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Signal-transducing mechanisms of ketamine-caused inhibition of *interleukin-1*β gene expression in lipopolysaccharide-stimulated murine macrophage-like Raw 264.7 cells

Ta-Liang Chen [a], Chia-Chen Chang [b], Yi-Ling Lin [b,c], Yune-Fang Ueng [b,d], Ruei-Ming Chen [b,c,e,*]

[a] *Department of Anesthesiology, Taipei Medical University, Taipei, Taiwan*
[b] *Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan*
[c] *Drug Abuse Research Center and Cell Physiology and Molecular Image Research Center, Taipei Medical University-Wan Fang Hospital, Taipei, Taiwan*
[d] *National Research Institute of Chinese Medicine, Taipei, Taiwan*
[e] *Department of Anesthesiology, Taipei Medical University-Wan Fang Hospital, Taipei, Taiwan*

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ABSTRACT

Ketamine may affect the host immunity. Interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) are pivotal cytokines produced by macrophages. This study aimed to evaluate the effects of ketamine on the regulation of inflammatory cytokine gene expression, especially IL-1β, in lipopolysaccharide (LPS)-activated murine macrophage-like Raw 264.7 cells and its possible signal-transducing mechanisms. Administration of Raw 264.7 cells with a therapeutic concentration of ketamine (100 μM), LPS, or a combination of ketamine and LPS for 1, 6, and 24 h was not cytotoxic to macrophages. Exposure to 100 μM ketamine decreased the binding affinity of LPS and LPS-binding protein but did not affect LPS-induced RNA and protein synthesis of TLR4. Treatment with LPS significantly increased *IL-1β*, *IL-6*, and *TNF-α* gene expressions in *Raw 264.7 cells*. Ketamine at a clinically relevant concentration did not affect the synthesis of these inflammatory cytokines, but significantly decreased LPS-caused increases in these cytokines. Immunoblot analyses, an electrophoretic mobility shift assay, and a reporter luciferase activity assay revealed that ketamine significantly decreased LPS-induced translocation and DNA binding activity of nuclear factor-kappa B (NFκB). Administration of LPS sequentially increased the phosphorylations of Ras, Raf, MEK1/2, ERK1/2, and IKK. However, a therapeutic concentration of ketamine alleviated such augmentations. Application of toll-like receptor 4 (TLR4) small interfering (si)RNA reduced cellular TLR4 amounts and ameliorated LPS-induced RAS activation and IL-1β synthesis. Co-treatment with ketamine and TLR4 siRNA synergistically ameliorated LPS-caused enhancement of IL-1β production. Results of this study show that a therapeutic concentration of ketamine can inhibit gene expression of *IL-1β* possibly through suppressing TLR4-mediated signal-transducing phosphorylations of Ras, Raf, MEK1/2, ERK1/2, and IKK and subsequent translocation and transactivation of NFκB.

Introduction

Ketamine is a widely used intravenous anesthetic agent, which is clinically applied to patients for inducing and maintaining anesthesia during surgical procedures (Himmelseher and Durieux, 2005). Because ketamine has fewer depressant effects on the cardiovascular system resulting in a better patient hemodynamic profile than barbiturates, inhalational anesthetics or propofol, it is often used for anesthesia in severely ill patients (White et al., 1982). Clinical analyses have implied that ketamine possesses immunomodulatory and anti-inflammatory characteristics (Molina et al., 2004). Previous studies showed that ketamine can suppress cocaine-caused immunotoxicity and lipopolysaccharide (LPS)-induced acute lung injury in rats (Rofael et al., 2003; Yang et al., 2005). In cardiac surgical patients, cardiopulmonary bypass-induced inflammation can be attenuated by ketamine administration (Bartoc et al., 2006). A variety of *in vitro* studies further reported that ketamine can suppress the activities of immune cells. For example, pretreatment with ketamine was shown to decrease leukocyte adherence in rat mesenteric venules (Schmidt et al., 1995). At clinically relevant concentrations, ketamine reduces phagocytotic activity and expressions of the adhesion molecules, CD18 and CD62L, in human neutrophils (Nishina et al., 1998; Weigand et al., 2000). Our previous study further showed that a therapeutic concentration of ketamine induces mitochondrial dysfunction and selectively suppresses the phagocytotic activity and oxidative ability of macrophages (Chang et al., 2005; Wu et al., 2008).

In the innate immune system, macrophages play pivotal roles in mediating a host's response to bacterial infection or tissue injuries

* Corresponding author. Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, 250 Wu-Xing St., Taipei 110, Taiwan. Fax: +886 2 86621119.
E-mail address: rmchen@tmu.edu.tw (R.-M. Chen).

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(Nathan, 1987; Lee et al., 2009a,b). During inflammation, a body of inflammatory cytokines is produced by macrophages to trigger activation of innate or adaptive-immune cells (Aderem, 2001). Interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) predominantly produced by macrophages participate in regulating innate host defenses and homeostasis (Wewers, 2004). Studies on disease regulation have implied that these inflammatory cytokines represent a common effector in the pathogenesis of inflammatory joint disorders such as rheumatoid arthritis (Firestein, 2004). Production of IL-1β by islet-infiltrating macrophages causes beta-cell dysfunction through nitric oxide production in human insulin-dependent diabetes mellitus (Sjoholm, 1998). A plethora of intrinsic and extrinsic factors can regulate *IL-1*β gene expression. LPS, a component of gram-negative bacterial cell walls, is a typical stimulator for regulating gene expression of inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in macrophages, and has been implicated as an important contributing factor to the pathogenesis of septic syndrome (Almeida and Gazzinelli, 2001; West and Heagy, 2004; Chen et al., 2005a,b). Previous studies showed that ketamine suppresses the biosyntheses of TNF-α and IL-6 in LPS-stimulated macrophages (Sun et al., 2004; Yang et al., 2005; Chang et al., 2005; Wu et al., 2008). Meanwhile, there are few studies on the effects of ketamine in regulating *IL-1*β gene expression.

During inflammation, LPS first binds to the LPS-binding protein (LBP) in the bloodstream (Wright et al., 1990). Then, the LPS–LBP complex induces certain gene expressions via toll-like receptor (TLR)-dependent mechanisms (Akira et al., 2006). TLRs, which are type I transmembrane proteins with extracellular domains comprised largely of leucine-rich repeats and intracellular signaling domains, have at least 12 members found in mammalian cells (Akira et al., 2006). TLR4 has been shown to be a major receptor in macrophages responsible for LPS stimulation (Beutler and Rietschel, 2003; Wu et al., 2009). After binding to the LPS–LBP complex, the alteration in TLR4's conformation induces cascade activation of intracellular protein kinases (Schroder et al., 2000). A recent study showed that the Ras protein can mediate the transduction of TLR signaling to diverse biological functions such as cell growth, survival, and differentiation (Kogut et al., 2007). After being phosphorylated by the Ras protein, activated Raf kinase sequentially triggers mitogen-activated protein kinase kinases (MEK) 1/2 and extracellular signal-regulated kinases (ERK) 1/2 (Kolch, 2005; Kholodenko, 2007). Activation of inhibitor kappaB kinase (IKK) by ERK1/2 then stimulates the translocation and transactivation of transcription factor nuclear factor-kappa B (NFκB), which induces the expressions of certain inflammatory genes (Dobrovolskaia et al., 2003; Siwak et al., 2005). NFκB-DNA binding elements are found in promoter regions of these inflammatory cytokine genes (Won et al., 2006). Previous studies showed that ketamine can alleviate LPS-induced NFκB activation (Sun et al., 2004; Yang et al., 2005). However, the role of the ERK1/2 cascade in ketamine-induced regulation of these inflammatory gene expressions is not well understood. Thus in this study, we attempted to evaluate the effects of ketamine on the biosynthesis of inflammatory cytokines, especially IL-1β, in LPS-activated macrophages, and its possible signal-transducing mechanisms in terms of activation of the TLR4-mediated Ras/Raf/MEK/ERK/IKK/NFκB cascade.

Materials and methods

Cell culture and drug treatment. A murine macrophage cell line, Raw 264.7, was purchased from the American Type Culture Collection (Rockville, MD, USA). Macrophages were cultured in RPMI 1640 medium (Gibco-BRL, Grand Island, NY, USA) 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% CO_2. Cells were allowed to grow to confluence prior to ketamine or LPS administration.

Ketamine and LPS 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). According to the clinical application, concentrations of ketamine at 1, 10, 100, and 1000 μM, which correspond to 0.01-, 0.1-, 1-, and 10-times the clinical plasma concentration (Domino et al., 1982; Grant et al., 1983), were chosen as the administered dosages in this study. Prior to the addition of ketamine, macrophages were washed with PBS buffer, and non-adherent cells were removed. Control macrophages received PBS only.

Assay of cell viability. Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Lee et al., 2009a,b). Briefly, macrophages (2×10^4 cells per well) were seeded in 96-well tissue culture plates overnight. After drug treatment, macrophages were cultured with new medium containing 0.5 mg/ml MTT 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.

Binding affinity of LPS to LBP. The effects of ketamine on the binding affinity of LPS to LBP were determined according to a previously described method (Lamping et al., 1996). Briefly, LPS (30 pg/ml) was dissolved in 20 mM EDTA and 100 mM Na_2CO_3 (pH 9.6), and coated onto 96-well plates at room temperature for 3 h. After washing and air-drying, the plates were blocked with 10 mg/ml bovine serum albumin in 150 mM NaC1 and 50 mM HEPES, pH 7.4, at 37 °C for 30 min. A competing reaction was begun following the addition of ketamine and LBP (50 μg/ml), purchased from Sigma. LPS-bound LBP was immunodetected using a rabbit polyclonal antibody against human LBP (Santa Cruz Biotechnology). After incubating with an anti-rabbit Ig alkaline phosphatase conjugate (Sigma), paranitrophenyl phosphate was used as the substrate for the enzymatic color reaction. The colorimetric products were detected at 405 nm.

Enzyme-linked immunosorbent assay (ELISA). The amounts of IL-1β in the culture medium of macrophages exposed to LPS, ketamine, or a combination of ketamine and LPS were determined according to a previously described method (Chen et al., 2005a,b). Briefly, after drug administration, the culture medium was collected and centrifuged. Levels of IL-1β in the culture medium were quantified following the standard protocols of the ELISA kits purchased from Endogen (Woburn, MA, USA).

Reverse-transcription- (RT) and quantitative polymerase chain reaction (PCR) assays. Messenger (m)RNA from macrophages exposed to LPS, ketamine, or a combination of ketamine and LPS were prepared for RT-PCR and quantitative PCR analyses of IL-1β, TLR4, and β-actin. Oligonucleotides for PCR analyses of IL-1β and β-actin were designed and synthesized by Clontech Laboratories (Palo Alto, CA, USA). The oligonucleotide sequences of upstream and downstream primers for these mRNA analyses were respectively 5′-ATGGCAACTGTTCCTGA-ACTCAACT-3′ and 5′-CAGGAC-AGGTATAGAATTCTTTCCTTT-3′ for IL-1β (Wu et al., 2003), 5′-ATGAAGTTCCTCTCTGCAAGAGACT-3′ and 3′-CACTAGGTTTGCCGAGTAGATCTC-5′ for IL-6 (33), 5′-ATGAGCACAGAA-AGCATGATCCGC-3′ and 3′-CTCAGGCCCGTCCAGATGAAACC-5′ for TNF-α (Wu et al., 2003), 5′-CAAGGGATAAGAACGCTGAGA-3′ and 5′-GCAATGTCTCTGGCAGGTGTA-3′ for TLR4 (Wolfs et al., 2002), and 5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGC-ACGATTTC-3′ for β-actin (Wu et al., 2003). The PCR reaction was carried out using 35 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 2 min. The PCR products were loaded onto a 1.8% agarose gel containing 0.1 μg/ml ethidium bromide and electrophoretically separated. DNA bands were visualized and photographed under UV-light exposure. The intensities of the DNA bands in the agarose gel were quantified with the aid of the UVIDOCMW version 99.03

Table 1
Effects of ketamine and lipopolysaccharide (LPS), and a combination of KTM and LPS on macrophage viability.

	Cell viability, O.D. values at 550 nm		
	1 h	6 h	24 h
Control	0.982 ± 0.110	1.034 ± 0.201	1.315 ± 0.297
KTM 1 μM	0.977 ± 0.153	1.011 ± 0.198	1.347 ± 0.273
10 μM	0.983 ± 0.211	0.998 ± 0.178	1.298 ± 0.301
100 μM	0.969 ± 0.217	0.976 ± 0.159	1.289 ± 0.217
1000 μM	0.955 ± 0.155	0.836 ± 0.141	0.625 ± 0.220*
LPS 1 ng/ml	0.995 ± 0.214	1.054 ± 0.203	1.309 ± 0.187
10 ng/ml	0.980 ± 0.192	1.024 ± 0.191	1.316 ± 0.235
100 ng/ml	0.977 ± 0.228	0.981 ± 0.169	1.293 ± 0.206
1 μM KTM + 100 ng/ml LPS	0.973 ± 0.231	0.987 ± 0.219	1.301 ± 0.183
10 μM KTM + 100 ng/ml LPS	0.981 ± 0.176	0.981 ± 0.208	1.285 ± 0.209
100 μM KTM + 100 ng/ml LPS	0.979 ± 0.201	0.973 ± 0.196	1.297 ± 0.301
1000 μM KTM + 100 ng/ml LPS	0.901 ± 0.188	0.813 ± 0.179	0.522 ± 0.217*

Macrophages were exposed to KTM, LPS, and a combination of KTM and LPS for 1, 6, and 24 h. Cell viability was determined by a colorimetric method. Each value represents the mean ± SEM for $n = 6$. * Indicates that a value significantly differs from the respective control, $p < 0.05$. OD, optical density.

digital imaging system (Uvtec, Cambridge, UK). Quantitative PCR analysis was carried out using iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad).

Extraction of nuclear proteins and immunodetection. The amounts of nuclear transcription factors were quantified following a previously described method (Tai et al., 2007). Briefly, after drug treatment, nuclear extracts of macrophages were prepared. Protein concentrations were quantified by a bicinchonic acid protein assay kit (Pierce, Rockford, IL, USA). Nuclear proteins (50 μg/well) were subjected to SDS-PAGE, and transferred to nitrocellulose membranes. After blocking, nuclear NFκB was immunodetected using a rabbit polyclonal antibody against mouse NFκB (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total NFκB was immunodetected as the internal standard. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

Electrophoretic mobility shift assay (EMSA). An EMSA was performed using a Dig gel shift kit (Roche Diagnostics, Mannheim, Germany) as described previously (Wu et al., 2008). Briefly, NFκB consensus or mutant oligonucleotides, purchased from Santa Cruz Biotechnology, were labeled with digoxigenin DIG. The nuclear extract (10 μg) was allowed to react with DIG-labeled oligonucleotides at room temperature for 25 min. The complex was subjected to non-denatured polyacrylamide gel electrophoresis (PAGE), and transferred to positively charged nylon membranes. After cross-linking at 120 mJ and blocking, the membranes were immunoreacted with the anti-DIG-AP. Following washing and chemiluminescent detection, the membranes were exposed to X-ray film. Intensities of the immunoreactive bands were determined using a digital imaging system (UVtec).

NFκB reporter assay. NFκB luciferase reporter plasmids (Sratagene, La Jolla, CA, USA) and pUC18 control plasmids were transfected into macrophages using a FuGene 6 transfection reagent (Roche Diagnostics) as described previously (Wu et al., 2009). After transfection, macrophages were exposed to ketamine and LPS. Then, the cells were harvested. The luciferase activity in cell lysates was measured using a dual luciferase assay system (Promega, Madison, WI, USA).

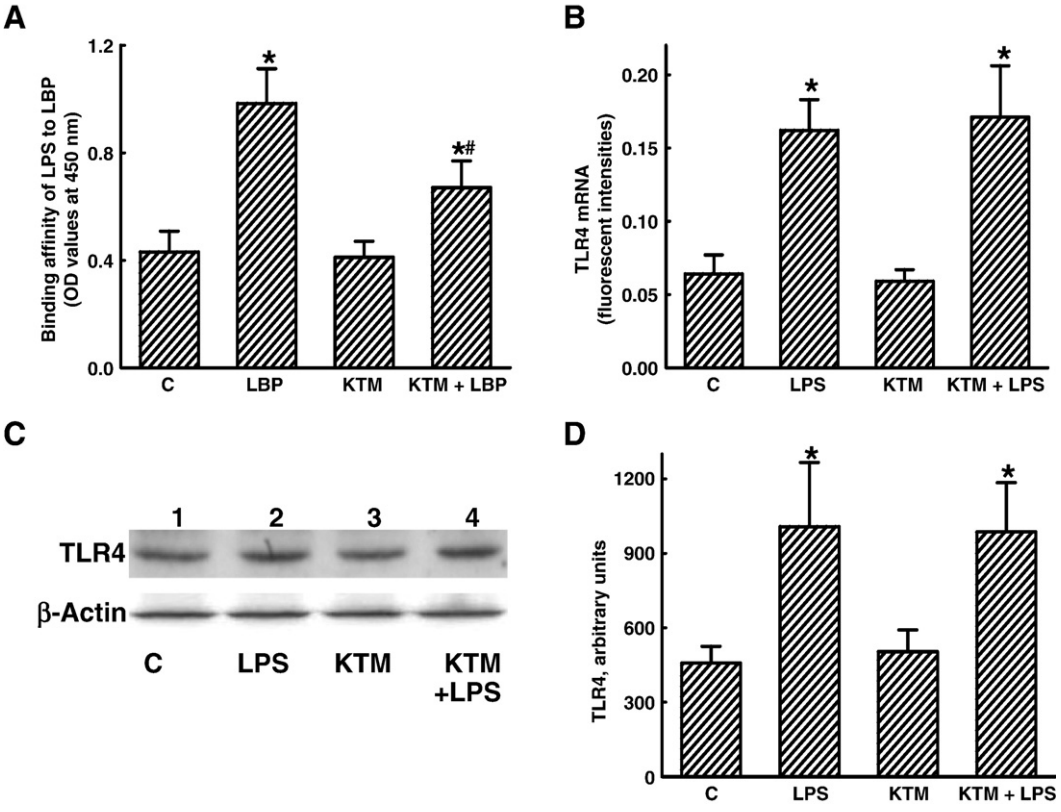

Fig. 1. Effects of ketamine (KTM) on the binding of lipopolysaccharide (LPS) to LPS-binding protein (LBP) and LPS-induced toll-like receptor (TLR)4 mRNA and protein syntheses. Competitive binding of KTM and LBP to LPS, which was coated onto 96-well plates, was analyzed as described in "Materials and methods" (A). After exposure to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS for 6 and 24 h, a quantitative PCR and immunoblot analysis were carried out for RNA and protein analyses of TLR4, respectively (B, C). β-Actin was immunodetected as the internal control (C, bottom panel). These protein bands were quantified and analyzed (D). Each value represents the mean ± SEM for $n = 6$. The symbol, *, indicates that a value significantly ($p < 0.05$) differs from the control group. OD, optical density.

Immunoblotting analyses of phosphorylated and nonphosphorylated Raf, MEK1/2, ERK1/2, and IKK. Cellular protein levels were immunodetected according a previously described method (Chen et al., 2007). Briefly, after drug treatment, cell lysates were prepared in ice-cold radioimmunoprecipitation assay buffer (25 mM Tris–HCl (pH 7.2), 0.1% 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). The proteins (50 μg per well) were subjected to sodium dodecylsulfate (SDS) PAGE, and transferred to nitrocellulose membranes. After blocking, phosphorylated Raf, MEK1/2, ERK1/2, and IKK were immunodetected using rabbit polyclonal antibodies against phosphorylated residues of Raf, MEK1/2, ERK1/2, or IKK protein (Cell Signaling, Danvers, MA, USA). Nonphosphorylated Raf, MEK2, ERK2, and IKK were immunodetected as the internal controls (Cell Signaling). These protein bands were quantified using a digital imaging system (UVtec).

Assay of Ras activity. Ras activity was determined by measuring the binding of only the activated form of Ras to the protein binding domain of Raf following a previously described method (Zhang et al., 2005). Briefly, cell lysates (200 μg) from control or drug-treated macrophages were incubated with glutathione S-transferase-Raf-1-protein binding domain agarose beads (Upstate Biotechnology, Lake Placid, NY, USA) to precipitate activated Ras. After electrophoretic separation and blotting, activation of Ras by Ang II was immunodetected using an anti-Ras monoclonal antibody (Upstate Biotechnology). These protein bands were quantified using a digital imaging system (UVtec).

TLR4 knockdown. Translation of TLR4 mRNA in macrophages was knocked down using an RNA interference (RNAi) method as described previously (Wu et al., 2008). TLR4 small interfering (si)RNA was purchased from Santa Cruz Biotechnology, which is a pool of 3 target-specific 20–25 nt siRNAs designed to knockdown TLR4 expression.

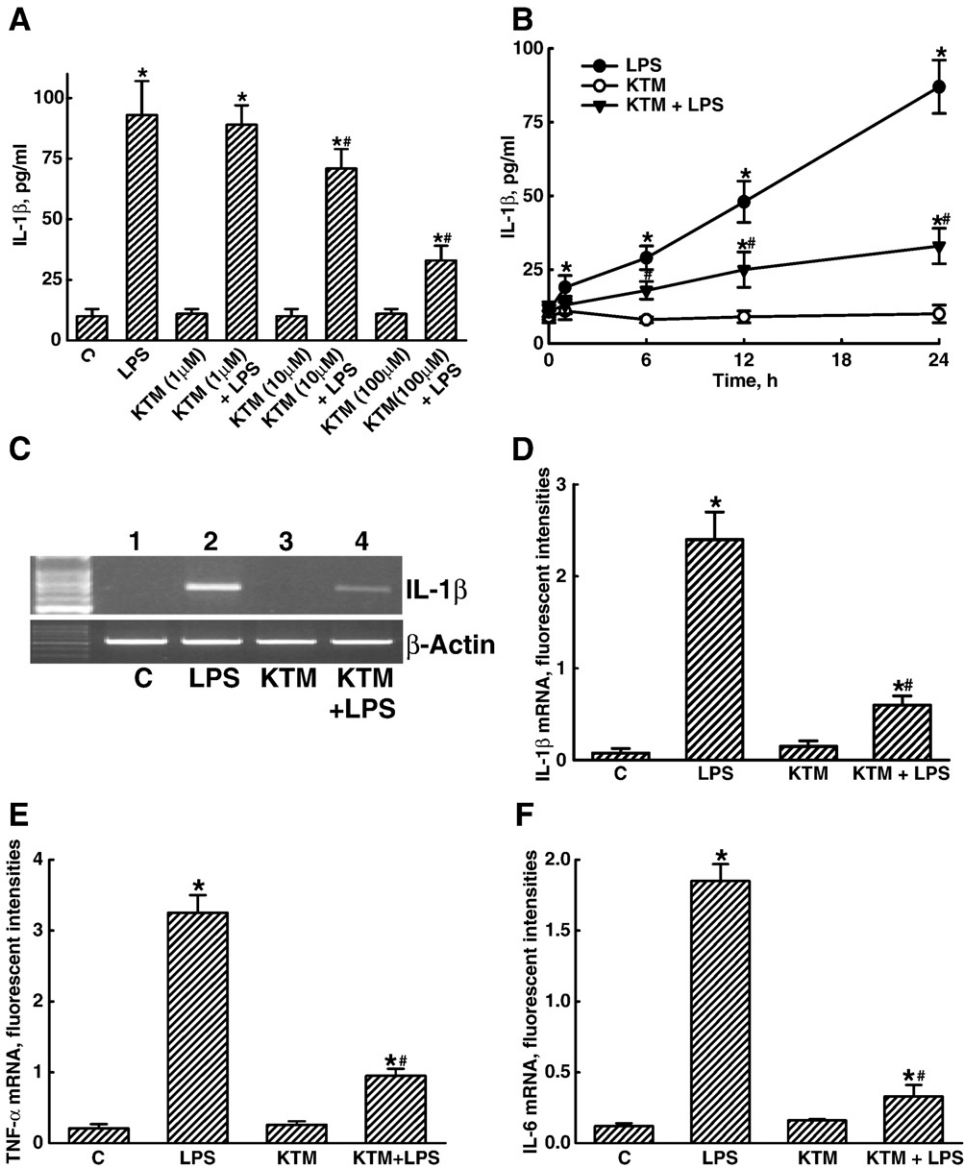

Fig. 2. Effects of ketamine (KTM) on lipopolysaccharide (LPS)-induced syntheses of interleukin (IL)-1b, IL-6, and tumor necrosis factor (TNF)-α mRNA or proteins. Raw 264.7 cells were exposed to 100 ng/ml LPS, 1, 10, and 100 μM KTM, or a combination of KTM and LPS for 24 h (A), or to 100 ng/ml LPS, 100 μM KTM, and a combination of KTM and LPS for 1, 6, and 24 h (B). Levels of IL-1β in the culture medium of Raw 264.7 cells were quantified using an ELISA. mRNA from Raw 264.7 cells exposed to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS for 6 h was prepared for RT-PCR and quantitative PCR analyses of IL-1β mRNA (C, D). β-Actin mRNA was detected as the internal standard (C, bottom panel). Effects of ketamine on the productions of TNF-α and IL-6 mRNA were also determined (E, F). 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 LPS-treated groups, respectively.

TLR4 siRNA was transfected into macrophages according to an siRNA transfection protocol provided by Santa Cruz Biotechnology. Briefly, after culturing macrophages in antibiotic-free RPMI medium at 37 °C in a humidified atmosphere of 5% CO_2 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 normal RPMI medium, and macrophages were treated with ketamine, LPS, or a combination of ketamine and LPS.

Statistical analysis. The statistical significance of differences between the control and ketamine-treated groups was evaluated using Student's *t*-test, and differences were considered statistically significant at p values of <0.05. Differences between ketamine- and LPS-treated macrophages were considered significant when the p value of Duncan's multiple-range test was <0.05. Statistical analysis between groups over time was carried out by two-way ANOVA.

Results

To evaluate the toxicity of ketamine, LPS, and the combination of ketamine and LPS to macrophages, cell viabilities were evaluated (Table 1). Treatment with 1, 10, and 100 μM ketamine for 1, 6, and 24 h did not affect cell viability. In 1- and 6-h-treated macrophages, exposure to 1000 μM ketamine did not cause cell death. However, after administration of 1000 μM ketamine for 24 h, cell viability was significantly reduced by 52%. Exposure to 1, 10, and 100 ng/ml LPS for 1, 6, and 24 h did not influence cell viability (Table 1). Macrophage viability was not influenced following treatment with a combination of ketamine at 1, 10, and 100 μM and LPS at 100 ng/ml. Meanwhile, a combination of 1000 μM ketamine and 100 ng/ml LPS decreased cell viability by 60% (Table 1).

The binding affinity of LPS to LBP and the syntheses of TLR4 RNA and protein were evaluated to determine the possible mechanism of ketamine-caused suppression of IL-1β production in LPS-activated macrophages (Fig. 1). In comparison to basal levels, the addition of LBP to wells coated with LPS caused a significant 2.3-fold increase in the binding affinity of LPS to LBP (Fig. 1A). Ketamine at a clinically relevant concentration decreased the affinity of LPS binding to LBP by 32%. Analysis of quantitative PCR revealed that TLR4 mRNA was detected in untreated macrophages (Fig. 1B). Administration of LPS increased TLR4 mRNA production by 2.5-fold. Treatment with ketamine did not change the basal or LPS-induced levels of TLR-4 mRNA. Exposure of macrophages to LPS obviously increased TLR4

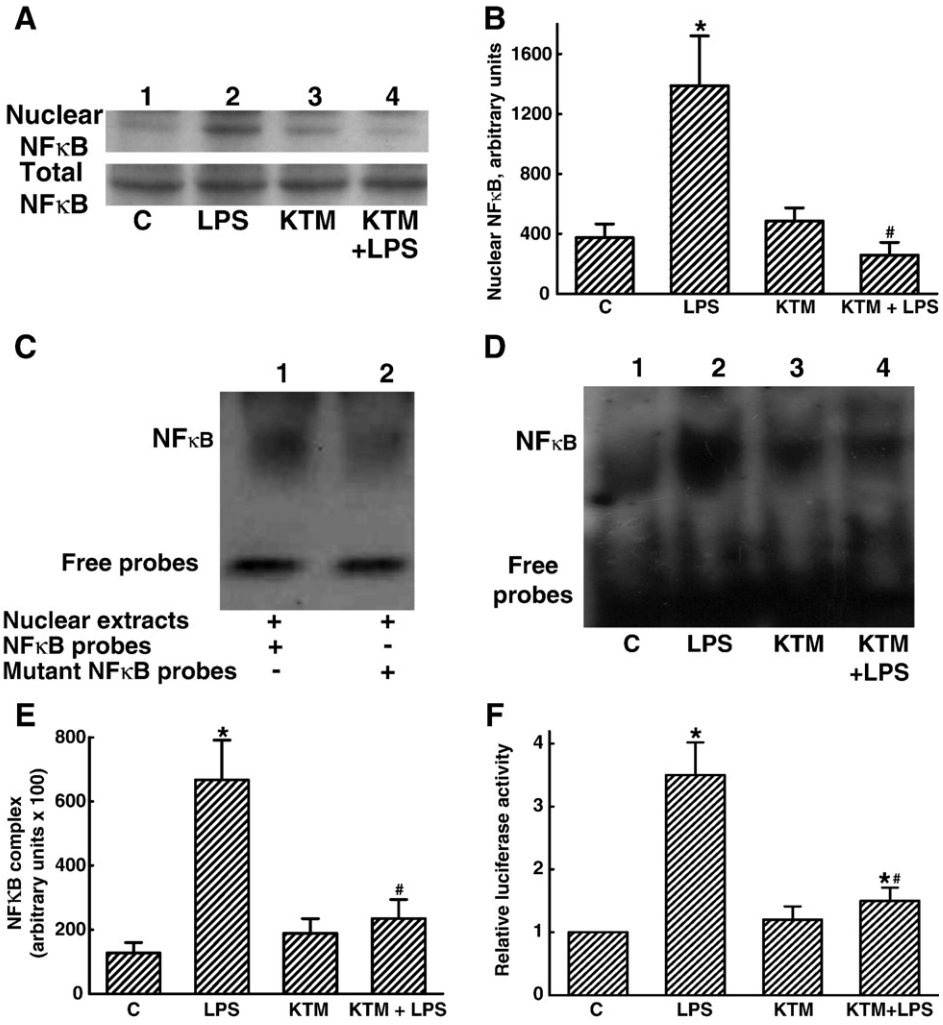

Fig. 3. Effects of ketamine (KTM) on lipopolysaccharide (LPS)-induced translocation and transactivation of NFkB. Raw 264.7 cells were exposed to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS. Nuclear NFκB was immunodetected (A, top panel), and total NFκB was determined as the internal control (A, bottom panel). These protein bands were quantified and analyzed (B). Nuclear extracts were reacted with normal or mutant NFκB consensus oligonucleotides, and the protein–DNA binding complex was electrophoretically separated for the EMSA analysis (C). After drug administration, nuclear extracts from Raw 264.7 cells exposed to 100 μM KTM, 100 ng/ml LPS, or a combination of KTM and LPS were prepared for the EMSA analysis of NFκB (D). These protein–DNA bands were quantified and analyzed using free probes as internal controls (E). An NFκB reporter assay was also carried out (F). 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 LPS-treated groups, respectively.

production (Fig. 1C, top panel, lane 2). Administration of ketamine did not change TLR4 synthesis in control or LPS-treated macrophages (lanes 3 and 4). β-Actin protein was immunodetected as the internal control (bottom panel). These protein bands were quantified (Fig. 1D). Exposure to LPS led to a significant 2.2-fold increase in TLR4 protein production. Treatment with ketamine did not influence LPS-induced augmentation of TLR4 synthesis.

Protein and RNA levels of IL-1β, IL-6, and TNF-α in LPS-activated macrophages were detected to determine the effects of ketamine on the expression of these inflammatory cytokines (Fig. 2). Exposure of macrophages to 100 ng/ml LPS for 24 h increased the amounts of IL-1β by 9.3-fold (Fig. 2A). Treatment with 1, 10, and 100 μM ketamine for 24 h did not affect cellular IL-1β levels. Ketamine at 1 μM did not change LPS-induced IL-1β production (Fig. 2A). At the concentrations of 10 and 100 μM, ketamine decreased LPS-induced IL-1β production by 24% and 65%, respectively. Administration of 100 ng/ml LPS into macrophages for 1, 6, 12, and 24 h significantly increased the amounts of IL-1β by 73%, and 2.6-, 4.4-, and 7.9-fold, respectively (Fig. 2B). Treatment with ketamine at a clinically relevant concentration of 100 μM for 1, 6, 12, and 24 h did not affect cellular IL-1β levels. Meanwhile, administration of 100 μM ketamine for 6, 12, and 24 h significantly alleviated LPS-caused increases in IL-1β synthesis by 38%, 50%, and 62%, respectively (Fig. 2B).

Analysis by RT-PCR revealed that low levels of IL-1β mRNA were detected in untreated macrophages (Fig. 2C, top panel, lane 1). Administration of LPS induced IL-1β mRNA (lane 2). Ketamine did not affect IL-1β mRNA production (lane 3). Treatment with ketamine obviously inhibited LPS-induced TNF-α mRNA (lane 4). The amounts of β-actin mRNA were detected as the internal control (Fig. 2C, bottom panel). Analysis by quantitative PCR further showed that the administration of LPS caused significant increases in IL-1β mRNA in macrophages (Fig. 2D). After ketamine administration, LPS-induced IL-1β mRNA synthesis was significantly ameliorated by 75%.

Exposure of macrophages to LPS significantly increased the levels of TNF-α mRNA by 17.8-fold (Fig. 2E). Ketamine at a therapeutic concentration did not change the production of TNF-α mRNA. Co-treatment of macrophages with ketamine and LPS significantly decreased this endotoxin-caused enhancement of TNF-α mRNA biosynthesis by 72% (Fig. 2E). Administration of macrophages with LPS caused an 11.2-fold increase in the amounts of IL-6 mRNA (Fig. 2F). A clinically relevant concentration of ketamine did not influence cellular IL-6 mRNA synthesis. Meanwhile, treatment with ketamine significantly decreased LPS-caused increases in IL-6 mRNA levels by 79% (Fig. 2F).

To determine the molecular mechanism of ketamine-involved regulation of *IL-1*β gene expression in LPS-activated macrophages, the translocation and transactivation of NFκB were evaluated (Fig. 3). Administration of macrophages with LPS increased nuclear NFκB levels (Fig. 3A, top panel, lane 2). Ketamine did not change nuclear NFκB amounts, but obviously decreased LPS-induced translocation of this transcriptional factor from the cytoplasm to nuclei (lanes 3 and 4). Levels of total NFκB in macrophages were immunodetected as the internal control (Fig. 3A, bottom panel). These protein bands were

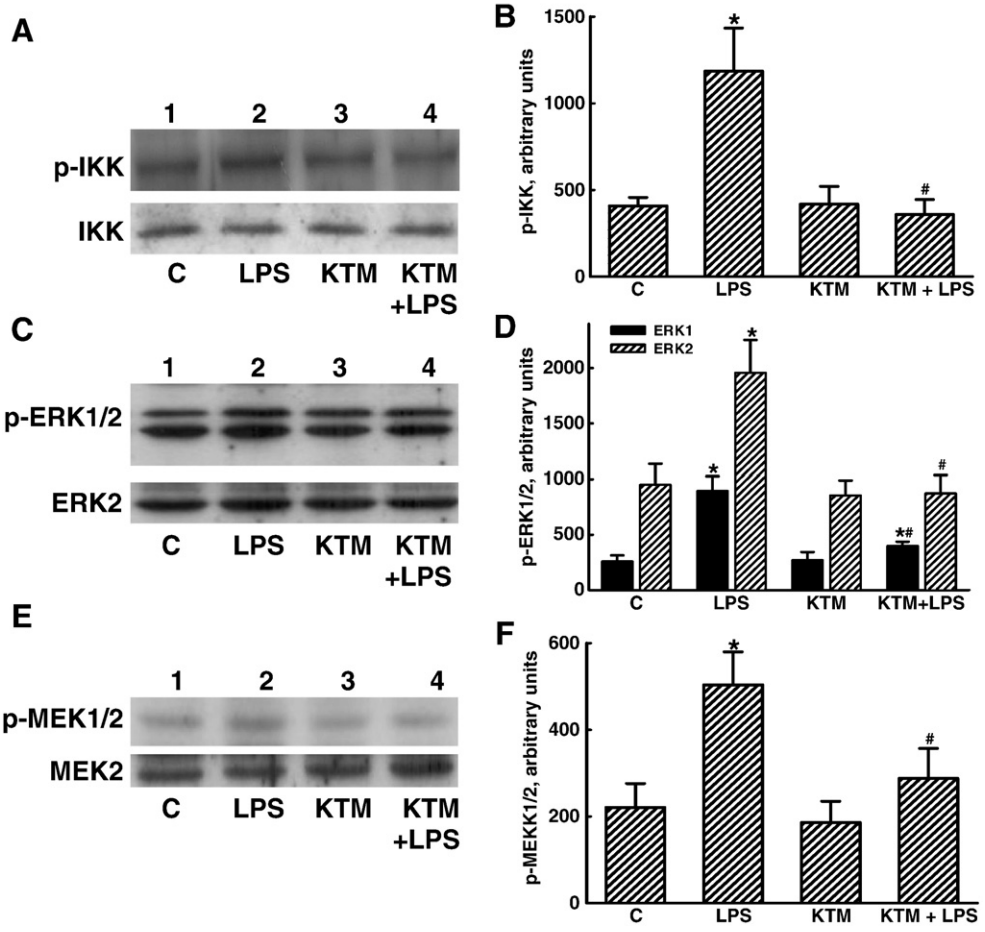

Fig. 4. Effects of ketamine (KTM) on lipopolysaccharide (LPS)-induced phosphorylations of IKK, ERK1/2, and MEK1/2. Raw 264.7 cells were exposed to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS. Phosphorylated IKK, ERK1/2, and MEK1/2 (p-IKK, p-ERK1/2, and p-MEK1/2) were immunodetected (A, C, E, top panels). Nonphosphorylated IKK, ERK1/2, and MEK1/2 were quantified as internal standards (A, C, E, bottom panels). These protein bands were quantified and analyzed (B, D, F). 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 LPS-treated groups, respectively.

quantified and analyzed (Fig. 3B). LPS increased nuclear NFκB levels by 3.7-fold. Exposure to a clinically relevant concentration of ketamine (100 μM) significantly ameliorated LPS-induced NFκB translocation by 82% (Fig. 3B).

EMSA results revealed that when nuclear extracts were reacted with NFκB consensus oligonucleotides, the NFκB mobility shift band appeared (Fig. 3C, lane 1). If mutated NFκB oligonucleotides were used instead of consensus ones, the NFκB complex band was obviously reduced (lane 2). Administration of LPS to macrophages increased the binding affinity of nuclear extracts to NFκB consensus oligonucleotides (Fig. 3D, top panel, lane 2). A therapeutic concentration of ketamine obviously decreased LPS-caused increases in the transactivation of NFκB with its consensus oligonucleotides (lane 4). The free probes were used as the internal control (Fig. 3D, bottom panel). These protein–DNA bands were quantified and analyzed (Fig. 3E). Exposure to LPS significantly enhanced the binding of nuclear extracts to NFκB consensus oligonucleotides by 5.3-fold. Administration of ketamine significantly decreased LPS-induced NFκB transactivation by 65% (Fig. 3E).

A reporter assay was carried out to further evaluate the role of NFκB in ketamine-induced suppression of IL-1β expression (Fig. 3F). Exposure of macrophages to LPS caused a significant 2.3-fold increase in luciferase activity. Administration of ketamine did not affect the activity of luciferase. Co-treatment of macrophages with LPS and ketamine significantly decreased the endotoxin-caused augmentation of the luciferase activity by 53% (Fig. 3F).

To further determine the signal-transducing mechanism of ketamine-involved suppression of LPS-induced IL-1β synthesis, the cascade phosphorylation of IKK, ERK1/2, and MEK1/2 was immunodetected (Fig. 3). Exposure to LPS increased IKK phosphorylation (Fig. 4A, top panel, lane 2). Ketamine did not change IKK phosphorylation but obviously decreased LPS-induced activation of this protein kinase (lanes 3 and 4). Nonphosphorylated IKK was immunodetected as the internal control (Fig. 4A, bottom panel). These immunoreactive protein bands were quantified and analyzed (Fig. 4B). LPS enhanced IKK phosphorylation by 2.9-fold. Ketamine significantly ameliorated LPS-induced MEK1/2 activation by 63% (Fig. 4B). Administration of LPS sequentially increased the phosphorylations of ERK1/2 and MEK1/2 (Figs. 4C, E, top panels, lane 2). Ketamine alleviated LPS-induced ERK1/2 and MEK1/2 phosphorylations (lanes 3 and 4). Nonphosphorylated ERK2 and MEK2 were immunodetected as the internal controls (Figs. 4C, E, bottom panels). These protein bands were quantified and analyzed (Figs. 4D, F). Exposure to LPS increased ERK1, ERK2, and MKK1/2 phosphorylations by 3.5-, 2.1-, and 2.3-fold, respectively. After ketamine administration, LPS-induced activations of ERK1, ERK2 and MEK1/2 were reduced by 44%, 55%, and 43%, respectively (Figs. 4D, F).

The cascade activation of Raf and Ras was evaluated to determine the upstream mechanism of ketamine-involved suppression of IL-1β synthesis in LPS-stimulated macrophages (Fig. 5). Exposure to LPS increased phosphorylation of Raf (Fig. 5A, top panel, lane 2). Ketamine at a clinically relevant concentration did not influence Raf phosphorylation (lane 3). Administration of ketamine obviously suppressed LPS-induced Raf phosphorylation (lane 4). The amounts of total Raf in macrophages were determined as the internal control (bottom panel). These protein bands were quantified and analyzed (Fig. 5B). LPS increased Ras phosphorylation by 3.5-fold. After ketamine treatment, the LPS-caused augmentation of Ras activation was reduced by 44%. Treatment of macrophages with LPS increased Ras activity (Fig. 5C, top panel, lane 2). Ketamine did not affect Ras activity (lane 3). The LPS-induced increase in Ras activity was ameliorated following ketamine administration (lane 4). Quantification of these protein bands showed that ketamine significantly ameliorated LPS-induced enhancement of Ras activity (Fig. 5D).

To investigate if ketamine-caused inhibition of *IL-1*β gene expression was TLR4-dependent in LPS-activated macrophages, TLR4 siRNA was applied to macrophages (Fig. 6). In untreated macrophages, TLR4 protein was immunodetected (Fig. 6A, top panel, lane 1). Application of TLR4 siRNA to macrophages obviously reduced cellular TLR4 protein levels (lane 2). The amounts of β-actin were immunodetected as the internal control (bottom panel). These protein bands were quantified and are presented in Fig. 6B. Application of TLR4 siRNA caused a

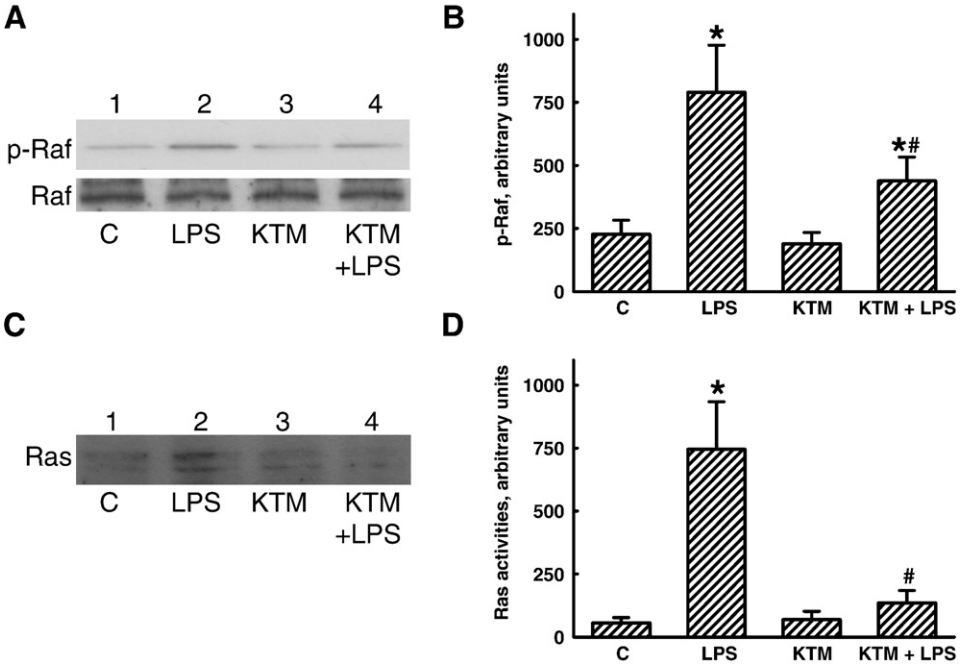

Fig. 5. Effects of ketamine (KTM) on lipopolysaccharide (LPS)-induced activation of Raf and Ras. Raw 264.7 cells were exposed to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS. Phosphorylated Raf (p-Raf) was immunodetected (A, top panel), and the amounts of Raf were quantified as an internal control (A, bottom panel). These protein bands were quantified and analyzed (B). Cell lysates were prepared for an assay of Ras activity (C). These protein bands were quantified and analyzed (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 LPS-treated groups, respectively.

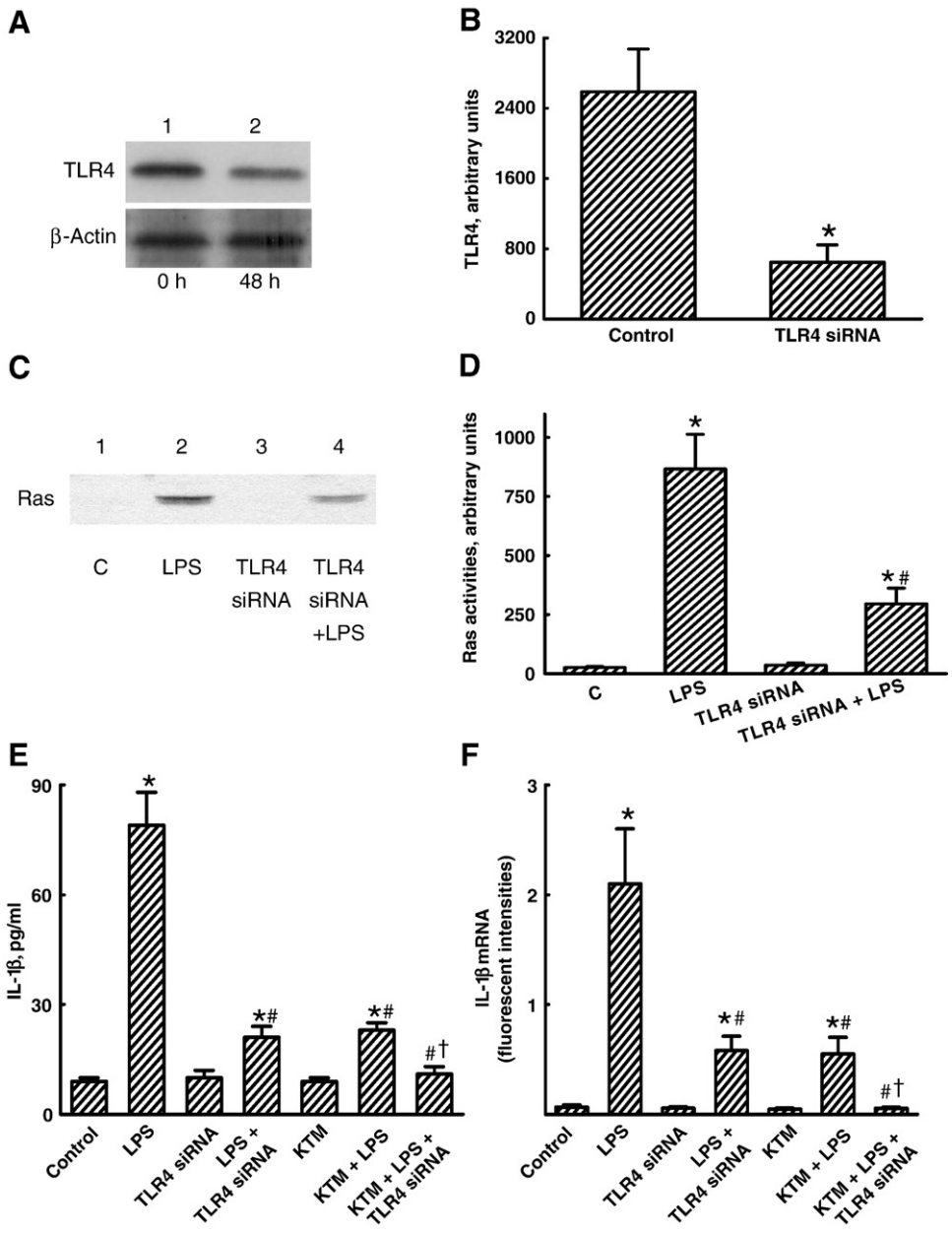

Fig. 6. Effects of toll-like receptor (TLR)4 small interfering (si)RNA on ketamine (KTM)-caused suppression of Ras activity and interleukin (IL)-1b expression. TLR4 siRNA was applied to Raw 264.7 cells for 48 h. Levels of TLR4 were immunodetected (A, top panel). β-Actin was quantified as the internal standard (A, bottom panel). These immunoreactive protein bands were quantified and analyzed (B). After application of TLR4 siRNA for 48 h, Raw 264.7 cells were then exposed to 100 ng/ml LPS, 100 μM KTM, or a combination of KTM and LPS. Cell lysates were prepared for an assay of Ras activity (C). These protein bands were quantified and analyzed (D). An ELISA was carried out to determine the levels of IL-1β in the culture medium of Raw 264.7 cells (E). Quantitative PCR analysis of IL-1β mRNA was conducted (F). Each value represents the mean ± SEM for $n = 6$. The symbols, *, #, and †, indicate that a value significantly ($p<0.05$) differs from the control, LPS-, and KTM + LPS-treated groups, respectively.

significant 75% decrease in TLR4 protein levels. Exposure of macrophages to LPS increased Ras activity (Fig. 6C, lane 2). Application of TLR4 siRNA to macrophages obviously decreased LPS-caused enhancement of Ras activity (lane 4). These protein bands were quantified and analyzed (Fig. 6D). LPS significantly increased Ras activity by 35-fold. After administration of TLR4 siRNA, the LPS-enhanced Ras activity decreased by 66%.

Exposure of macrophages to LPS significantly enhanced the amount of IL-1β by 8.6-fold (Fig. 6E). Ketamine at a therapeutic concentration of 100 μM alleviated LPS-induced augmentation of IL-1β production by 63%. Co-treatment of ketamine and TLR4 siRNA completely recovered LPS-induced IL-1β synthesis (Fig. 6E). Analysis of quantitative PCR revealed that TLR4 knockdown lowered LPS-induced IL-1β mRNA production by 71% (Fig. 6F). Administration of ketamine decreased LPS-induced IL-1β mRNA by 65%. LPS-induced IL-1β mRNA synthesis was synergistically inhibited following co-treatment with ketamine and TLR4 siRNA (Fig. 6F).

Discussion

This study showed that a therapeutic concentration (100 μM) of ketamine can decrease cellular IL-1β, IL-6, and TNF-α levels in LPS-activated macrophages. Exposure of macrophages to LPS increased cellular IL-1β production in concentration- and time-dependent manners. After ketamine administration, the LPS-caused enhancement of IL-1β synthesis was concentration-dependently decreased. In addition, the expression of IL-6 and TNF-α mRNA was inhibited by ketamine. Ketamine at 20–120 μM corresponds to clinical plasma

concentrations (Domino et al., 1982; Grant et al., 1983). Ketamine at 100 μM, which is within the range of relevantly clinical concentrations, was not toxic to Raw 264.7 cells. Thus, a concentration of 100 μM was chose as the administered dosage to evaluate the effects of ketamine at a clinical level on macrophage activities. Ketamine at such a therapeutic concentration led to a time-dependent reduction in LPS-caused increases in the amounts of cellular IL-1β, IL-6, and TNF-α. Clinically, ketamine is a widely used intravenous anesthetic agent for induction and maintenance of anesthesia but usually induces immunomodulation (Himmelseher and Durieux, 2005; White et al., 1982; Molina et al., 2004). Our previous study showed that a therapeutic concentration of ketamine can induce mitochondrial dysfunction and selectively suppress the phagocytotic activity and oxidative ability of macrophages (Chang et al., 2005; Wu et al., 2008). During inflammation, IL-1β, IL-6, and TNF-α are critical cytokines predominantly produced by macrophages and have been reported to regulate innate host defense and homeostasis (Wewers, 2004). The ketamine-induced suppression of IL-1β production partially explains why this anesthetic agent has immunosuppressive effects when it is clinically applied.

The mechanism of ketamine-caused suppression of IL-1β biosynthesis occurs at the transcriptional level. An assay of cell viability revealed that ketamine at a therapeutic concentration (100 μM) was not cytotoxic to macrophages. Meanwhile, exposure to 1000 μM ketamine caused significant death of macrophages. The present results are similar to those of our previous study that ketamine at only 1000 μM could induce death of human umbilical vein cells via an apoptotic mechanism (Chen et al., 2005a,b). Thus, ketamine can ameliorate LPS-induced IL-1β production by a mechanism that is not due to the cytotoxic effects of this anesthetic agent against macrophages. In parallel with decreases in protein levels, ketamine significantly inhibited LPS-induced IL-1β mRNA production. LPS activated the transcription factor, NFκB, in macrophages. NFκB-DNA binding elements are found in the promoter regions of the *IL-1*β gene (Won et al., 2006). After ketamine administration, the LPS-induced activation of NFκB and consequent syntheses of IL-1β mRNA and protein were lowered by ketamine. Thus, the mechanism of ketamine-caused suppression of IL-1β biosynthesis is via a transcriptional event rather than due to its cytotoxic effects.

Ketamine reduces the extracellular binding affinity of LPS to LBP. During inflammation, LPS in the bloodstream first binds to LBP and then triggers sequential inflammatory reactions (Wright et al., 1990). When LBP is heat-denatured, LPS cannot induce TNF-α mRNA and protein production in macrophages (Mathison et al., 1992). Thus, the binding of LPS to LBP has been implicated as playing a key role in LPS-induced regulation of inflammatory gene expressions. Our ELISA analysis revealed that when LBP was added to plates coated with LPS, the binding of LPS and LBP was obvious. However, the binding affinity of LPS to LBP significantly decreased following ketamine application. Our results indicate that ketamine can interfere with LPS binding to LBP. Therefore, one possible reason to explain how ketamine suppresses LPS-induced IL-1β production is the competitive effects of this intravenous anesthetic agent on the binding affinity of LPS to LBP.

The ketamine-involved inhibition of *IL-1*β gene expression may be TLR4-dependent. In macrophages, the structure of TLR4 is conformationally changed following binding to the LPS-LBP complex, and this alteration can transform extracellular LPS stimulation into intracellular signals (Beutler and Rietschel, 2003; Schroder et al., 2000). This study showed that application of TLR4 siRNA to macrophages obviously knocked down the translational rate of this receptor. In parallel with decreases in TLR4 protein levels, LPS-induced IL-1β biosynthesis was suppressed. Co-treatment with ketamine and TLR4 siRNA synergistically alleviated LPS-induced IL-1β production. Thus, the ketamine-caused suppression of IL-1β production in LSP-activated macrophages may be TLR4-dependent. In lamina propria T cells, LPS has been reported to upregulate TLR4 expression (Ince et al., 2006). In the present study, we showed that LPS induced TLR4 mRNA production. Meanwhile, ketamine at a therapeutic concentration did not affect LPS-induced TLR4 mRNA synthesis. Therefore, TLR4 may be involved in regulating ketamine-caused suppression of IL-1β biosynthesis in LPS-activated macrophages, but ketamine does not affect TLR4 expression.

Ketamine suppressed activations of Ras and Raf in LPS-stimulated macrophages. Exposure of macrophages to LPS significantly increased Ras activity. Meanwhile, application of TLR4 siRNA caused a significant decrease in LPS-induced augmentation of Ras activity. Kogut et al. (2007) reported that the Ras protein can be a mediator of the transduction of TLR signaling to diverse cell activities, including growth, survival, and differentiation (Kogut et al., 2007). In this study, we further showed that the Ras protein is a downstream effector following TLR4 activation in LPS-stimulated macrophages. By comparison, administration of a therapeutic concentration of ketamine also lowered LPS-induced Ras activation. Our data reveal that TLR4 has a critical role in mediating the ketamine-caused reduction in Ras activity. Administration of ketamine significantly decreased LPS-caused phosphorylation of Raf kinase. Ras activation can stimulate Raf phosphorylation (Kolch, 2005). Thus, the ketamine-caused downregulation of LPS-induced Raf activation is due to its suppressive effects on Ras activity. In bone marrow macrophages, *IL-1*β gene expression is regulated by a Ras/Raf cascade (Ganesan et al., 2006). Therefore, ketamine at a clinically relevant concentration possibly inhibits *IL-1*β gene expression in LPS-activated macrophages through suppression of the TLR4-mediated activations of Ras and Raf proteins.

Ketamine induces decreases in the phosphorylation cascades of MEKK1/2, ERK, and IKK. In parallel with reductions in Ras and Raf activations, ketamine at a therapeutic concentration ameliorated LPS-induced MEK1/2 phosphorylation. Simultaneously, LPS-induced ERK1/2 phosphorylation was reduced following ketamine administration. MEK1/2 has been shown to be a downstream kinase that mediates signals from Ras/Raf in oxidative cell death (Yagoda et al., 2007). After activation, MEK1/2 consequentially phosphorylates ERK1/2, which has been implicated in regulating cell development (Schubbert et al., 2007). Thus, the ketamine-involved suppressive effects on LPS-induced Ras/Raf activation may be one of the principle reasons explaining how this intravenous anesthetic agent can lower MEK1/2 and ERK1/2 phosphorylations in LPS-activated macrophages. LPS increased IKK phosphorylation. Meanwhile, ketamine at a clinically relevant concentration reduced LPS-induced IKK activation. The ERK1/2-involved IKK activation was shown to cause transient stimulation in contracting adult rat skeletal muscle (Ho et al., 2005). Therefore, our assays of kinase activation revealed that in LPS-activated macrophages, ketamine induces suppression of the cascade of Ras/Raf/MEK/ERK/IKK phosphorylations. And, these reduced effects may be involved in regulating ketamine-involved inhibition of *IL-1*β gene expression.

Ketamine decreased translocation of NFκB in LPS-activated macrophages. In response to LPS stimulation, the levels of nuclear NFκB were significantly augmented. Meanwhile, the total levels of cellular NFκB in macrophages did not change following LPS administration. Thus, the increases in the levels of nuclear NFκB in macrophages exposed to LPS were due to enhanced translocation of this transcription factor from the cytoplasm to nuclei. Treatment of macrophages with ketamine at a therapeutic concentration significantly alleviated LPS-induced translocation of NFκB from the cytoplasm to nuclei. In mouse skin, resveratrol was reported to block IKK activity, which then inhibited phorbol ester-induced activation of NFκB (Kundu et al., 2006). Our present study shows that a clinically relevant concentration of ketamine decreased LPS-induced enhancement of IKK activity. Thus, the ketamine-caused down-regulation of NFκB's translocation is due to the suppressive effects of this intravenous anesthetic agent on IKK activation. IKK-mediated

NFκB activation contributes to the regulation of many physiological processes, including innate- and adaptive-immune responses, cell death, and inflammation (Perkins, 2007). Therefore, the ketamine-induced suppression of IKK-mediated NFκB activation can explain the immunomodulation which occurs in the clinical use of this intravenous anesthetic agent.

Downregulation of NFκB transactivation participates in ketamine-induced suppression of *IL-1*β gene expression. Occurring simultaneously with the enhanced NFκB translocation from the cytoplasm to nuclei, the binding affinity of this transcription factor to its consensus DNA oligonucleotides increased after LPS administration. However, exposure to a therapeutic concentration of ketamine significantly ameliorated the LPS-induced transactivation of NFκB with its specific DNA binding elements in macrophages. When the IKK/NFκB signal was reduced in mouse skin, the expression of cyclooxygenase-2 was downregulated in parallel and this promoted skin tumorigenesis (Kundu et al., 2006). NFκB-DNA binding elements exist in the promoter region of the *IL-1*β gene (Won et al., 2006). Thus, the ketamine-induced suppression of NFκB transactivation in LPS-activated macrophages can directly inhibit expression of the *IL-1*β gene in LPS-activated macrophages. Production and secretion of IL-1β by macrophages play important roles in regulating innate host defense and homeostasis (Wewers, 2004). In addition, IL-1β has been implicated as being a two-edged sword in immune-related diseases (Marx, 1988; Firestein, 2004). Therefore, the ketamine-involved inhibition of *IL-1*β gene expression in LPS-activated macrophages can explain why this anesthetic agent has immunosuppressive effects when it is applied clinically.

In conclusion, the present study shows that a clinically relevant concentration (100 μM) of ketamine decreases IL-1β biosynthesis in LPS-activated macrophages at the transcriptional level. Ketamine decreases the extracellular binding affinity of LPS to LBP. Furthermore, the ketamine-caused suppression of *IL-1*β expression was shown to be TLR4-dependent. Sequentially, a therapeutic concentration of ketamine downregulated LPS-induced increases in Ras activity and Raf phosphorylation. In parallel with these decreases in Ras/Raf activations, ketamine ameliorated LPS-induced phosphorylations of MEK1/2. ERK1/2, and IKK. Consequentially, the translocation and transactivation of NFκB enhanced by LPS were significantly lowered after exposure to ketamine. Our present data suggest that ketamine reduces IL-1β biosynthesis in LPS-stimulated macrophages through suppression of the cascade of extracellular LPS binding to LBP and intracellular TLR4-dependent activations of Ras/Raf/MEK/ERK/IKK as well as translocation and transactivation of the NFκB transcription factor. The ketamine-induced suppression of IL-1β production partially contributes to the immunomodulation, which is often observed during the clinical application of this intravenous anesthetic agent.

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References

Aderem, A., 2001. Role of Toll-like receptors in inflammatory response in macrophages. Crit. Care Med. 29, S16–S18.
Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.
Almeida, I.C., Gazzinelli, R.T., 2001. Proinflammatory activity of glycosylphosphatidylinositol anchors derived from *Trypanosoma cruzi*: structural and functional analyses. J. Leukoc. Biol. 70, 467–477.
Bartoc, C., Frumento, R.J., Jalbout, M., Bennett-Guerrero, E., Du, E., Nishanian, E., 2006. A randomized, double-blind, placebo-controlled study assessing the anti-inflammatory effects of ketamine in cardiac surgical patients. J. Cardiothor. Vasc. Anesth. 20, 17–22.
Beutler, B., Rietschel, E.T., 2003. Innate immune sensing and its roots: the story of endotoxin. Nat. Rev. Immunol. 3, 169–176.
Chang, Y., Chen, T.L., Sheu, J.R., Chen, R.M., 2005. Suppressive effects of ketamine on macrophage functions. Toxicol. Appl. Pharmacol. 204, 27–35.
Chen, R.M., Chen, T.G., Chen, T.L., Lin, L.L., Chang, C.C., Chang, H.C., Wu, C.H., 2005a. Anti-inflammatory and antioxidative effects of propofol on lipopolysaccharide-activated macrophages. Ann. N.Y. Acad. Sci. 1042, 262–271.
Chen, R.M., Chen, T.L., Lin, Y.L., Chen, T.G., Tai, Y.L., 2005b. Ketamine reduces nitric oxide biosynthesis in human umbilical vein endothelial cells by down-regulating endothelial nitric oxide synthase expression and intracellular calcium levels. Crit. Care Med. 33, 1044–1049.
Chen, T.G., Chen, T.L., Chang, H.C., Tai, Y.T., Cherng, Y.G., Chang, Y.T., Chen, R.M., 2007. Oxidized low-density lipoprotein induces apoptotic insults to mouse cerebral endothelial cells via a Bax-mitochondria-caspase protease pathway. Toxicol. Appl. Pharmacol. 219, 42–53.
Dobrovolskaia, M.A., Medvedev, A.E., Thomas, K.E., Cuesta, N., Toshchakov, V., Ren, T., Cody, M.J., Michalek, S.M., Rice, N.R., Vogel, S.N., 2003. Induction of in vitro reprogramming by Toll-like receptor (TLR)2 and TLR4 agonists in murine macrophages: effects of TLR "homotolerance" versus "heterotolerance" on NF-kappa B signaling pathway components. J. Immunol. 170, 508–519.
Domino, E.F., Zsigmond, E.K., Domino, L.E., Domino, K.E., Kothary, S.P., Domino, S.E., 1982. Plasma levels of ketamine and two of its metabolites in surgical patients using a gas chromatographic mass fragmentographic assay. Anesth. Analg. 61, 87–92.
Firestein, G.S., 2004. The T cell cometh: interplay between adaptive immunity and cytokine networks in rheumatoid arthritis. J. Clin. Invest. 114, 471–474.
Ganesan, L.P., Joshi, T., Fang, H., Kutala, V.K., Roda, J., Trotta, R., Lehman, A., Kuppusamy, P., Byrd, J.C., Carson, W.E., Caligiuri, M.A., Tridandapani, S., 2006. FcgammaR-induced production of superoxide and inflammatory cytokines is differentially regulated by SHIP through its influence on PI3K and/or Ras/Erk pathways. Blood 108, 718–725.
Grant, I.S., Nimmo, W.S., McNicol, L.R., Clements, J.A., 1983. Ketamine disposition in children and adults. Br. J. Anaesth. 55, 1107–1110.
Himmelseher, S., Durieux, M.E., 2005. Ketamine for perioperative pain management. Anesthesiology 102, 211–220.
Ho, R.C., Hirshman, M.F., Li, Y., Cai, D., Farmer, J.R., Aschenbach, W.G., Witczak, C.A., Shoelson, S.E., Goodyear, L.J., 2005. Regulation of IkappaB kinase and NF-kappaB in contracting adult rat skeletal muscle. Am. J. Physiol. 289, C794–C801.
Ince, M.N., Elliott, D.E., Setiawan, T., Blum, A., Metwali, A., Wang, Y., Urban, J.F.J., Weinstock, J.V., 2006. *Heligmosomoides polygyrus* induces TLR4 on murine mucosal T cells that produce TGFbeta after lipopolysaccharide stimulation. J. Immunol. 176, 726–729.
Kholodenko, B.N., 2007. Untangling the signalling wires. Nat. Cell Biol. 9, 247–249.
Kogut, M.H., Genovese, K.J., He, H., 2007. Flagellin and lipopolysaccharide stimulate the MEK-ERK signaling pathway in chicken heterophils through differential activation of the small GTPases, Ras and Rap1. Mol. Immunol. 44, 1729–1736.
Kolch, W., 2005. Coordinating ERK/MAPK signalling through scaffolds and inhibitors. Nat. Rev. Mol. Cell Biol. 6, 827–837.
Kundu, J.K., Shin, Y.K., Kim, S.H., Surh, Y.J., 2006. Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-kappaB in mouse skin by blocking IkappaB kinase activity. Carcinogenesis 27, 1465–1474.
Lamping, N, Hoess, A, Yu, B, Park, T.C., Kirschning, C.J., Pfeil, D., Reuter, D., Wright, S.D., Herrmann, F., Schumann, R.R., 1996. Effects of site-directed mutagenesis of basic residues (arg 94, lys 95, lys 99) of lipopolysaccharide (LPS)-binding protein on binding and transfer of LPS and subsequent immune cell activation. J. Immunol. 157, 4648–4656.
Lee, C.J., Tai, Y.T., Lin, Y.L., Chen, R.M., 2009a. Molecular mechanisms of propofol-involved suppression of nitric oxide biosynthesis and *inducible nitric oxide synthase* gene expression in lipopolysaccharide-stimulated macrophage-like Raw 264.7 cells. Shock. doi:10.1097/SHK.0b013e3181a6eaf5.
Lee, S.T., Wu, T.T., Yu, P.Y., Chen, R.M., 2009b. Apoptotic insults to human HepG2 cells induced by S-(+)-ketamine occurs through activation of a Bax-mitochondria-caspase protease pathway. Br. J. Anaesth. 102, 80–89.
Marx, J.L., 1988. Cytokines are two-edged swords in disease. Science 239, 257–258.
Mathison, J.C., Tobias, P.S., Wolfson, E., Ulevitch, R.J., 1992. Plasma lipopolysaccharide (LPS)-binding protein. A key component in macrophage recognition of gram-negative LPS. J. Immunol. 149, 200–206.
Molina, P.E., Zambell, K.L., Zhang, P., Vande, S.C., Carnal, J., 2004. Hemodynamic and immune consequences of opiate analgesia after trauma/hemorrhage. Shock 21, 526–534.
Nathan, C.F., 1987. Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J. Clin. Invest. 80, 1550–1560.
Nishina, K., Akamatsu, H., Mikawa, K., 1998. The inhibitory effects of thiopental, midazolam, and ketamine on human neutrophil functions. Anesth. Analg. 86, 159–165.
Perkins, N.D., 2007. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat. Rev. Mol. Cell Biol. 8, 49–62.
Rofael, H.Z., Turkall, R.M., Abdel-Rahman, M.S., 2003. Effect of ketamine on cocaine-induced immunotoxicity in rats. Toxicology 188, 101–114.
Schmidt, H., Ebeling, D., Bauer, H., Bach, A., Bohrer, H., Gebharn, M.M., Martin, E., 1995. Ketamine attenuates endotoxin-induced leukocyte adherence in rat mesenteric venules. Crit. Care Med. 23, 2008–2014.
Schroder, N.W., Opitz, B., Lamping, N., Michelsen, K.S., Zahringer, U., Gobel, U.B., Schumann, R.R., 2000. Involvement of lipopolysaccharide binding protein, CD14, and Toll-like receptors in the initiation of innate immune responses by Treponema glycolipids. J. Immunol. 165, 2683–2693.

Schubbert, S., Shannon, K., Bollag, G., 2007. Hyperactive Ras in developmental disorders and cancer. Nat. Rev. Cancer 7, 295–308.

Siwak, D.R., Shishodia, S., Aggarwal, B.B., Kurzrock, R., 2005. Curcumin-induced antiproliferative and proapoptotic effects in melanoma cells are associated with suppression of IkappaB kinase and nuclear factor kappaB activity and are independent of the B-Raf/mitogen-activated/extracellular signal-regulated protein kinase pathway and the Akt pathway. Cancer 104, 879–890.

Sjoholm, A., 1998. Aspects of the involvement of interleukin-1 and nitric oxide in the pathogenesis of insulin-dependent diabetes mellitus. Cell Death Differ. 5, 461–468.

Sun, J., Wang, X.D., Liu, H., 2004. Ketamine suppresses endotoxin-induced NF-kappaB activation and cytokines production in the intestine. Acta Anaesth. Scand. 48, 317–321.

Tai, Y.T., Cherng, Y.G., Chang, C.C., Hwang, Y.P., Chen, J.T., Chen, R.M., 2007. Pretreatment with low nitric oxide protects osteoblasts from high nitric oxide-induced apoptotic insults through regulation of c-Jun N-terminal kinase/c-Jun-mediated *Bcl-2* gene expression and protein translocation. J. Orthop. Res. 25, 625–635.

Weigand, M.A., Schmidt, H., Zhao, Q., Plaschke, K., Martin, E., Bardenheuer, H.J., 2000. Ketamine modulates the stimulated adhesion molecule expression on human neutrophils in vitro. Anesth. Analg. 90, 206–212.

West, M.A., Heagy, W., 2004. Endotoxin tolerance: a review. Crit. Care Med. 30, S64–S73.

Wewers, M.D., 2004. IL-1beta: an endosomal exit. Proc. Natl. Acad. Sci. U.S.A. 101, 10241–10242.

White, P.F., Way, W.L., Trevor, A.J., 1982. Ketamine: its pharmacology and therapeutic uses. Anesthesiology 56, 119–136.

Wolfs, T.G., Buurman, W.A., van Schadewijk, A., de Vries, B., Daemen, M.A., Hiemstra, P.S., van ' t Veer, C., 2002. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. J. Immunol. 168, 1286–1293.

Won, J.H., Im, H.T., Kim, Y.H., Yun, K.J., Park, H.J., Choi, J.W., Lee, K.T., 2006. Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages via the NF-kappaB inactivation. Br. J. Pharmacol. 148, 216–225.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., Mathison, J.C., 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249, 1431–1433.

Wu, C.H., Chen, T.L., Chen, T.G., Ho, W.P., Chiu, W.T., Chen, R.M., 2003. Nitric oxide modulates pro- and anti-inflammatory cytokines in lipopolysaccharide-activated macrophages. J. Trauma. 55, 540–545.

Wu, G.J., Chen, T.L., Ueng, Y.F., Chen, R.M., 2008. Ketamine inhibits tumor necrosis factor-α and interleukin-6 gene expressions in lipopolysaccharide-stimulated macrophages through suppression of toll-like receptor 4-mediated c-Jun N-terminal kinase phosphorylation and activator protein-1 activation. Toxicol. Appl. Pharmacol. 228, 105–113.

Wu, J.G., Chen, T.L., Chang, C.C., Chen, R.M., 2009. Propofol suppresses tumor necrosis factor-α biosynthesis in lipopolysaccharide-stimulated macrophages possibly through downregulation of nuclear factor-kappa B-mediated *toll-like receptor 4* gene expression. Chem.-Biol. Interact. 180, 465–471.

Yagoda, N., von Rechenberg, M., Zaganjor, E., Bauer, A.J., Yang, W.S., Fridman, D.J., Wolpaw, A.J., Smukste, I., Peltier, J.M., Boniface, J.J., Smith, R., Lessnick, S.L., Sahasrabudhe, S., Stockwell, B.R., 2007. RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. Nature 447, 864–868.

Yang, J., Li, W., Duan, M., Zhou, Z., Lin, N., Wang, Z., Sun, J., Xu, J., 2005. Large dose ketamine inhibits lipopolysaccharide-induced acute lung injury in rats. Inflamm. Res. 54, 133–137.

Zhang, L., Ma, Y., Zhang, J., Cheng, J., Du, J., 2005. A new cellular signaling mechanism for angiotensin II activation of NF-kappaB: an IkappaB-independent, RSK-mediated phosphorylation of p65. Arterioscler Throm. Vasc. Biol. 25, 1148–1153.