrosis); B1-5. TUNEL (apoptosis); C1-3. Note the brown nuclei in the positive cells (arrows: positive cells, bar = 20 microns, magnification × 400). The numbers of MPO- and TUNEL-positive cells per square millimeter were counted at 4 h (B5 and C3). The values in the bar graphs represent the mean ± standard error (n = 4 rats per time-point per group). * $P < 0.05$ versus positive control (PH/LPS).

response in the liver (4). Sepsis is a major cause of death and is associated with hypotension (i.e., septic shock) and multiple organ failure, including liver failure (1). However, the etiology of sepsis has not been completely elucidated and there is no specific treatment. Therefore, determination of the cause of sepsis is especially important in a clinical situation.

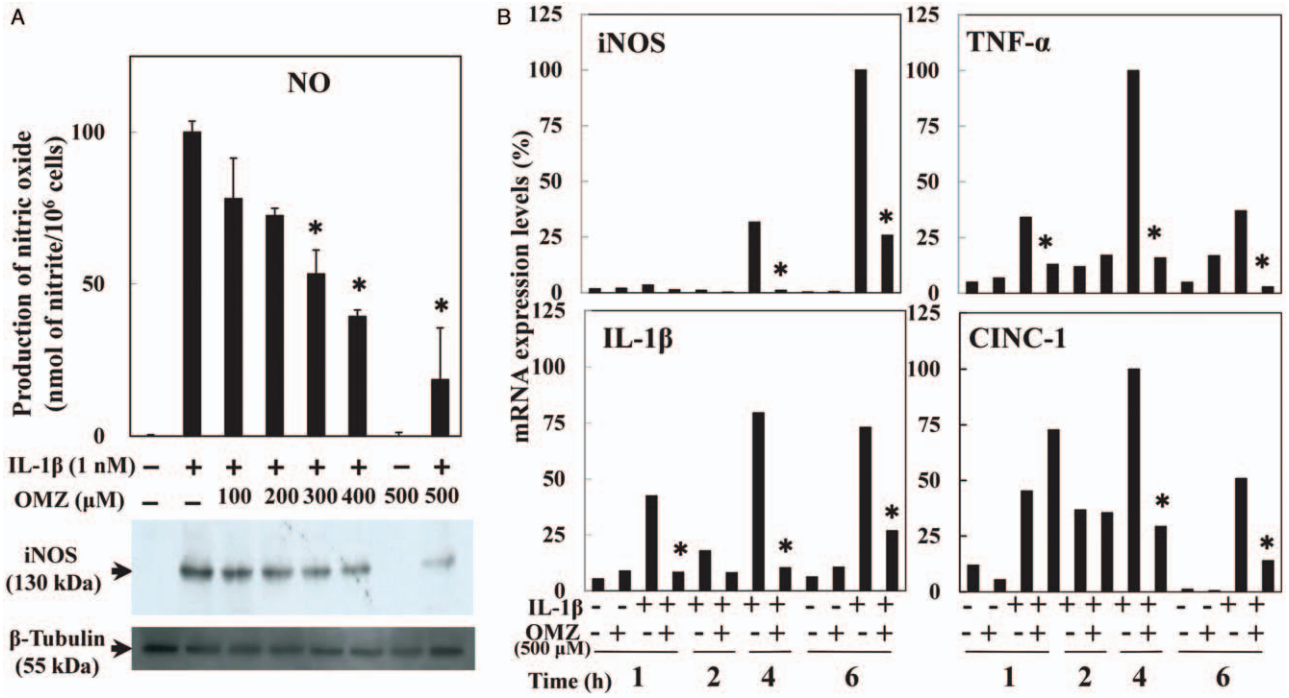

FIG. 8. **Effects of omeprazole on nitric oxide production, iNOS protein expression, and inflammatory mediator mRNA expression in rat primary cultured hepatocytes.** A, nitric oxide (NO, upper) and iNOS protein (middle). B, mRNA expression of iNOS, TNF-α, IL-1β, and CINC-1. * $P < 0.05$ versus positive control (IL-1β). OMZ, omeprazole.

In rats, GalN-treatment or 70% hepatectomy with a sublethal dose of LPS increases the sensitivity to endotoxin. Therefore, these rats induce liver failure (32). NO in the serum starts to increase at 3 h and further increases until 6 h after LPS injection (3). NF-κB, a transcription factor involved in inflammation and apoptosis (33), mediates this induction, including iNOS stimulation (3, 5). The resulting cytokine storm provokes multiple organ failure, including liver failure, which is the result of apoptosis of hepatocytes induced by TNF-α (34, 35). Upregulation of iNOS, TNF-α, and other inflammatory mediators in inflamed hepatocytes is central to liver inflammation. In response to interactions with pathogenic bacteria, inflammatory cells increase the production of these proinflammatory mediators, which in turn activate other processes that promote inflammation.

In rat models of GalN/LPS and PH/LPS, we reduced the doses of LPS after GalN treatment and 70% hepatectomy to 2.5 μg/kg and 25 μg/kg, respectively. In these positive controls (without OMZ), lower LPS did not have any effect on survival (less than 10% or approximately 0%) in the GalN/LPS model, but increased survival (20%–40%) in the PH/LPS model (Fig. 1). Under such conditions, 180 mg/kg and 100 mg/kg OMZ enhanced the cumulative survival of GalN/LPS and PH/LPS rats, respectively (Fig. 2). Biochemical analyses showed that OMZ inhibited the activation of NF-κB (Fig. 3), decreased the mRNA expression of inflammatory mediators (TNF-α, iNOS, CINC-1, IL6, and IL-1β), increased IL-10 mRNA expression in the liver (Fig. 4), and decreased the production of NO, ALT/AST, TNF-α, IL-6, and IL-1β in serum (Fig. 5). Further histopathological analyses in the liver also showed that OMZ reduced the areas of focal necrosis with inflammatory cell infiltration and massive hemorrhage in GalN/LPS and PH/LPS rats (Figs. 6 and 7), whereas MPO experiments demonstrated that OMZ reduced necrosis in both models (Figs. 6B5 and 7B5). However, in TUNEL staining (apoptosis), OMZ reduced apoptosis in GalL/LPS but not PH/LPS rats.

In both models, OMZ had similar liver-protective effects. However, there were some differences between these models. For example, in the case of EMSA (NF-κB activation), positive control rats in the PH/LPS model exhibited less effective increases in NF-κB activation at 4 h, and OMZ had no effect (Fig. 3B). In contrast, at both 1 and 6 h in the GalN/LPS model, high increases in NF-κB activation were observed, which were inhibited by OMZ, and IL-6 and IL-1β were increased at 1 h in PH/LPS and at 6 h in GalN/LPS rats, respectively, which was also inhibited by OMZ. These differences may demonstrate an important indicator for the clinical use of OMZ in the future.

In the liver, the activation of Kupffer cells represents a central mechanism of inflammatory liver injury involving the production of two important inflammatory mediators, namely TNF-α and NO by iNOS (6, 7). Kupffer cells produce and secrete TNF-α and other cytokines in part through the activation of NF-κB, which in turn activate hepatocytes and Kupffer cells themselves via their receptors (36). In the partial hepatectomy model in other studies, indigenous Kupffer cells are activated, and in the dead bacteria model, a large number of macrophages infiltrate the liver and presumably are to be activated. Activated hepatic macrophages have been reported to cause endothelial damage in sinusoids, coagulation in sinusoids, and hepatic necrosis via microcirculatory disturbances (37).

There have also been many studies on the role of Kupffer cells in liver regeneration, and mechanisms such as the

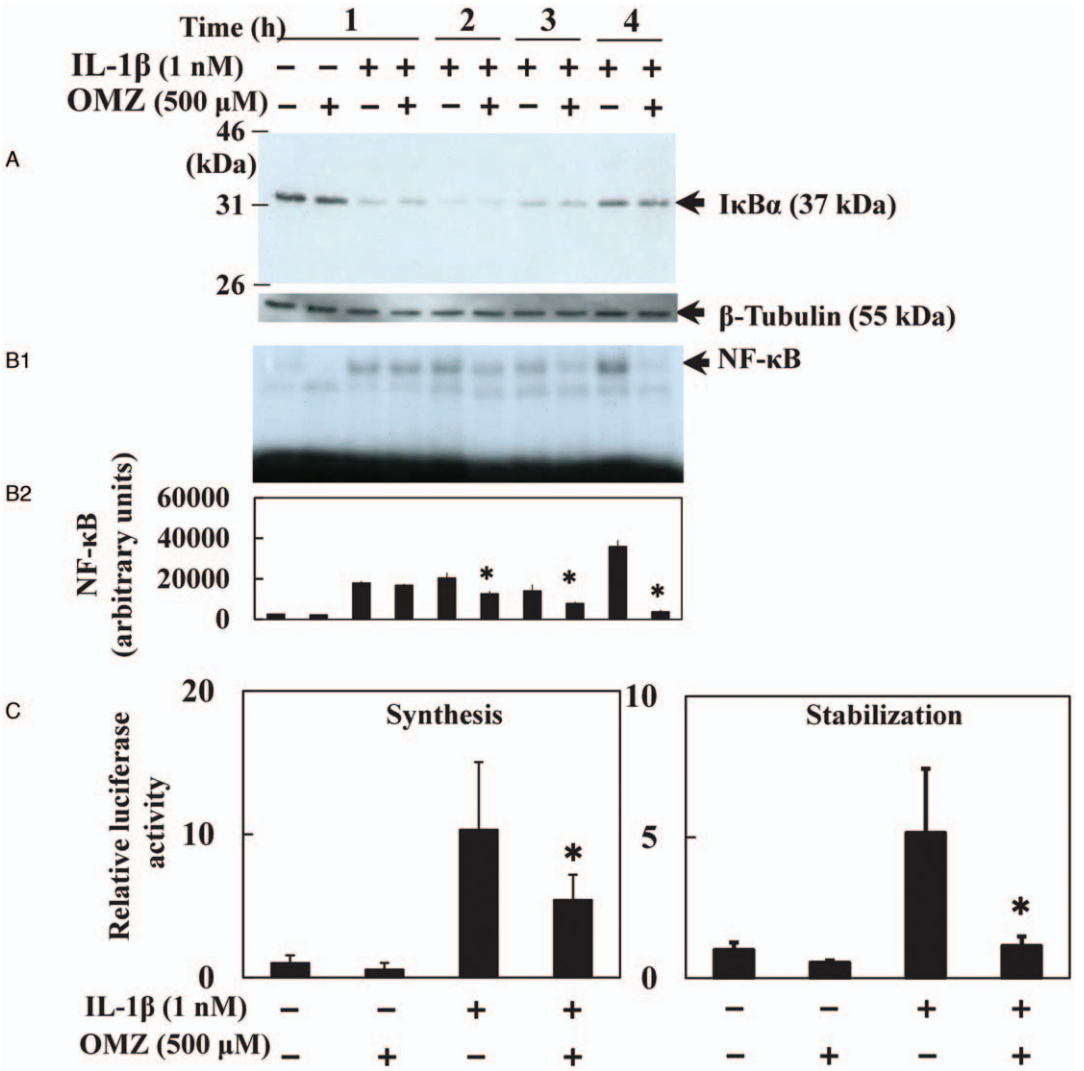

FIG. 9. **Effects of omeprazole on NF-κB activation and iNOS mRNA levels in primary cultured rat hepatocytes.** (A) IκBα degradation, (B) NF-κB activation (electrophoretic mobility shift assay (B1) and densitometry (B2)), and (C) transfection experiments: iNOS mRNA synthesis (pRiNOS-Luc-SVpA) and its stabilization (pRiNOS-Luc-3'UTR). * $P < 0.05$ versus positive control (IL-1β). OMZ, omeprazole.

regulation of hepatocyte proliferation by cytokine secretion from Kupffer cells have already been shown. TNF-α released by activated macrophages in the liver have important roles in hepatocyte necrosis and liver regeneration (38, 39). Blood TNF-α levels increase early after partial hepatectomy and are thought to be involved in promoting liver regeneration, and the increased TNF-α after partial hepatectomy may have an inhibitory effect on apoptosis (40). The effects of OMZ on TNF-α and Kupffer cells are the subject of future research.

The dose and administration method of OMZ (40 mg/kg–240 mg/kg, i.p.; single administration) used in this study differed to the standard clinical use (20 mg/50 kg, i.v.; single administration). These doses were calculated according to those previously used in our experimental studies (100 mg/kg LPZ) (4). Lower doses of OMZ in this study were comparable to doses in other animal models (41, 42). Higher doses of OMZ were more effective. No adverse effects were reported with higher doses of OMZ in other research reports. However, while this study was a single dose, other studies pointed out the side effects from long-term administration of OMZ, and it has been pointed out that another *in vivo* model tends to change to a high-fat diet as a result of long-term PPIs administration (43). Further studies are needed on the side effects of high-dose OMZ.

In addition, from the results obtained in *in vitro* primary cultured rat hepatocytes (Figs. 8 and 9), we confirmed that OMZ inhibited the induction of iNOS in a dose-dependent manner, followed by the blockade of excess NO production, which is one of the factors involved in organ injury including that of the liver (6–8). *In vitro* experiments also revealed that OMZ reduced the mRNA expression of other proinflammatory mediators (TNF-α, IL-1β, and CINC-1), in part through the inhibition of NF-κB activation. OMZ decreased the expression of iNOS mRNA and protein through the inhibition of both promoter transactivation (mRNA synthesis) and mRNA stabilization. These findings are consistent with the results of previous studies (25).

Inflammatory cytokines such as TNF-α are mainly produced by hepatic macrophages. However, it has been shown that TNF-α is produced in primary cultures of rat hepatocytes (44). In this study, we used hepatocytes, which make up the majority of liver tissue, and also focused on the changes in NO produced by hepatocytes. NF-κB was inhibited, and iNOS and cytokines were decreased, which was also shown in the results of primary culture using hepatocytes, leading to hepatoprotection. In general, the anti-inflammatory effects of OMZ are associated with increased anti-inflammatory cytokines, anti-apoptotic effects, and increased gastric blood flow (31, 32, 45, 46). Therefore, the effects from other parts of the body, such as antioxidant and anti-inflammatory effects due to anti-apoptotic effects and increased gastric blood flow, are subject to further research.

The regulation of inflammatory reactions during the perioperative period is important to prevent organ damage and complications. In this study, we investigated the hepatoprotective effects of OMZ using *in vivo* and *in vitro* liver injury models. Our experiments showed that OMZ prevents proinflammatory mediator expression (iNOS, TNF-α, CINC-1, IL-6, and IL-1β) by suppressing NF-κB activation. In addition, OMZ increased survival in GalN/LPS and PH/LPS rats. These results suggest that OMZ may have a role in preventing liver injury, and further in-depth studies are needed to explore its possible therapeutic applications (Supplementary material http://links.lww.com/SHK/B386).

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