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ONO 3403, a synthetic serine protease inhibitor, inhibits lipopolysaccharide-induced tumor necrosis factor-α and nitric oxide production and protects mice from lethal endotoxic shock

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ONO 3403, a new synthetic serine protease inhibitor, is a derivative of camostat mesilate and has a higher protease-inhibitory activity. The effect of ONO 3403 on lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)-α and nitric oxide (NO) production in RAW 264.7 macrophage-like cells was examined. ONO 3403 significantly inhibited LPS-induced TNF-α production at a lower concentration than camostat mesilate. It also inhibited LPS-induced NO production. Their inhibition was responsible for the reduced mRNA expression of TNF-α and inducible NO synthase. In LPS-stimulated cells, ONO 3403 prevented the augmentation of MyD88 expression and inhibited the phosphorylation of IκB-α, stress-activated protein kinase (SAPK) and IRF-3, and the production of interferon-β. ONO 3403 abolished the elevation of the extracellular serine protease activity in response to LPS. Further, it reduced the circulating TNF-α level, hepatic injury and mortality in mice receiving an injection of D-galactosamine and LPS. ONO 3403 was suggested to inhibit LPS-induced inflammatory responses via inactivation of MyD88-dependent and independent pathways.

Keywords: ONO 3403, synthetic serine protease inhibitor, LPS, TNF-α, NO, endotoxic shock

Introduction

The biological functions and therapeutic effect of synthetic serine protease inhibitors have been studied.[1,2] They are known to inhibit the production of pro-inflammatory cytokines and mediators in response to various pathogenic agents and exhibit anti-inflammatory activity.[3,4] Among them, camostat mesilate (FOY 305) and gabexate mesilate (FOY 307) have been used clinically for the treatment of pancreatitis and disseminated intravascular coagulation.[5,6] Camostat mesilate (FOY 305) suppresses the growth of autochthonous solid tumors and inhibits the metastasis of carcinomas in mice through interference with the protease activity.[7,8] Recently, orally active ONO 3403 has been produced as an analog of camostat mesilate (FOY 305) with more potent protease-inhibitory activity against various proteases, such as trypsin, plasmin, thrombin, urokinase-type plasminogen activator and plasma kallikrein.[9,10] However, there is no report on the anti-inflammatory action of ONO 3403 with a higher protease-inhibitory activity. It is of interest to characterize the relationship between anti-protease-inhibitory activity and anti-inflammatory activity of ONO 3403.

Bacterial lipopolysaccharide (LPS) is present on the outer membranes of all Gram-negative bacteria and stimulates macrophages to produce a variety of

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pro-inflammatory cytokines, free radicals and mediators.[11] Finally, LPS causes systemic inflammatory response syndrome, sepsis, disseminated intravascular coagulation and lethal endotoxic shock in animals.[12,13] Lipopolysaccharide stimulates macrophages via Toll-like receptor (TLR) 4 and activates MyD88-dependent and independent pathways downstream of TLR4.[14,15] A series of protease inhibitors has been examined for treatment of endotoxic shock. Urinary trypsin inhibitor prevents LPS-induced systemic inflammatory response and organ injury through inhibiting the production of pro-inflammatory cytokines and chemokines, like gabexate mesilate.[16–18] Some serine protease inhibitors inhibit LPS-induced nuclear factor (NF)-κB activation and subsequent production of pro-inflammatory mediators.[19,20] Moreover, secretory leukoprotease inhibitor is reported to play an important role in innate immunity by attenuating excessive inflammatory responses.[21] In the present study, we examined the effect of ONO 3403 as a new synthetic protease inhibitor on the production of TNF-α and NO in LPS-stimulated RAW 264.7 macrophage-like cells and on the development of lethal endotoxic shock in mice receiving D-galactosamine (D-GalN) and LPS. Here, we report that ONO 3403 may reduce LPS-induced TNF-α and NO production and protect mice from the lethal endotoxic shock.

Materials and Methods

Reagents

ONO 3403 was obtained from Ono Pharmaceutical Co. (Osaka, Japan). Lipopolysaccharide from *Escherichia coli* O55 was purchased from Sigma Chemical Co. (St Louis, MO, USA). Antibodies to IκB-α, stress-activated protein kinase (SAPK/JNK), IRF-3, and their phosphorylated forms were obtained from Cell Signaling (Beverly, MA, USA). Antibodies to the mouse inducible-type of NO synthase (iNOS) and phycoerythrin (PE)-conjugated TLR-4 were purchased from Transduction Laboratories (Lexington, KY, USA). As a series of TLR ligands Pam_3Cys was obtained from Calbiochem (San Diego, CA, USA), and polyI:C, CpG DNA and imiquimod were purchased from Invivogen (San Diego, CA, USA). D-Galactosamine was obtained from Wako Pure Chemicals (Osaka, Japan).

Cell culture and LPS stimulation

The murine macrophage cell line RAW 264.7 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in RPMI 1640 medium containing 5% fetal bovine serum and antibiotics at 37°C with 5% CO_2. RAW 264.7 cells were seeded into 35-mm culture dishes or a 96-well microplate for experiments. Peritoneal macrophages were obtained by washing the peritoneal cavity of mouse receiving an intraperitoneal injection of 4% thioglycolate solution (1 ml). The cells were suspended in the culture medium and plated in a 96-well plastic microplate at a cell concentration of 3×10^5/100 µl. The cells were pretreated with various concentrations of ONO 3403 *in vitro* for various time periods (hours) and stimulated with LPS (100 ng/ml) for the indicated periods. ONO 3403 was dissolved in dimethylsulfoxide at 20 mg/ml and diluted in the medium. Mock-treated RAW cells were cultured with dimethylsulfoxide alone.

Measurement of TNF-α and interferon (IFN)-γ levels

The culture supernatants were obtained 1 h after LPS stimulation of cells pretreated with or without ONO 3403. The sera were obtained 1 h (TNF-α and 5 h (IFN-γ after the administration of D-GalN with LPS into mice pretreated with or without ONO 3403. The concentrations of TNF-α and IFN-γ were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Cambridge, MA, USA).

Determination of nitrite concentration

Nitrite was measured as the end product, nitrite, by using Griess reagent as described elsewhere.[22] The cells were pretreated with ONO 3403 for various periods and stimulated with LPS at 100 ng/ml for 24 h. The culture supernatant (100 µl) was mixed with 100 µl of Griess reagent for 10 min, and the absorbance at 570 nm was measured in a microplate reader.

Immunoblotting

RAW 264.7 cells were seeded into 35-mm plastic dishes (5×10^5 cells/dish). The cells were pretreated with ONO 3403 (20 µg/ml) for 20 min and stimulated with LPS (100 ng/ml) for various hours. Briefly, the cells were lysed with a lysis buffer containing protease inhibitors. The protein concentration of the samples was determined by the BCA protein assay reagent (Pierce, Rockford, IL, USA). Each cell lysate containing 20 µg of protein was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis with 6–12% gel under reducing conditions and transferred to the membranes. The membranes were blocked with 5% skimmed milk and treated with appropriately diluted antibodies overnight at 4°C. The immune complexes were detected with a 1 : 2000 dilution of horseradish peroxidase-conjugated

secondary antibodies. The bands were visualized with a chemiluminescent reagent (Pierce).

Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using a RNeasy minikit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed to cDNA using a RT system with random hexamers (Toyobo, Tokyo, Japan). The level of mRNA was analyzed with StepOne real-time PCR, according to the manufacturer's instruction (Applied Biosystems, Foster City, CA, USA). The reaction mixture consists of SYBR green PCR master mix (Toyobo, Japan) and following sequence specific primers: TNF-α sense (TGTTGCCTCCTCTTTTGCTT), antisense (TGGTCACCAAATCAGCGTTA), iNOS sense (GTCTTGCAAGCTGATGGTCA), antisense (ACCACTCGTACTTGGGATGC), IFN-β sense (CCAGCCACCTAGAGATCAG), antisense (TGTCCAGCGGTGTGTGTTACAT), and β-actin sense (ATGACCCAGATCATGTTTGA), antisense (TACGACCAGAGGCATACAG). All primer sets were obtained from Invitrogen (Carlsbad, CA, USA). The level of mRNA in each sample is expressed as the fold increase over non-treated controls in the ratio of each target gene to β-actin.

Protease assay

RAW 264.7 cells (7.5×10^4/ml) were resuspended in freshly prepared RPMI 1640 (phenol-red free) containing 2% fetal bovine serum in 96-well plates. The cells were pretreated with or without ONO 3403 (20 μg/ml) for 20 min and stimulated with LPS (1 μg/ml) for 3 min. The samples (30 μl) were removed and mixed with Chromozym TH (170 μl) at 1.25 mg/ml, which was dissolved in phenol-red free RPMI 1640 containing 2 mM L-glutamine. Chromozym TH was obtained from Roche (Mannheim, Germany) and used as substrate for the determination of serine protease activity in aqueous solutions. Absorbance readings at 405 nm were performed with a spectrophotometer in 15, 30 and 60 min incubation at ambient temperature. Results are expressed as increase in absorbance units with respect to time zero, per time point. Since LPS at 1 μg/ml inhibited the protease activity more markedly than LPS at 100 ng/ml, LPS was used at a concentration of 1 μg/ml in the experiment.

Lethality studies

Female BALB/c mice, 8–10 weeks old, were obtained from Japan SLC (Hamamatsu, Japan). The study protocol was approved by the Animal Care Committee and carried out under the guidance for care and use of laboratory animals, Aichi Medical University. The LPS-induced lethal shock was performed by an intraperitoneal (i.p.) injection with a mixture of LPS (1 μg) and D-GalN (10 mg) per mouse. Based on the results from preliminary experiments, ONO 3403 (0.5–1 mg per mouse) was injected i.p. 30 min before the challenge of D-GalN and LPS. The control mice were injected with ONO 3403 alone. Six mice were used in each experimental group and the experiment was performed twice. The lethality was monitored until 3 d after the administration of LPS.

Histology

Livers were removed, fixed in 10% formalin and subsequently embedded in paraffin. Sections were stained with hematoxylin and eosin and observed under a microscope.

Statistical analysis

Experimental values are presented as mean ± SD from at least 3 independent experiments unless otherwise stated. The significance of differences between experimental and control groups was determined with the Student's *t*-test. A value of $P < 0.01$ was considered statistically significant.

RESULTS

Effect of ONO 3403 on the production of TNF-α and the expression of TNF-α mRNA in response to LPS

First, the cytotoxic action of ONO 3403 was examined by using RAW 264.7 cells. The cells were treated with ONO 3403 at concentrations of 1–150 μg/ml for 24 h and the cell viability was determined with a MTT assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Chemicon, Temecula, CA, USA). ONO 3403 did not show any cytotoxic action at the concentration range 5–20 μg/ml. However, ONO 3403 exhibited a cytotoxic action at concentrations higher than 100 μg/ml as in the previous report of Ohkoshi *et al.*[10]

To examine the effect of ONO 3403 on the production of TNF-α in response to LPS, the cells were stimulated with ONO 3403 at 5–20 μg/ml for 20 min and then stimulated with 100 ng/ml LPS for 1 h. As shown in Figure 1A, pretreatment with ONO 3403 (20 μg/ml) significantly inhibited LPS-induced TNF-α production, although LPS alone induced the production of a large amount of TNF-α. Subsequently, the inhibitory action of

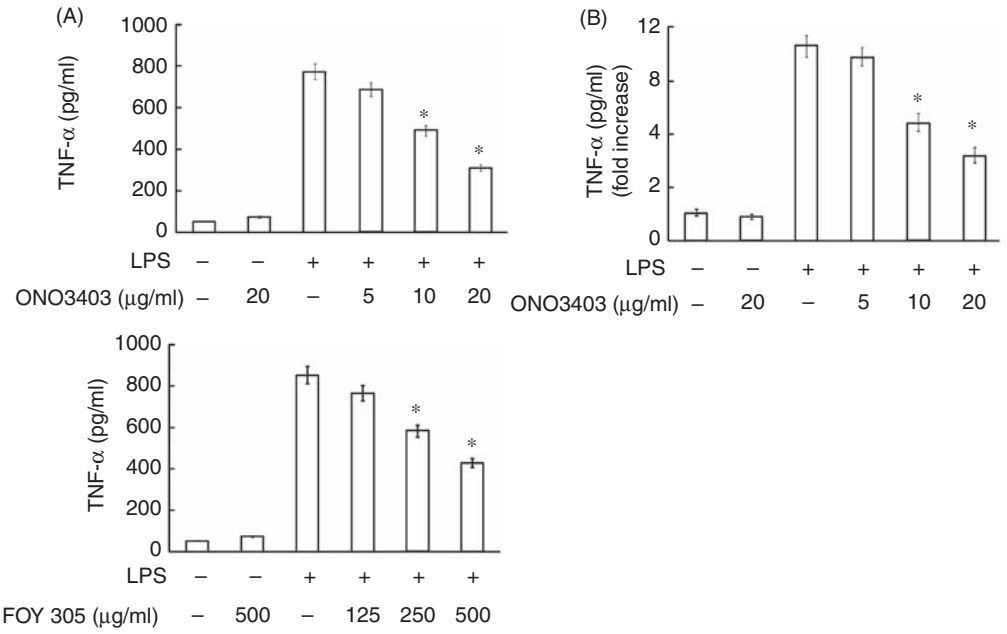

Fig. 1. Effect of ONO 3403 on the production of TNF-α and the expression of TNF-α mRNA in response to LPS. RAW 264.7 cells were pretreated with ONO 3403 (5–20 μg/ml) or camostat mesilate (125–500 μg/ml) for 20 min and stimulated with LPS (100 ng/ml) for 1 h. The concentration of TNF-α (A) and the expression of TNF-α mRNA (B) was determined by ELISA and real-time PCR, respectively. $*P < 0.01$ versus LPS alone.

ONO 3403 was compared with that of camostat mesilate. ONO 3403 inhibited LPS-induced TNF-α production at lower concentrations than camostat mesilate, suggesting that the inhibitory action of ONO 3403 on LPS-induced TNF-α production was much stronger than that of camostat mesilate. The effect of ONO 3403 on the expression of TNF-α mRNA was also examined by analyzing the cell lysates with semiquantitative real-time PCR (Fig. 1B). Lipopolysaccharide markedly augmented the expression of TNF-α mRNA whereas the augmentation was significantly reduced by ONO 3403 at 10 μg/ml and 20 μg/ml. In addition, the effect of ONO 3403 on LPS-induced interleukin (IL)-6 production was examined with ELISA. Untreated normal control cells produced a small amount of IL-6 (25 ± 3.2 pg/ml) and the cells treated with LPS (100 ng/ml) for 6 h produced a high amount of IL-6 (252.3 ± 13.4 pg/ml). However, pretreatment with ONO 3403 at 10 μg/ml and 20 μg/ml reduced the LPS-induced IL-6 production to 122.5 ± 4.3 (52% reduction) and 97.2 ± 5.1 pg/ml (62% reduction), respectively.

Effect of ONO 3403 on LPS-induced NO production

The effect of ONO 3403 on LPS-induced NO production was examined (Fig. 2). The cells were pretreated with ONO 3403 (20 μg/ml) for various times and then incubated with LPS (100 ng/ml) for 24 h. Pretreatment of ONO 3403 for 10 min and 20 min inhibited LPS-induced NO production, although LPS significantly

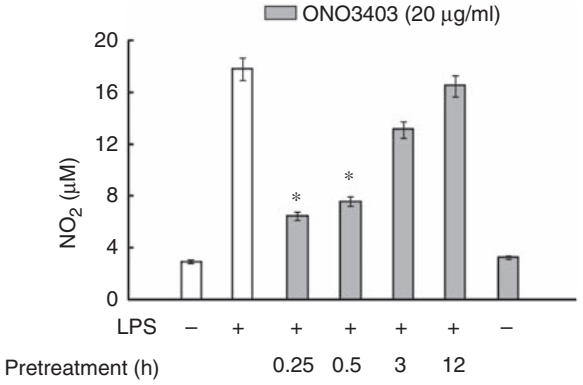

Fig. 2. Effect of ONO 3403 on the production of NO in response to LPS. RAW 264.7 cells were treated with ONO 3403 (20 μg/ml) for the indicated time and stimulated with LPS (100 ng/ml) for 24 h. $*P < 0.01$ versus LPS alone.

augmented the NO production in non-treated cells. Furthermore, neither simultaneous nor post-treatment with ONO 3403 inhibited NO production. In addition, ONO 3403 alone did not affect NO production in RAW 264.7 cells. Next, the effect of ONO 3403 on LPS-induced TNF-α and NO production was examined in peritoneal macrophages. ONO 3403 exhibited an inhibitory action on LPS-induced TNF-α and NO production in peritoneal macrophages (~65% inhibition) as well as RAW 263.7 cells.

Further, the effect of ONO 3403 on the TLR-independent NO production was examined. The 24-h

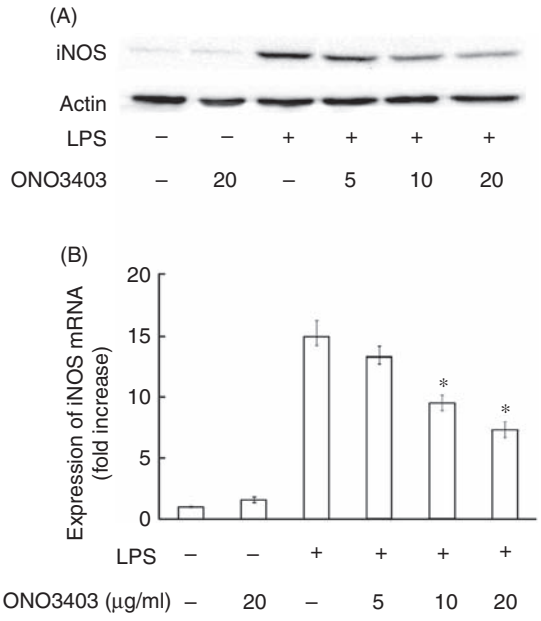

Fig. 3. Effect of ONO 3403 on the expression of iNOS protein and mRNA in response to LPS. RAW 264.7 cells were pretreated with ONO 3403 at 5–20 μg/ml for 20 min and stimulated with LPS at 100 ng/ml. (A) The expression of iNOS protein was determined with immunoblotting 16 h after LPS treatment. A typical experiment of three independent experiments is shown. (B) The expression of iNOS mRNA was determined with real-time PCR 8 h after LPS treatment. $*P < 0.01$ versus LPS alone.

treatment of IFN-γ (100 U/ml) and the combination of TNF-α (25 ng/ml), A23187 (Ca ionophore, 100 nM) and phorbol-12-myristate-13-acetate (10 nM) led to NO production at 21.3 ± 2.6 μM and 10.3 ± 1.4 μM, respectively, when it was 4.0 ± 0.8 μM in the non-stimulated cells. However, pretreatment with ONO 3403 (20 μg/ml) showed no significant inhibitory effect.

Effect of ONO 3403 on the expression of inducible type NO synthase (iNOS) protein and mRNA in response to LPS

The effect of ONO 3403 on the expression of iNOS protein and mRNA was examined with immunoblotting and real-time PCR, respectively. RAW 264.7 cells were pretreated with ONO 3403 at 5–20 μg/ml for 20 min and stimulated with LPS at 100 ng/ml for 16 h. The high expression of iNOS protein was detected in LPS-treated cells. On the other hand, pretreatment with ONO 3403 at 20 μg/ml significantly reduced the expression of iNOS protein (Fig. 3A). The effect of ONO 3403 on iNOS mRNA expression was examined 8 h after LPS treatment (Fig. 3B). Lipopolysaccharide markedly augmented the level of iNOS mRNA in stimulated cells whereas the pretreatment of ONO 3403 at 20 μg/ml definitely inhibited the augmentation.

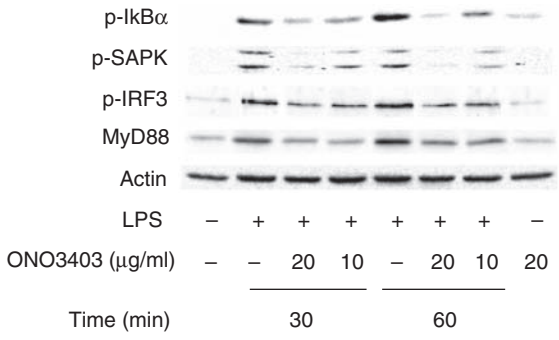

Fig. 4. Effect of ONO 3403 on LPS-induced expression of various signaling molecules in MyD88-dependent and -independent pathways. RAW 264.7 cells were pretreated with ONO 3403 (10–20 μg/ml) for 20 min and stimulated with LPS (100 ng/ml) for 30 min and 60 min. The expression of MyD88 and the phosphorylated forms of IκB-α (pIκB-α), SAPK (pSAPK) and IRF-3 (pIRF-3) proteins was determined by immunoblotting. A typical experiment of three independent experiments is shown.

Effect of ONO 3403 on the activation of several signaling molecules required for LPS-induced TNF-α and NO production

The production of TNF-α and NO is induced by LPS through triggering MyD88-dependent and independent (TRIF-dependent) signaling pathways.[15,23] Therefore, we examined the effects of ONO 3403 on LPS-induced activation of signaling molecules that are required for the production of iNOS and TNF-α (Fig. 4). First, the effect of ONO 3403 on the expression of MyD88 in LPS-stimulated RAW 264.7 cells was examined. ONO 3403 prevented LPS-induced MyD88 augmentation although LPS significantly augmented it. Further, LPS significantly augmented the phosphorylation of IκB-α protein and SAPK/JNK in normal control cells whereas their phosphorylation was inhibited in ONO 3403-pretreated cells 30 min and 60 min after LPS stimulation.

Next, the effect of ONO 3403 on the activation of IRF-3 and IFN-β belonging to the MyD88-independent pathway[24] was examined. The phosphorylation of IRF-3 protein in LPS-stimulated cells in the presence and absence of ONO 3403 was determined by immunoblotting. IRF-3 protein was clearly phosphorylated 30 min and 60 min after LPS treatment, whereas ONO 3403 clearly inhibited it (Fig. 4). The effect of ONO 3403 on LPS-induced IFN-β expression was examined. Lipopolysaccharide induced a high level of IFN-β mRNA expression whereas pretreatment with ONO 3403 (20 μg/ml) for 20 min significantly reduced it. ONO 3403 was suggested to inhibit both MyD88-dependent and -independent signaling pathways in LPS signaling. In addition, ONO 3403 alone did not affect the expression of those signal molecules.

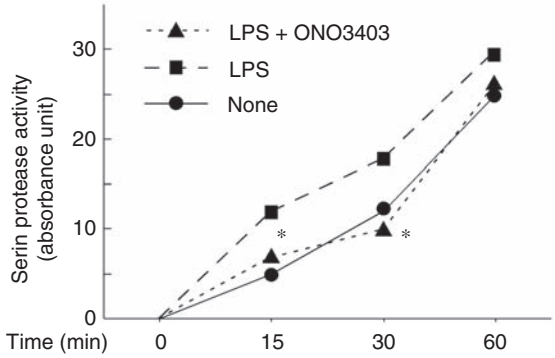

Fig. 5. Effect of ONO 3403 on the extracellular serine protease activity in response to LPS. RAW 264.7 cells (7.5×10^4/ml) were pretreated with ONO 3403 (20 μg/ml) for 20 min and stimulated with LPS (1 μg/ml) for 3 min. The experimental results are shown as increase of absorbance units per individual sample various time after measurement. $*P < 0.01$ versus LPS alone.

Effect of ONO 3403 on the expression of TLR4 in RAW 264.7 cells

The effect of ONO 3403 on the cell surface expression of TLR4 and CD14 as LPS receptors was examined with laser flow cytometric analysis. The pretreatment of ONO 3403 did not alter the cell surface expression of TLR4 and CD14.

Effect of ONO 3403 on the extracellular serine protease activity in response to LPS

Since the inhibition of an extracellular serine protease activity by serine protease inhibitors is reported to lead to LPS-induced NF-κB inactivation,[25] the effect of ONO 3403 on the extracellular serine protease activity in LPS-stimulated RAW 264.7 cells was examined (Fig. 5). The cells were stimulated with LPS (100 ng/ml) for 3 min after ONO 3403 pretreatment for 20 min. The protease activity was examined using a substrate that is specifically cleaved by thrombin-like serine proteases. As shown in Figure 5, a significant increase in the protease activity was observed in response to LPS. On the other hand, ONO 3403 inhibited the elevation of the serine protease activity in LPS-treated cells at 15 min and 30 min after the reaction.

In vivo *effect of ONO 3403 on the level of circulating TNF-α and development of lethal endotoxic shock in mice*

Based on the preceding results, the protective effect of ONO 3403 on LPS-induced lethality was examined by using D-GalN-sensitized mice. ONO 3403 (0.5 mg and 1 mg per mouse) was injected i.p. 30 min before the administration of D-GalN (10 mg) and LPS (1 μg) into mouse. The injection of D-GalN and LPS killed all 6 mice within 24 h. As shown in Figure 6A, ONO 3403 pretreatment definitely reduced the mortality in mice receiving D-GalN and LPS and the pretreatment with ONO 3403 (1 mg/mouse) was more effective for the protection than that (0.5 mg/mouse). The LPS-induced lethality is mainly mediated by TNF-α.[26,27] The effect of ONO 3403 on the level of circulating TNF-α in the lethal shock was examined (Fig. 5B). The level of serum TNF-α was reduced to approximately 70% in ONO 3403-pretreated mice compared to that in mock-treated mice ($P < 0.05$). In livers of mice receiving D-GalN and LPS, there were severe hepatic lesions accompanied by vascular congestion, inflammatory cell infiltration and hepatocyte death (Fig. 6C). Pretreatment of ONO 3403 definitely prevented the development of hepatic lesions. In addition, the effect of ONO 3403 on IFN-γ production was examined with ELISA 5 h after treatment with D-GalN and LPS. The level of circulating IFN-γ was 56.4 ± 4.2 pg/ml in untreated control mice and 104.3 ± 6.2 pg/ml in D-GalN and LPS-treated mice. Pretreatment with ONO 3403 did not affect the level of circulating IFN-γ (98.5 ± 3.4 pg/ml).

DISCUSSION

In the present study, we demonstrate that ONO 3403 significantly inhibits LPS-induced TNF-α and NO production and protects mice from LPS-mediated lethal shock. A series of serine protease inhibitors are reported to inhibit the production of pro-inflammatory cytokines in response to LPS and protect LPS-induced tissue injury.[17,18] This has been confirmed by the present study using ONO 3403 as a new synthetic serine protease inhibitor. ONO 3403 is produced as a derivative of camostat mesilate (FOY 305) with more potent protease-inhibitory activity.[9] The present study demonstrates that ONO 3403 may exhibit a stronger anti-inflammatory action than camostat mesilate (FOY 305). This is the first report concerning a protective effect of ONO 3403 against LPS-mediated toxicity. ONO 3403 inhibits the growth of normal and transformed cells,[10,28] and induces apoptosis-like cell death via stimulating calpain activity.[29] However, the cytotoxic action is not observed at concentrations of 2–20 μg/ml in our study, excluding the possibility that the inhibition of pro-inflammatory mediators by ONO 3403 might be dependent on the cytotoxic action.

ONO 3403 inhibits the augmentation of MyD88 expression in LPS-stimulated RAW 264.7 cells. Moreover, it inhibits the phosphorylation of IκB-α, suggesting the subsequent inactivation of NF-κB. ONO 3403 also inhibits the phosphorylation of SAPK,

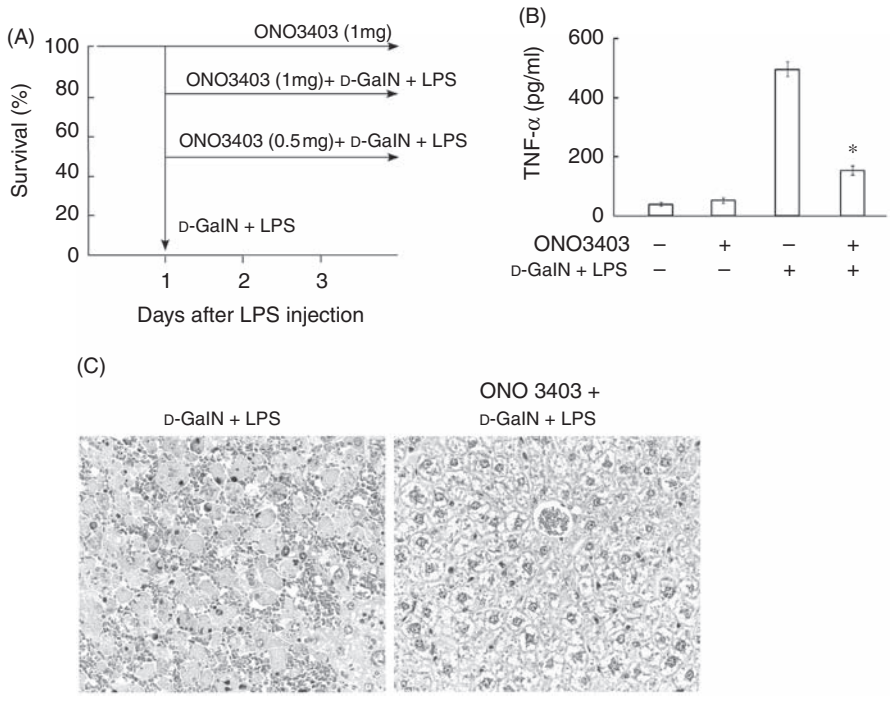

Fig. 6. Effect of ONO 3403 on the mortality of lethal endotoxic shock and the level of circulating TNF-α in mice injected with D-GalN and LPS. (A) Mice were injected i.p. with ONO 3403 (0.5 mg and 1 mg per mouse) 0.5 h before the injection of D-GalN (10 mg) and LPS (1 μg). The mortality was determined for 3 days after the LPS challenge. (B) The effect of ONO 3403 on the level of serum TNF-α in mice injected with a mixture of D-GalN and LPS. The sera were taken 1 h after the LPS challenge. The level of serum TNF-α was determined by ELISA. $*P<0.01$ versus LPS alone. (C) Hepatic lesions in D-GalN- and LPS-treated mice with or without pretreatment of ONO 3403. Livers were removed 6 h after D-GalN and LPS treatment (×100).

a member of mitogen-activated protein kinases. Therefore, ONO 3403 is suggested to inhibit the MyD88-dependent pathway. Furthermore, ONO 3403 inhibits the phosphorylation of IRF-3 and the production of IFN-β, which belong to the MyD88-independent pathway in LPS signaling. Taken together, ONO 3403 is suggested to inhibit the whole LPS signaling just downstream of TLR-4. Gabexate mesilate (FOY 307) is reported to inhibit NF-κB activation by inhibiting IκB-α degradation.[30] On the other hand, the present study demonstrates that ONO 3403 inhibits both MyD88-dependent and -independent pathways, excluding the possibility that the target molecule of ONO 3403 is IκB-α. ONO 3403 did not affect the expression of TLR4. Therefore, it might be hypothesized that ONO 3403 inhibited the intracellular LPS signaling downstream of TLR4. However, the precise inhibitory mechanism of ONO 3403 is still unclear. Moreover, ONO 3403 significantly inhibits NO production in response to TLR agonists, such as Pam_3Cys, CpG DNA, and polyI:C,[31] but not IFN-γ and the combination of TNF-α, Ca ionophore, and phorbol-12-myristate-13-acetate. Therefore, ONO 3403 possibly inhibits some signal event, which is common to the activation of a series of TLRs.

ONO 3403, a derivative of camostat mesilate, has a higher protease-inhibitory activity than camostat mesilate (FOY 305).[9] ONO 3403 exhibited anti-inflammatory activity at a lower concentration than camostat mesilate (FOY 305). ONO 3403 almost completely inhibits augmentation of the serine protease activity in response to LPS. Therefore, the anti-inflammatory action of ONO 3403 might be responsible for the high anti-protease inhibitory activity. It was also effective in the *in vivo* reduction of circulating TNF-α levels in LPS-injected mice. Considering that the lethal shock with D-GalN and LPS is thought of as an experimental endotoxic shock,[32] it would be of interest to study the therapeutic effectiveness of ONO 3403, a synthetic serine protease inhibitor, on patients with septic shock, in comparison with other serine protease inhibitors.[17,33,34]

Proteases play an essential role in modulating the turnover of extracellular matrix and regulating fundamental cellular functions such as apoptosis, cell growth and activation, protein secretion and phagocytosis.[35] Proteases are classified based on their catalytic mechanisms into serine, aspartic, metallo, threonine and cysteine proteases and are localized extracellularly, at the cellular surface, in the cytoplasm of cells or within specific subcellular structures such as lysosomes.[36] As shown in the human macrophage-like cell line THP-125, the present study suggested a high basal thrombin-like protease activity in RAW 264.7 cells. Lipopolysaccharide augments the

serine protease activity within several minutes after the stimulation. It is unclear whether or not the augmentation is dependent on TLR 4 signaling because the augmentation occurs within a few minutes.

The known mammalian TLR-signaling cascade is analogous to the *Drosophila* Toll signaling pathway.[37,38] In *Drosophila*, a proteolytically processed product of a Spaetzle gene activates the Toll receptor and the Spaetzle gene needs to be processed by serine protease to activate the Toll receptor.[39,40] It has been suggested that LPS may first activate mammalian serine proteases, which generate a product required for further signaling, but there is no direct evidence for this.[25]

Conclusions

We have demonstrated, for the first time, that ONO 3403, a new synthetic serine protease inhibitor, decreases the level of pro-inflammatory mediators in LPS-treated macrophage-like cells. The anti-inflammatory action of ONO 3403 might be caused by the high anti-protease inhibitory activity via inactivation of TLR4-mediated LPS signaling. Further, pretreatment of ONO 3403 protects mice from lethal endotoxic shock.

Acknowledgements

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