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LIPOTEICHOIC ACID–INDUCED *TNF-α* AND *IL-6* GENE EXPRESSIONS AND OXIDATIVE STRESS PRODUCTION IN MACROPHAGES ARE SUPPRESSED BY KETAMINE THROUGH DOWNREGULATING TOLL-LIKE RECEPTOR 2–MEDIATED ACTIVATION OF ERK1/2 AND NFκB

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ABSTRACT—Lipoteichoic acid (LTA), a gram-positive bacterial outer membrane component, can cause septic shock. Our previous studies showed that ketamine has anti-inflammatory and antioxidant effects on gram-negative LPS-induced macrophage activation. In this study, we further evaluated the effects of ketamine on the regulation of LTA-induced *TNF-α* and *IL-6* gene expressions and oxidative stress production in macrophages and its possible mechanisms. Exposure of macrophages to a therapeutic concentration of ketamine (100 μM) inhibited LTA-induced TNF-α and IL-6 expressions at protein or mRNA levels. In parallel, ketamine at 100 μM reduced LTA-stimulated phosphorylation of extracellular signal–regulated kinase 1/2 (ERK1/2). Sequentially, ketamine reduced the LTA-triggered translocation of nuclear factor-κB (NFκB) from the cytoplasm to nuclei and its transactivation activity. Pretreatment with PD98059, an inhibitor of ERK, decreased LTA-enhanced NFκB activation and TNF-α and IL-6 mRNA syntheses. Cotreatment with ketamine and PD98059 synergistically suppressed the LTA-induced translocation and transactivation of NFκB and biosyntheses of TNF-α and IL-6 mRNA. Application of toll-like receptor 2 (TLR2) small interfering RNA (si)RNA into macrophages decreased the levels of this receptor, and simultaneously ameliorated LTA-augmented NFκB transactivation and consequent production of TNF-α and IL-6 mRNA. Cotreatment with ketamine and TLR2 siRNA synergistically lowered TNF-α and IL-6 mRNA syntheses in LTA-activated macrophages. Ketamine and TLR2 siRNA could reduce the LTA-induced increases in production of nitrite and intracellular reactive oxygen species in macrophages, and their combination had better effects than a single exposure. Thus, this study shows that one possible mechanism involved in ketamine-induced inhibition of LTA-induced *TNF-α* and *IL-6* gene expressions and oxidative stress production is through downregulating TLR2-mediated phosphorylation of ERK1/2 and the subsequent translocation and transactivation of NFκB.

KEYWORDS—Ketamine, macrophages, LTA, TLR2, inflammatory cytokines, oxidative stress

INTRODUCTION

Ketamine, an intravenous anesthetic agent, has more stable hemodynamics than other anesthetic agents, so it is often applied as an inducer of anesthesia in critically ill patients (1). Ketamine can also be a competitive *N*-methyl-D-aspartate receptor antagonist and modulates central sensitization induced by tissue damage or perioperative analgesics (2). Clinically, induction of anesthesia with ketamine is usually associated with increases in cardiac output, arterial blood pressure, and heart rate (3, 4). In both patients and experimental animals, ketamine has been shown to possess anti-inflammatory effects (5–7). Previous studies provided *in vitro* data demonstrating that ketamine can induce dysfunction of lymphocytes, natural killer cells, and neutrophils (8–10). Recent studies in our laboratory further showed that therapeutic concentrations of ketamine selectively suppressed the macrophage functions of phagocytosis, oxidative ability, and cytokine production via a mitochondrion-dependent mechanism (11, 12).

During recent decades, the incidence of sepsis and septic shock has been increasing (13). Although endotoxin-mediated events are clearly important in gram-negative infection, gram-positive bacteria also have crucial roles (14). The increasing prevalence of sepsis from gram-positive bacterial pathogens necessitates re-evaluation of many of the basic assumptions about the molecular pathogenesis of septic shock. Lipoteichoic acid (LTA), an outer membrane component of gram-positive bacteria, was shown to be one of the critical factors participating in the pathogenesis of sepsis (15). In response to stimuli, LTA can stimulate macrophages to produce massive amounts of inflammatory factors that exhibit systemic effects into the general circulation (16). TNF-α and IL-6, typical and critical inflammatory cytokines predominantly produced by macrophages, have pleiotropic effects on regulating the immune response and acute-phase reactions (17). TNF-α has been reported to contribute to the progression of myocardial infarction, rheumatoid arthritis, and macrophage-mediated tumor cytotoxicity (18, 19). IL-6 can be a regulator of macrophage development and antitumor activities (20, 21). Effects of ketamine on LTA-induced TNF-α and IL-6 biosyntheses are still unknown.

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Toll-like receptors (TLRs) are type I transmembrane proteins with extracellular leucin-rich domains and intracellular signaling domains (22). There are at least 12 member proteins found in mammalian cells. Toll-like receptor 2–dependent mechanisms were shown to participate in LTA-induced inflammation (23). Toll-like receptor 2 is a major receptor in macrophages responsible for LTA stimulation (24). After binding to LTA, the alteration in TLR2's conformation induces cascade activation of intracellular protein kinases and transcriptional factors (25). Our previous studies have shown that after exposure of macrophages to LPS, a gram-negative outer membrane component, ketamine can inhibit inflammatory cytokine gene expression via a TLR4-dependent pathway (12). Intracellular reactive oxygen species (ROS) is one of the key inflammatory factors (16). A previous study executed in our laboratory showed that LPS can increase oxidative stress to macrophages because of its inducing effects on *iNOS* gene, consequently producing massive amounts of NO (26). Meanwhile, studies about the effects of ketamine on gram-positive bacteria-induced inflammation, especially in the molecular mechanisms, are limited. Thus, this study was designed to evaluate the effects of ketamine on TNF-α and IL-6 biosynthesis and ROS production in LTA-activated macrophages and its possible mechanisms from the viewpoint of TLR2-mediated signal-transducing phosphorylation of extracellular signal–regulated kinase (ERK) and the consequent translocation and transactivation of transcription factor nuclear factor-κB (NFκB).

MATERIALS AND METHODS

Cell culture and drug treatment

Murine macrophage-like Raw 264.7 cells, purchased from American Type Culture Collection (Rockville, Md), were cultured following a previous method (27). Macrophages were cultured in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal calf serum, L-glutamine, penicillin (100 IU/mL), and streptomycin (100 μg/mL) in 75-cm^2 flasks at 37°C in a humidified atmosphere of 5% carbon dioxide. Ketamine and LTA were dissolved in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4). Clinically relevant plasma concentrations of ketamine of 1, 10, and 100 μM were chosen as the treatment concentrations in this study (28). Control macrophages received PBS only.

Assay of cell viability

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (29). Briefly, macrophages (2×10^4 cells/well) were seeded in 96-well tissue culture plates overnight. After drug treatment, macrophages were cultured with a new medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for a further 3 h. The blue formazan products in macrophages were dissolved in dimethyl sulfoxide and spectrophotometrically measured at a wavelength of 550 nm.

Enzyme-linked immunosorbent assay

Levels of TNF-α and IL-6 in the culture medium of macrophages were determined according to a previously described method (5). Briefly, macrophages (2×10^4 cells/well) were seeded in 96-well tissue culture plates overnight. After drug treatment, the medium was collected and centrifuged. The amounts of TNF-α and IL-6 were quantified following the standard protocols of the enzyme-linked immunosorbent assay kits purchased from Endogen (Woburn, Mass).

Quantitative reverse-transcriptase polymerase chain reaction assay

Messenger RNAs from macrophages exposed to drugs were prepared for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analyses of TNF-α, IL-6, and β-actin mRNA as previously described (12). The oligonucleotide sequences of the upstream and downstream primers for these mRNA analyses were 5′-ATGAGCACAGAAAGCATGATCCGC-3′ and 3′-CTCAGGCCCGTCCAGATGAAACC-5′ for TNF-α, 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′ and 3′-CACTAGGTTTGCCGAGTAGATCTC-5′ for IL-6, and 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′ for β-actin (30). A qRT-PCR analysis was carried out using iQSYBR Green Supermix (Bio-Rad, Hercules, Calif) and the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad).

Immunoblotting analyses of phosphorylated and nonphosphorylated ERK1/2

Protein levels were immunodetected according to a previously described method (31). After drug treatment, cell lysates were prepared in an ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, and 1 mM EDTA). Protein concentrations were quantified using a bicinchonic acid protein assay kit (Pierce, Rockford, Ill). The proteins (50 μg/well) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Phosphorylated ERK1/2 was immunodetected using a rabbit polyclonal antibody against phosphorylated residues of ERK1/2 (Cell Signaling, Danvers, Mass). Nonphosphorylated ERK2 was immunodetected as the internal controls (Cell Signaling).

Immunodetection of NFκB

Amounts of NFκB were quantified following a previously described method (32). After drug treatment, nuclear extracts of macrophages were prepared. Protein concentrations were quantified by a bicinchonic acid protein assay kit (Pierce). Nuclear proteins (50 μg/well) were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking, NFκB was immunodetected using a rabbit polyclonal antibody against mouse NFκB (Santa Cruz Biotechnology, Santa Cruz, Calif). Total NFκB was immunodetected as the internal standard.

NFκB reporter assay

The NFκB luciferase reporter plasmids (Stratagene, La Jolla, Calif) and pUC18 control plasmids were transfected into macrophages using a FuGene 6 transfection reagent (Roche Diagnostics) as previously described (26). After transfection, macrophages were exposed to ketamine and LTA. Cells were then harvested. The luciferase activity in cell lysates was measured using a dual luciferase assay system (Promega, Madison, Wis). Briefly, after dispensing 100 μL of the Luciferase Assay Reagent (Promega) into luminometer tubes, the cell lysates (20 μL) were added into the tubes and briefly vortexed. The luminometer was programmed to perform a 2-s measurement delay followed by a 10-s measurement read for luciferase activity.

TLR2 knockdown

Translation of TLR2 mRNA in macrophages was knocked down using an RNA interference method following a small-interfering RNA (siRNA) transfection protocol provided by Santa Cruz Biotechnology (Santa Cruz, Calif) as previously described (12). The TLR2 siRNA was purchased from Santa Cruz Biotechnology and is a pool of three target-specific 20 to approximately 25-nt siRNAs designed to knock down TLR2 expression. Briefly, after culturing macrophages in an antibiotic-free RPMI medium at 37°C in a humidified atmosphere of 5% carbon dioxide for 24 h, the siRNA duplex solution, which was diluted in the siRNA transfection medium (Santa Cruz Biotechnology), was added to the macrophages. After transfecting for 24 h, the medium was replaced with a normal Dulbecco's modified Eagle medium, and macrophages were treated with ketamine, LTA, or a combination of ketamine and LTA.

Determination of cellular nitrite production

Cellular nitrite production was determined according to the Griess reaction as previously described (33). After drug administration, the culture medium of macrophages was collected and centrifuged. The supernatant fractions were reacted with nitrate reductase. After a reaction of the supernatant with sulfanilamide and *N*-1-napthylethylenediamine, a colorimetric azo compound was formed and quantified using an Anthos 2010 microplate photometer (Anthos Labtec Instruments, Lagerhausstrasse, Wals/Salzburg, Austria).

Determination of cellular oxidative stress

Levels of intracellular ROS were quantified to evaluate cellular oxidative stress to macrophages after exposure to ketamine and LTA according to a previously described method (11). Briefly, macrophages (5×10^5 cells/well) were cultured in 12-well tissue culture plates overnight and then cotreated with drugs and 2′,7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, Oreg), a ROS-sensitive dye. After drug treatment, macrophages were harvested and suspended in 1× PBS buffer. Relative fluorescent intensities

TABLE 1. **Effects of KTM and LTA on macrophage viability**

	Cell viability, optical density values at 550 nm		
	1 h	6 h	24 h
Control	1.073 ± 0.210	1.088 ± 0.251	1.332 ± 0.288
KTM 1 μM	0.998 ± 0.172	0.997 ± 0.274	1.328 ± 0.281
KTM 10 μM	1.012 ± 0.200	1.021 ± 0.187	1.281 ± 0.275
KTM 100 μM	0.969 ± 0.196	0.998 ± 0.201	1.264 ± 0.303
LTA 10 μg/mL	1.015 ± 0.208	0.988 ± 0.221	1.299 ± 0.208
1 μM KTM + 10 μg/mL LTA	0.987 ± 0.244	0.981 ± 0.189	1.312 ± 0.299
10 μM KTM + 10 μg/mL LTA	0.977 ± 0.201	0.979 ± 0.190	1.297 ± 0.230
100 μM KTM + 10 μg/mL LTA	0.968 ± 0.174	0.968 ± 0.214	1.287 ± 0.287

Macrophages were exposed to 1, 10, and 100 μM ketamine (KTM), 10 μg/mL LTA, or a combination of KTM and LTA for 1, 6, and 24 h. Cell viability was determined following a colorimetric method. Each value represents the mean ± SEM for n = 6.

in macrophages were quantified using a flow cytometer (Becton Dickinson, San Jose, Calif).

Statistical analysis

The statistical significance of differences among control, ketamine-, LTA-, and ketamine + LTA–treated macrophages were evaluated using nonparametric ANOVA followed by Duncan multiple-range test, and differences were considered statistically significant at values of $P < 0.05$.

RESULTS

Toxicity of ketamine and LTA to macrophages

Exposure of macrophages to 1, 10, and 100 μM ketamine for 1, 6, and 24 h did not affect cell viability (Table 1). After treatment with 10 μg/mL LTA for 1, 6, and 24 h, the viability of macrophages was not influenced. Cotreatment with ketamine and LTA for 1, 6, and 24 h did not cause cell death (Table 1).

Ketamine inhibits LTA-induced TNF-α and IL-6 expressions at protein or mRNA levels

Exposure of macrophages to 10 μg/mL LTA for 24 h increased the levels of TNF-α in the culture medium of macrophages by 4.8-fold (Fig. 1A). Treatment with 1 μM ketamine did not affect LTA-enhanced TNF-α biosynthesis. Meanwhile, when the concentrations reached 10 and 100 μM, ketamine decreased the LTA-augmented TNF-α production by 27% and 53%, respectively (Fig. 1A). Treatment of macrophages with 10 μg/mL LTA for 1, 6, and 24 h caused significant 2.4-, 3.3-, and 5-fold increases in the amounts of TNF-α, respectively (Fig. 1B). Treatment with 100 μM ketamine alone for 1, 6, and 24 h did not affect the basal levels of TNF-α. However, exposure to 100 μM ketamine for 1, 6, and 24 h reduced LTA-induced enhancements in the levels of TNF-α by 100%, 42%, and 52%, respectively (Fig. 1B). Quantitative PCR analyses revealed that exposure to 10 μg/mL LTA for 6 h led to 7.2-fold induction of TNF-α mRNA production (Fig. 1C). Ketamine at a clinically relevant concentration (100 μM) did not change TNF-α mRNA synthesis. However, cotreatment with 100 μM ketamine and LTA for 6 h significantly inhibited LTA-induced TNF-α mRNA production by 49% (Fig. 1C). Exposure to 10 μg/mL LTA for 6 h caused significant 5.2-fold increases in IL-6 mRNA production (Fig. 1D). Ketamine at 100 μM inhibited LTA-induced IL-6 mRNA synthesis by 45%.

Activation of ERK1/2 is involved in ketamine-induced suppression of TNF-α and IL-6 expressions in LTA-stimulated macrophages

Exposure of macrophages to LTA increased ERK1/2 phosphorylation (Fig. 2A, top panel, lane 2). A therapeutic concentration of ketamine did not change ERK1/2 phosphorylation (lane 3). However, treatment of macrophages with ketamine obviously decreased LTA-induced phosphorylation of this protein kinase (lane 4). Nonphosphorylated ERK2 was immunodetected as the internal controls (Fig. 2A, bottom panel). These immunoreactive protein bands were quantified and statistically analyzed (Fig. 2B). Lipoteichoic acid enhanced ERK1/2 phosphorylation by 3.9-fold. Ketamine at 100 μM significantly reduced LTA-induced ERK1/2 activation by 54% (Fig. 2B). Exposure of macrophages to LTA caused 5.2- and 5.5-fold induction of TNF-α and IL-6 mRNA syntheses (Fig. 2, C and D). Pretreatment with PD98059, an inhibitor of ERK, did not affect the basal levels of TNF-α and IL-6 mRNA but caused significant decreases in LTA-induced expressions of these two inflammatory cytokines by 54% and 53%, respectively. Ketamine inhibited TNF-α and IL-6 mRNA productions in LTA-activated macrophages by 50% and 42%, respectively. Cotreatment with ketamine and PD98059 had synergistic effects on the suppression of LTA-induced TNF-α and IL-6 mRNA productions by 69% and 73%, respectively (Fig. 2, C and D).

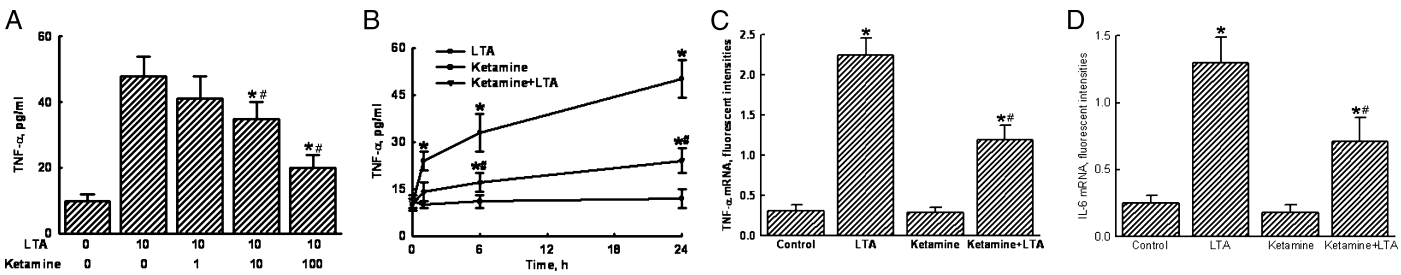

FIG. 1. **Effects of ketamine on LTA-induced syntheses of TNF-α and IL-6**. Macrophages were exposed to 1, 10, and 100 μM ketamine, 10 μg/mL LTA, and a combination of ketamine and LTA for 24 h (A) or to 100 μM ketamine, 10 μg/mL LTA, and a combination of ketamine and LTA for 1, 6, and 24 h (B). Levels of TNF-α and IL-6 in the culture medium of macrophages were quantified by an enzyme-linked immunosorbent assay. Messenger RNA from macrophages exposed to 100 μM ketamine, 10 μg/mL LTA, and a combination of ketamine and LTA for 6 h was prepared for a quantitative PCR (C, D). Each value represents the mean ± SEM for n = 6. The symbols, * and #, indicate that a value significantly ($P < 0.05$) differs from the control and LTA-treated groups, respectively.

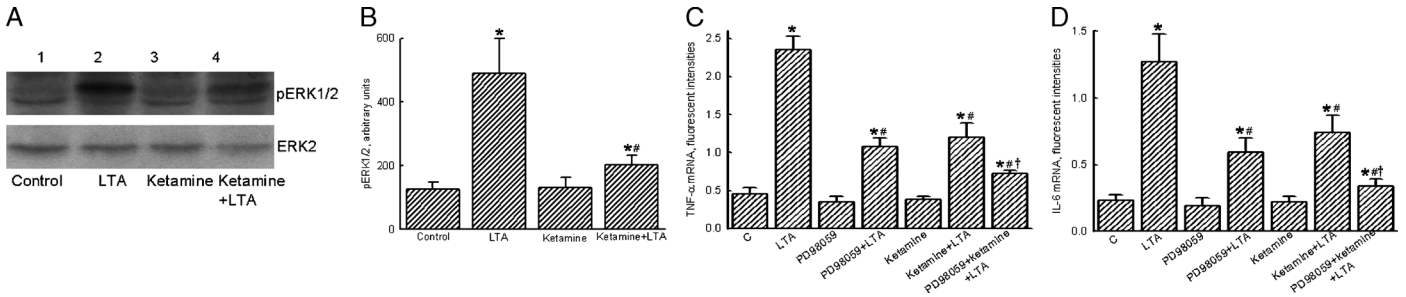

FIG. 2. **Effects of ketamine on LTA-induced phosphorylation of ERK1/2**. Macrophages were exposed to a therapeutic concentration (100 μM) of ketamine, 10 μg/mL LTA, and a combination of ketamine and LTA. Phosphorylated ERK1/2 (pERK1/2) was immunodetected (A, top panel). Nonphosphorylated ERK2 was quantified as the internal standard (A, bottom panel). These protein bands were quantified and analyzed (B). Macrophages were pretreated with 30 μM PD98059, an inhibitor of ERK, for 1 h then exposed to ketamine or LTA. Messenger RNA was prepared for quantitative PCR analyses of TNF-α and IL-6 mRNA (C, D). Each value represents the mean ± SEM for n = 6. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

Exposure of macrophages to LTA caused a significant 5.5-fold increase in the levels of TNF-α mRNA (Fig. 3A). Pretreatment with PD98059 decreased LTA-induced enhancement of TNF-α mRNA production. Exposure to 1 μM ketamine did not affect PD98059-induced inhibition of TNF-α mRNA synthesis in LTA-activated macrophages (Fig. 3A). Meanwhile, when the concentrations reached 10 and 100 μM, ketamine synergistically decreased LTA-induced TNF-α mRNA production with LTA by 30% and 52%,

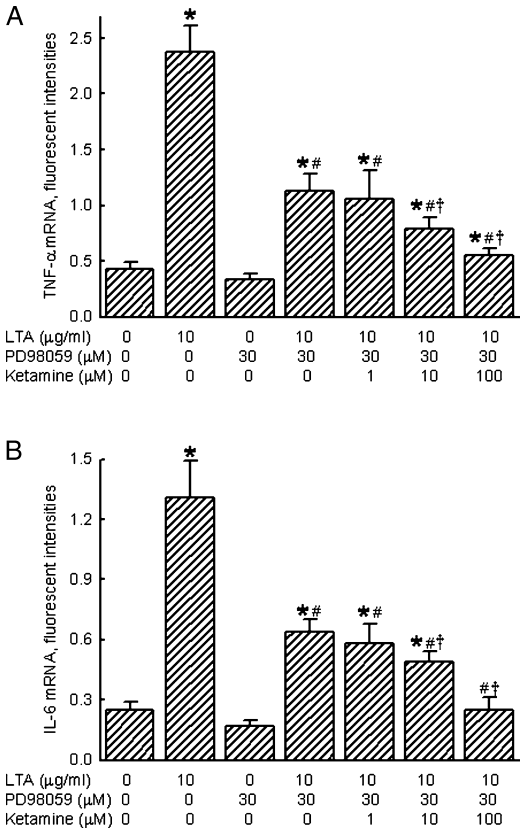

FIG. 3. **Concentration-dependent effects of ketamine on PD98059-induced inhibition of TNF-a and IL-6 mRNA syntheses in LTA-activated macrophages**. Macrophages were pretreated with 30 μM PD98059 for 1 h then exposed to 1, 10, and 100 μM ketamine or 10 μg/mL LTA. Messenger RNA was prepared for qRT-PCR analyses of TNF-α (A) and IL-6 mRNA (B). Each value represents the mean ± SEM for n = 3. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

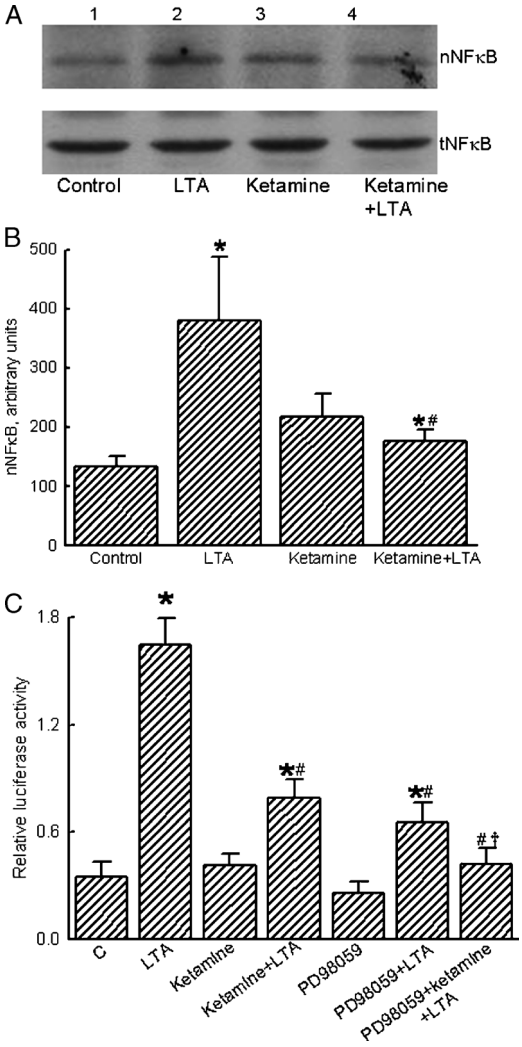

FIG. 4. **Effects of ketamine on LTA-induced translocation and transactivation of NFκB**. Macrophages were exposed to a therapeutic concentration (100 μM) of ketamine, 10 μg/mL LTA, and a combination of ketamine and LTA. Nuclear NFκB was immunodetected (A, top panel), and total NFκB was determined as the internal control (A, bottom panel). These protein bands were quantified and analyzed (B). A reporter gene assay was carried out to evaluate the transactivation activity of NFκB (C). Each value represents the mean ± SEM for n = 6. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

respectively (Fig. 3A). Ketamine at 1 μM did not affect PD98059-induced inhibition of IL-6 mRNA synthesis in LTA-activated macrophages (Fig. 3B). Meanwhile, exposure to 10 and 100 μM ketamine had synergistic effects with PD98059 on downregulating LTA-induced IL-6 mRNA production by 34% and 61%, respectively (Fig. 3B).

The ketamine-induced inhibition of TNF-α *gene expression is mediated by translocation and transactivation of NFκB*

In untreated macrophages, nuclear NFκB was detected (Fig. 4A, top panel, lane 1). Exposure to LTA increased the levels of nuclear NFκB (lane 2). Ketamine at a clinically relevant concentration (100 μM) did not affect the amounts of nuclear NFκB in macrophages (lane 3). However, treatment with ketamine decreased the LTA-involved increase in nuclear NFκB levels (lane 4). Amounts of total NFκB were immunodetected as the internal controls (Fig. 4A, bottom panel). These protein bands were quantified and analyzed (Fig. 4B). Treatment of macrophages with LTA significantly increased the translocation of NFκB from the cytoplasm to nuclei by 2.9-fold. After exposure to ketamine, the LTA-triggered augmentation in nuclear NFκB decreased by 54%.The results of reporter gene assays revealed that exposure of macrophages to LTA caused a significant 4.7-fold increase in the DNA-binding activity of NFκB (Fig. 4C). Both ketamine and PD98059 did not affect such DNA-binding activity. However, exposure to ketamine and PD98059 significantly lowered LTA-enhanced DNA-binding activity of NFκB by 52% and 60%, respectively. Cotreatment with ketamine and PD98059 caused a 75% decrease of NFκB transactivation in LTA-stimulated macrophages (Fig. 4C).

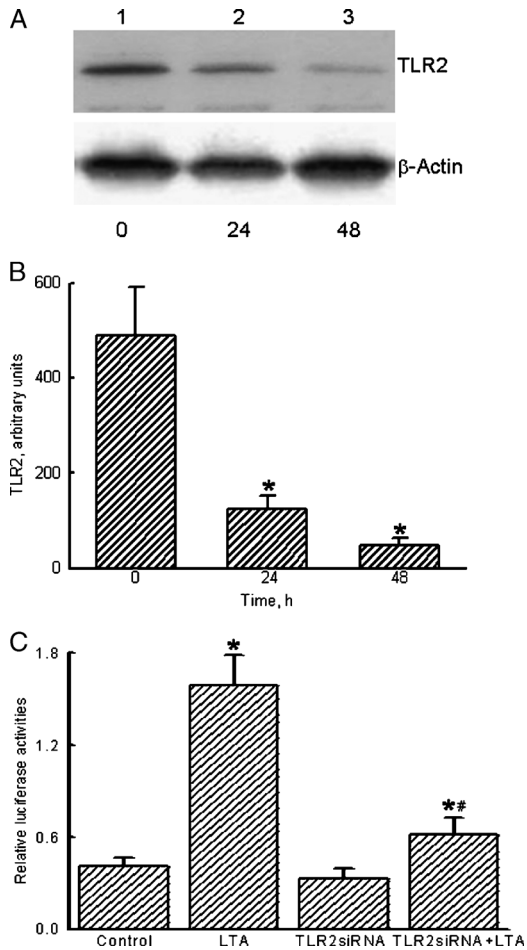

FIG. 5. **Effects of TLR2 on ketamine-involved reduction of LTA-induced transactivation of NFκB**. Macrophages were applied to TLR2 siRNA for 24 and 48 h, and the amounts of this membrane receptor were immunodetected (A, top panel). Levels of β-actin were detected as the internal standard (bottom panel). These immunoreactive protein bands were quantified and analyzed (B). Macrophages were exposed to TLR2 siRNA for 48 h and then treated with a therapeutic concentration (100 μM) of ketamine or 10 μg/mL LTA. A reporter gene assay was carried out to evaluate the transactivation activity of NFκB (C). Each value represents the mean ± SEM for n = 6. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

TLR2 participates in LTA-induced NFκB transactivation

In untreated macrophages, TLR2 could be detected (Fig. 5A, top panel, lane 1). Application of TLR2 siRNA to macrophages for 24 and 48 h obviously decreased the levels of TLR2 (lanes 2 and 3). Amounts of β-actin were immunodetected as the internal controls (bottom panel). These immunoreactive protein bands were quantified and analyzed (Fig. 5B). Application of TLR2 siRNA to macrophages significantly reduced cellular TLR2 protein levels by 57% and 79%, respectively. Reporter gene assay showed that exposure of macrophages to LTA caused a 3.9-fold increase in the DNA-binding activity of NFκB (Fig. 5C). Application of TLR2 siRNA to macrophages for 48 h did not affect NFκB transactivation. However, treatment with TLR2 siRNA significantly decreased LTA-enhanced DNA-binding activity of NFκB by 61% (Fig. 5C).

Ketamine-involved suppression of LTA-induced TNF-α and IL-6 mRNA expression involves TLR2

Exposure of macrophages to LTA induced TNF-α mRNA by 7.8-fold (Fig. 6A). Ketamine and TLR2 siRNA alone did not influence the basal levels of TNF-α mRNA in macrophages. Treatment with ketamine at a therapeutic concentration and TLR2 siRNA caused 49% and 58% decreases in LTA-induced TNF-α mRNA synthesis, respectively. However, cotreatment with ketamine and TLR2 siRNA completely inhibited TNF-α mRNA production in LTA-activated macrophages (Fig. 6A). Lipoteichoic acid could induce IL-6 mRNA production in macrophages by 4.7-fold (Fig. 6B). Exposure to ketamine or TLR2 siRNA alone did not affect the basal amounts of IL-6 mRNA. Treatment of macrophages with ketamine and TLR2 siRNA caused significant 53% and 62% decreases in LTA-induced IL-6 mRNA production, respectively. However, cotreatment with ketamine and TLR2 siRNA completely lowered LTA-stimulated IL-6 mRNA synthesis (Fig. 6B).

TLR2 mediates ketamine-induced reduction of LTA-enhanced nitrite and intracellular ROS productions

Exposure of macrophages to LTA increased nitrite production in the culture medium by 7-fold (Fig. 7A). Treatment with ketamine or TLR2 siRNA alone did not change the basal levels of nitrite. Treatment of macrophages with ketamine and TLR2 siRNA caused significant 43% and 52% reductions in LTA-enhanced nitrite production, respectively. Meanwhile,

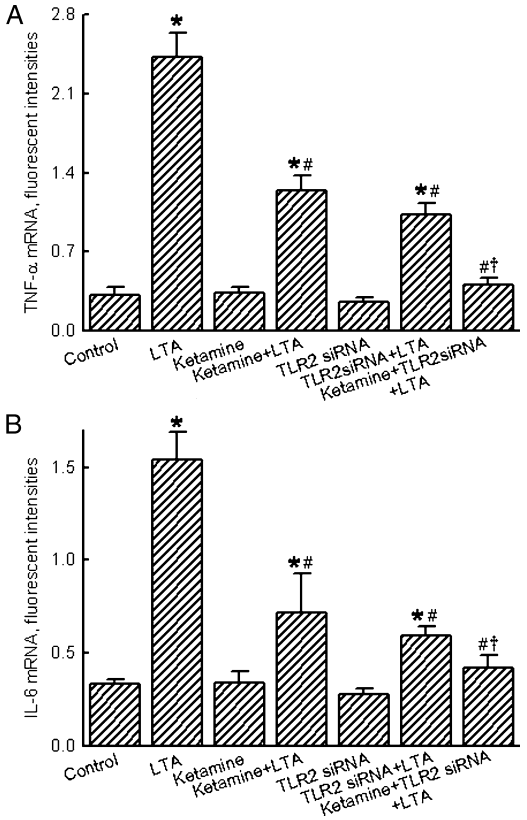

FIG. 6. **Effects of TLR2 on ketamine-involved suppression of LTA-induced TNF-α and IL-6 mRNA productions**. Macrophages were exposed to TLR2 siRNA for 48 h and then treated with a therapeutic concentration (100 μM) of ketamine or 10 μg/mL LTA. Messenger RNA was prepared for quantitative PCR analyses of TNF-α mRNA (A) and IL-6 mRNA (B). Each value represents the mean ± SEM for n = 6. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

cotreatment of ketamine and TLR2 siRNA synergistically alleviated nitrite production in the culture medium of macrophages exposed to LTA by 72% (Fig. 7A). The levels of intracellular ROS in macrophages were significantly augmented by 10.8-fold (Fig. 7B). Treatment of macrophages with ketamine or TLR2 siRNA did not affect the basal levels of intracellular ROS. Ketamine and TLR2 siRNA could reduce LTA-stimulated intracellular ROS production by 46% and 61%, respectively. However, cotreatment of ketamine and TLR2 siRNA had synergistic effects on decreasing the amounts of intracellular ROS in LTA-activated macrophages by 74% (Fig. 7B).

DISCUSSION

Ketamine can suppress the biosyntheses of TNF-α and IL-6 at protein or mRNA levels in LTA-activated macrophages. LPS at 1 to 100 ng/mL can increase the amounts of TNF-α and IL-6 in macrophages (12, 30). This study further reported that LTA at 10 μg/mL enhanced the biosyntheses of TNF-α and IL-6. Thus, LPS and LTA have different effective concentrations to stimulate inflammatory cytokine production in macrophages. Treatment of macrophages with ketamine concentration- and time-dependently decreased LTA-induced augmentation in the levels of TNF-α. In parallel with the decrease in TNF-α production, ketamine at 100 μM significantly inhibited TNF-α mRNA expression in LTA-activated macrophages. The LTA-induced IL-6 mRNA synthesis was significantly inhibited by ketamine. Ketamine at 100 μM is within the range of clinical plasma concentrations (28). Exposure of macrophages to a combination of 100 μM ketamine and LTA did not affect cell viability. Thus, ketamine at a clinically relevant plasma concentration can downregulate LTA-induced *TNF-α* and *IL-6* expressions, but such suppression is not caused by its cytotoxicity. Our previous study reported that ketamine can decrease syntheses of TNF-α and IL-6 in macrophages exposed to LPS, a gram-negative endotoxin (12). In this study, we further show that in gram-positive endotoxin LTA-activated macrophages, ketamine also suppressed TNF-α and IL-6 productions. Lipoteichoic acid has been reported to be one of the major causes involved in the pathogenesis of sepsis (15). Therefore, ketamine may have the potential to prevent septic shock induced by gram-positive and -negative bacteria via downregulation of TNF-α and IL-6 biosyntheses.

The ketamine-induced inhibition of *TNF-α* and *IL-6* gene expressions occurs through transcriptional events. Exposure of macrophages to a therapeutic concentration of ketamine significantly decreased LTA-induced enhancement of TNF-α protein synthesis. Analyses by quantitative PCR further showed that ketamine at a clinically relevant concentration inhibited TNF-α mRNA production in LTA-stimulated macrophages.

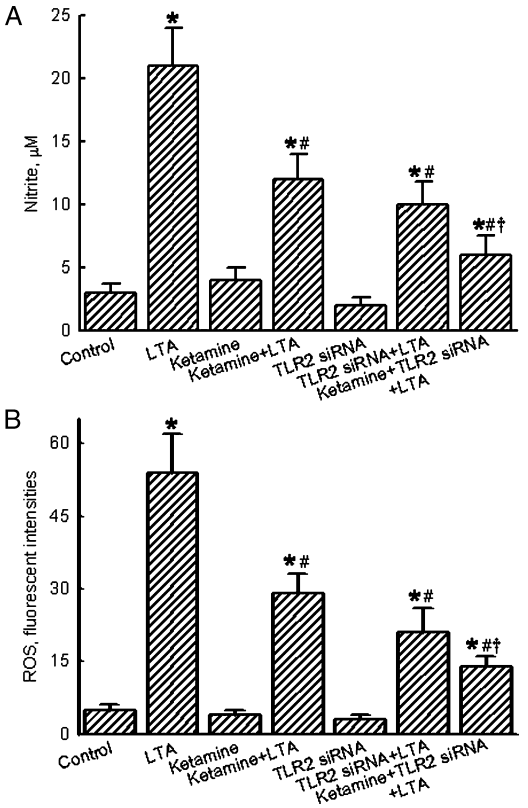

FIG. 7. **Effects of ketamine and TLR2 siRNA on LTA-induced augmentation of nitrite and intracellular ROS productions**. Macrophages were exposed to TLR2 siRNA for 48 h and then treated with a therapeutic concentration (100 μM) of ketamine or 10 μg/mL LTA. Amounts of nitrite in the culture medium of macrophages were detected using the Griess reaction (A). Levels of intracellular ROS were quantified by a flow cytometer (B). Each value represents the mean ± SEM for n = 6. The symbols, *, #, and †, indicate that a value significantly ($P < 0.05$) differs from the control, LTA-, and ketamine + LTA–treated groups, respectively.

The LTA-induced IL-6 mRNA expression was also inhibited by ketamine. In response to stimuli, LTA has been reported to activate two kinds of transcriptional factors, NFκB and activator protein 1, to regulate inflammation-related genes (34, 35). In the promoter regions of the *TNF-α* and *IL-6* genes, there are certain binding sites for the transcription factor NFκB (36, 37). In this study, exposure to LTA increased the translocation of NFκB from the cytoplasm to nuclei. Meanwhile, a therapeutic concentration of ketamine alleviated this translocation. Analysis by a reporter gene assay further revealed that ketamine decreased LTA-induced transactivation of NFκB in macrophages. Therefore, the present results show that ketamine at a clinically relevant concentration can inhibit *TNF-α* and *IL-6* gene expressions at the transcriptional level, and that this involves NFκB.

Ketamine at a therapeutic concentration decreased activation of ERK1/2 to inhibit *TNF-α* and *IL-6* gene expressions. Exposure to a clinically relevant concentration of ketamine reduced LTA-enhanced ERK1/2 phosphorylation. Extracellular signal–regulated kinase 1/2 is an upstream protein kinase for activating inhibitor of NFκB (IκB) kinase (IKK) (38). After activation, ERK1/2 can phosphorylate IKK and activate this protein kinase. Nuclear factor-κB is characterized by its sequestration in the cytoplasm as inactive complexes with an inhibitory molecule of IκB (38). The ERK1/2-activated IKK can phosphorylate IκB at two conserved serine residues in the N-terminus, triggering the degradation of this inhibitor and allowing for the rapid translocation of NFκB into nuclei where it avidly binds to DNA (39). In addition, when combining with PD98059, an inhibitor of ERK, ketamine had synergistic effects on inhibiting LTA-induced TNF-α and IL-6 mRNA productions. Ketamine also showed concentration-dependent effects on PD98059-induced suppression of TNF-α and IL-6 mRNA productions in LTA-activated macrophages. Thus, the ketamine-induced suppression of NFκB translocation and transactivation and *TNF-α* and *IL-6* expressions in LTA-stimulated macrophages is caused by downregulation of ERK1/2 phosphorylation. Therefore, one of the possible reasons to explain the ketamine-involved suppression of NFκB activation and *TNF-α* and *IL-6* gene expressions in LTA-activated macrophages is caused by the sequential downregulation of the phosphorylation of ERK1/2 and IKK by this intravenous anesthetic agent.

Toll-like receptor 2 may contribute to the ketamine-induced suppression of *TNF-α* and *IL-6* gene expressions in LTA-activated macrophages. Application of TLR2 siRNA to macrophages significantly knocked down translation of this receptor. In parallel with the decrease in the amounts of TLR2, the transactivation of NFκB was consequently decreased in LTA-stimulated macrophages. Previous studies showed that TLRs can use Tpl2 to activate the ERK1/2-dependent activation of NFκB (25). Toll-like receptor 2 is a major receptor in macrophages responsible for LTA stimulation (24, 25). In corneal keratocytes, LTA selectively mediates the Raf/ERK pathway to induce serum response element–enhanced gene transcription (40). In this study, we showed that LTA can activate NFκB. Meanwhile, the LTA-induced activation of NFκB was lowered by knocking down TLR2 translation. Treatment of macrophages with ketamine could also downregulate NFκB activation. After exposure to TLR2 siRNA, the expression of cellular TNF-α and IL-6 mRNA significantly decreased in LTA-activated macrophages. Cotreatment with ketamine and TLR2 siRNA synergistically inhibited LTA-induced enhancement of TNF-α and IL-6 mRNA productions. In rat microglia, highly purified LTA induces the biosyntheses of proinflammatory cytokines through TLR2-mediated signals (41). Thus, this study provides *in vitro* data showing that the ketamine-involved suppression of ERK/NFκB activation and *TNF-α* and *IL-6* gene expressions may be TLR2 dependent. Meanwhile, this study cannot rule out the possibility that the other pathways are also likely for ketamine-induced reduction in the ERK/NFκB activation.

The ketamine-induced reduction in the levels of nitrite and intracellular ROS may involve TLR2. Exposure of macrophages to LTA significantly increased nitrite and intracellular ROS productions. Our previous study showed that LTA can induce *iNOS* gene expression and subsequent overproduction of NO (26). NO is one of the intracellular ROS (42). Overproduction of NO contributes to the enhancement of intracellular ROS in LTA-activated macrophages. Application of TLR2 siRNA decreased LTA-augmented production of nitrite and intracellular ROS in macrophages. Transcription factor NFκB is a downstream target of TLR2-mediated activation of protein kinases (39). Nuclear factor-κB is involved in the regulation of *NADPH oxidase* and *xathine oxidase* gene expressions, which are two major sources of intracellular ROS (43). This study demonstrates that cotreatment with ketamine and TLR2 siRNA significantly and synergistically reduced translocation and transactivation. Our previous study reported that LPS-enhanced oxidative stress to macrophages could be ameliorated by propofol, the other intravenous anesthetic agent, possibly via activation of TLR4-NFκB (26). Therefore, the ketamine-induced reduction in LTA-stimulated productions of NO and intracellular ROS occurs via downregulating TLR2-mediated activation of ERK1/2-NFκB.

In conclusion, the present study shows that a clinically relevant concentration of ketamine (100 μM) decreased the biosyntheses of TNF-α in LTA-activated macrophages. In parallel with decreases in cellular TNF-α levels, exposure of macrophages to ketamine significantly inhibited TNF-α mRNA. Ketamine at a therapeutic concentration could also inhibit LTA-induced IL-6 mRNA synthesis. Analysis by RNA interference further revealed that the ketamine-involved inhibition of *TNF-α* and *IL-6* gene expression was TLR2 dependent. After exposure to ketamine, the LTA-induced phosphorylation of ERK1/2 and subsequent translocation of NFκB from the cytoplasm to nuclei and its transactivation to consensus DNA elements were significantly ameliorated. Our present data suggest that ketamine reduces the biosynthesis of TNF-α or IL-6 in LTA-activated macrophages by inhibiting *TNF-α* or *IL-6* gene expression. This suppression occurs through NFκB-involved transcriptional regulation. The TLR2-mediated signal-transducing activation of ERK1/2 may be one of the multiple pathways that

regulate ketamine-involved downregulation of NFκB activation and *TNF-α* and *IL-6* gene expressions. The ketamine-induced suppression of TNF-α– and IL-derived inflammation can partially explain its anti-inflammatory and immunosuppressive effects in clinical applications. Because murine macrophage-like Raw 264.7 cells are not similar to normal human macrophages, the use of this cell line in this study has its experimental limitations.

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