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Inhibition of lipopolysaccharide-induced inflammatory responses by piperine

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ABSTRACT

Piperine, a main component of *Piper longum* Linn. and *Piper nigrum* Linn., is a plant alkaloid with a long history of medical use. Piperine exhibits anti-inflammatory activity; however, the underlying mechanism remains unknown. We examined the effects of piperine on lipopolysaccharide (LPS)-induced inflammatory responses. Administration of piperine inhibited LPS-induced endotoxin shock, leukocyte accumulation and the production of tumor necrosis factor-α (TNF-α), but not of interleukin (IL)-1β and IL-6. In peritoneal macrophages, piperine inhibited LPS/poly (I:C)/CpG-ODN-induced TNF-α production. Piperine also inhibited LPS-induced endotoxin shock in TNF-α knockout (KO) mice. To clarify the inhibitory mechanism of LPS-induced endotoxin shock, type 1 interferon (IFN) mRNA expression was determined. Piperine inhibited LPS-induced expression of type 1 IFN mRNA. Piperine inhibited the levels of interferon regulatory factor (IRF)-1 and IRF-7 mRNA, and the phosphorylation and nuclear translocation of IRF-3. Piperine also reduced activation of signal transducer and activator of transcription (STAT)-1. In addition, activation of STAT-1 was inhibited in IFN-α/β-treated cells by piperine. These results suggest that piperine inhibits LPS-induced endotoxin shock through inhibition of type 1 IFN production.

1. Introduction

Piperine is a phenolic component of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). Black pepper and long pepper are important medicinal plants which are used in traditional medicine by many people in Asia and the Pacific islands, especially in Indian medicine (Singh, 1992). *In vitro* and *in vivo* studies have functionally implicated piperine as an antidepressant, hepatoprotective, anti-metastatic, antithyroid, immunomodulatory, and antitumor compound (Srinivasan, 2007). It has also been reported that piperine inhibits nitric oxide (NO), tumor necrosis factor-α (TNF-α), and pro-inflammatory gene expression *in vitro*, as well as *in vivo* (Pradeep and Kuttan, 2003; Kumar et al., 2007; Pradeep and Kuttan, 2004).

Macrophages respond to lipopolysaccharide (LPS) early in infection, and thus play a pivotal role in host defense. However, at high concentrations, LPS abnormally stimulates macrophages to release massive amounts of pro-inflammatory mediators, such as interleukin (IL)s, TNF-α, superoxide, and NO (Su, 2002; Dos Santos and Slutsky, 2000). NO and pro-inflammatory mediators, such as TNF-α, IL-6, and IL-12 can disturb normal cellular function, and this disruption can lead to multiple organ dysfunction syndromes or lethal septic shock (Marshall, 2001; Shen et al., 2004). Sepsis is a systemic response to serious infection, and has a poor prognosis when it is associated with organ dysfunction, hypoperfusion, or hypotension (Parrillo, 1996). Therefore, investigation of LPS-induced signaling pathways in macrophages is important and necessary for discovering potential therapeutic targets and drugs.

The activation of toll-like receptors (TLRs) transmits a signal to the cell interior through the Toll/IL-1R (TIR) domain that is found in the cytoplasmic region of each TLR. The TLR4, which is activated by bacterial LPS, is linked to MyD88-dependent and -independent pathways (Pålsson-McDermott and O'Neill, 2004). The former pathway involves downstream molecules, including MyD88, TIR domain-containing protein (TIRAP), IL-1R-associated kinase (IRAK), and TNF receptor-associated factor (TRAF)-6. The transmitted signal triggers Iκ-B kinase (IKK) and mitogen-activated protein kinase (MAPK) cascades, thereby resulting in the activation of transcription factors, NF-κB and AP-1, respectively (Schorey and Cooper, 2003). The MyD88-dependent pathway regulates the production of TNF-α, IL-1, IL-6, and IL-12 through activation of MAPK and NF-κB. The latter pathway is involved in interferon regulatory factor (IRF) activation and induction of the interferon (IFN)-α/β gene. IRF-1 expression is induced by LPS. Expression of IRF-1 and IRF-7 is positively regulated by interferon type 1 receptor signaling, whereas IRF-3 is constitutively expressed

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(Paun and Pitha, 2007). Type 1 IFN (IFN-α, IFN-β) is the key autocrine and paracrine immunoregulatory cytokine produced by macrophages and plasmocytoid dendritic cells after exposure to pathogens. Although first associated with the ability to combat viral infections, the overall activity of type 1 IFN in innate immunity extends to other pathogens (Baccala et al., 2007). Type 1 IFN is also a critical factor in LPS-induced endotoxin shock (Sakaguchi et al., 2003).

Although piperine has an excellent spectrum of therapeutic importance, the molecular mechanisms underlying the compound's anti-inflammatory response are not well defined. We determined the effect of piperine on LPS-induced inflammatory responses. We have shown that piperine inhibits LPS-induced production of type 1 IFN. This finding may account for the inhibitory action of piperine against endotoxin shock.

2. Materials and methods

2.1. Chemicals and reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). Enzyme-linked immunosorbant assay (ELISA) kits for mouse IL-1β, IL-6, and TNF-α

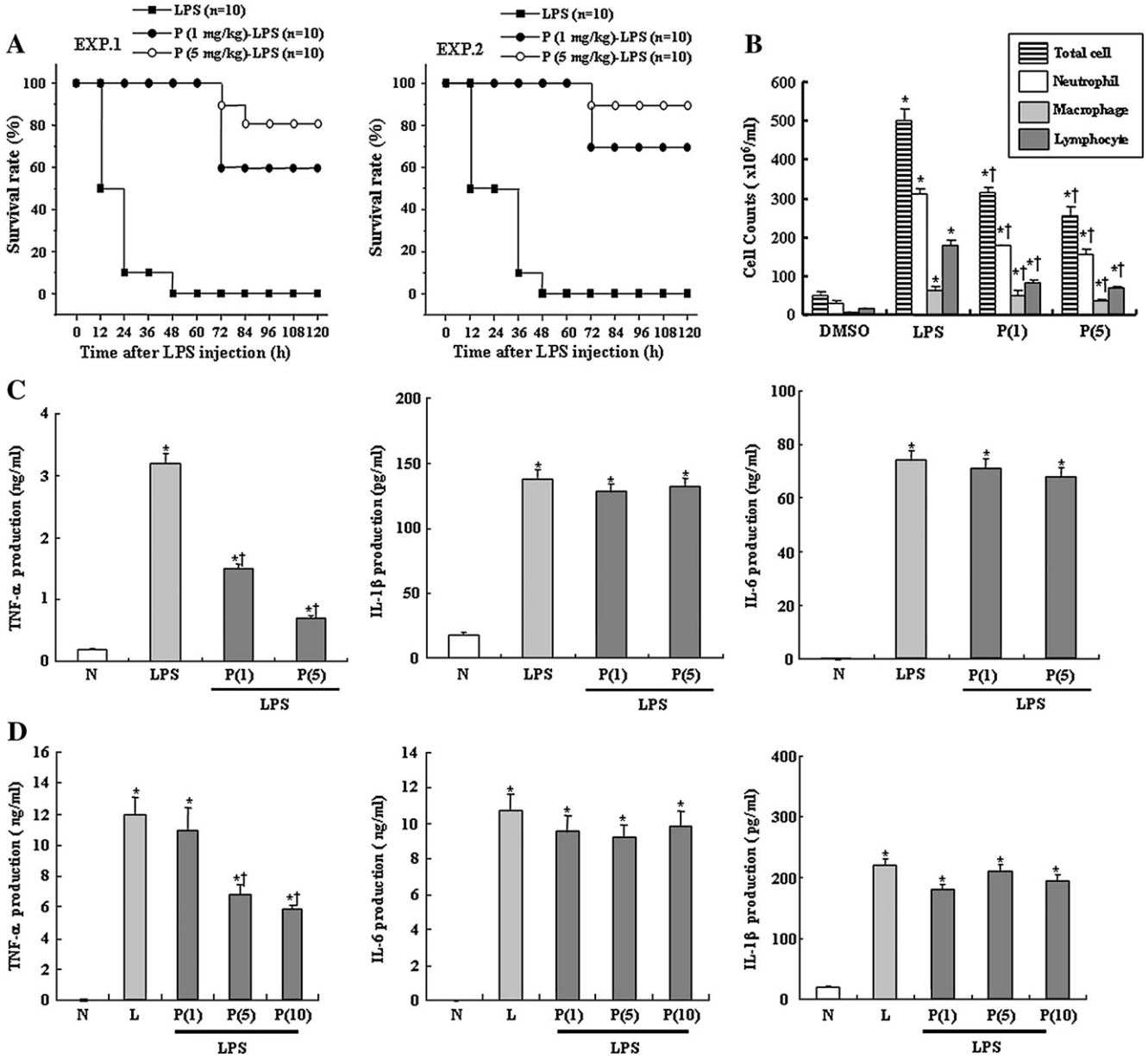

Fig. 1. Effect of piperine on LPS-induced endotoxin shock and cytokine production. Endotoxin shock was induced in female, 6–8-week-old C57BL/6 mice, as described in the Materials and methods. A. The effects of piperine on the survival rate of endotoxin shock mice ($n = 10$ per group) were monitored for survival for 120 h. To examine the effect of piperine on LPS-induced TNF-α, IL-1β, and IL-6 production *in vivo*, piperine was administered i.p. to mice ($n = 6$ per group) at 1 or 5 mg/kg. B. Peritoneal lavages were obtained from the mice 3 h after challenge with 37.5 mg/kg of LPS. The identification of leukocyte number was performed by immunohistochemical staining of cytospin preparation. C. Serum samples were obtained from the mice 3 h after challenge with 37.5 mg/kg of LPS for TNF-α, IL-1β, and IL-6 detection. D. Peritoneal macrophages were pretreated with 1, 5, or 10 μM piperine for 30 min prior to stimulation with 500 ng/ml of LPS for 24 h. Culture supernatants were harvested for detection of TNF-α, IL-1β, and IL-6. The amounts of IL-1β, TNF-α, and IL-6 were measured as described in the Materials and methods. E. The kinetics of LPS-induced TNF-α transcription was examined by real time RT-PCR. To examine the effect of piperine on LPS-induced TNF-α mRNA expression, cells were pretreated with 10 μM piperine for 30 min prior to stimulation with 500 ng/ml of LPS for the indicated time points. F. The expression of the TLR-4/MD2 complex was measured by flow cytometry. The cells were treated with piperine (10 μM) for 30 min and then analyzed. Representative histograms of at least three separate experiments are shown. *, $P<0.05$ vs. DMSO treatment; †, $P<0.05$ vs. LPS treatment alone. Data represent the mean ± S.E.M. of three independent experiments.

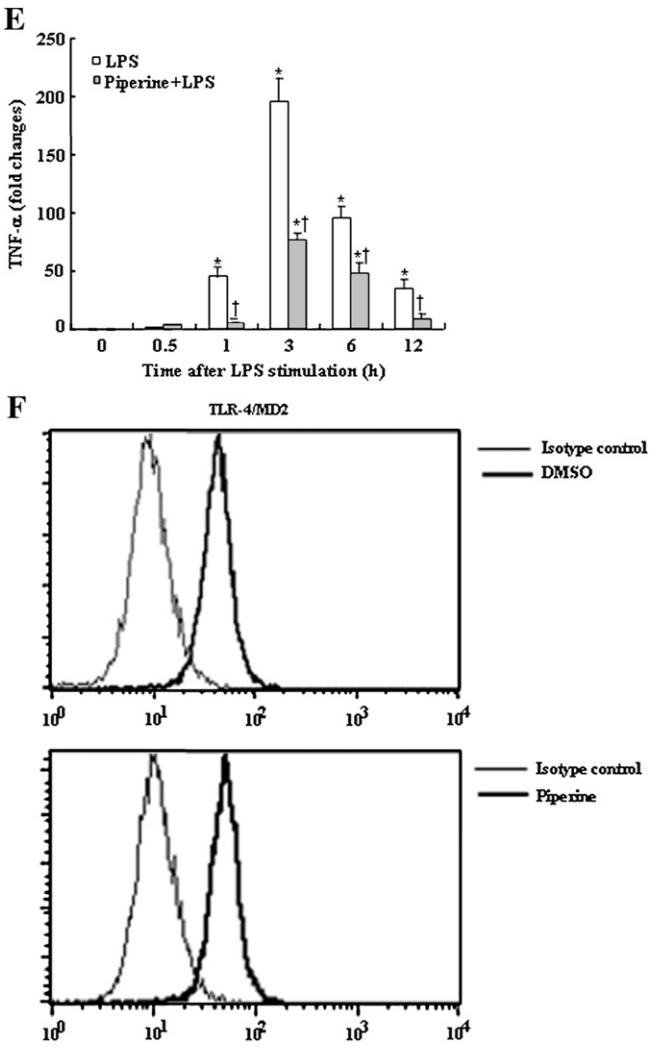

Fig. 1 (*continued*).

detection were purchased from R&D Systems (Minneapolis, MN, USA). LPS from *Escherichia coli* 055:B5, piperine, poly (I:C), and CpG-ODN (5′-TCC ATG ACG TTC CTG AAT GCT-3′) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Recombinant IFN-α/IFN-β was purchased from PBL Interferon Source (Piscataway, NJ, USA). Thioglycollate (TG) was purchased from BD Pharmingen (San Diego, CA, USA). Antibodies (Abs) against total and phosphospecific MAPKs, IRF-1, IRF-3, phosphospecfic STAT-1, and STAT-1 were from Cell Signaling Technology (Beverly, MA, USA). IRF-3 was from Zymed (San Francisco, CA, USA). IκB-α monoclonal Abs (mAbs) and peroxidase-conjugated secondary Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TLR-4/MD-2 complex and isotype control (rat IgG_{2a}, κ) were purchased from BD Pharmingen (San Diego, CA, USA). Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) markers were from Bio-Rad (Hercules, CA, USA). TRIzol reagent, Moloney murine leukemia virus reverse transcriptase (MML-V), and the polymerase chain reaction (PCR) reaction kit were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Inducible IL-6, TNF-α, IL-1β, and β-actin oligonucleotide primers were purchased from Genotech (Daejeon, Republic of Korea). TNF-α KO (B6; 129S6-TnftmlGkl/J) and control mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6 mice were purchased from Orient Bio Co. (Sungnam, KyungKiDo, Republic of Korea).

2.2. Animal model of endotoxin shock

Endotoxin shock was induced in female, 6–8-week-old C57BL/6 mice by intraperitoneal (i.p.) injection of bacterial endotoxin (LPS from *E. coli* serotype O55:B5, 37.5 mg/kg). The three groups of mice ($n=10$ per group) were injected i.p. with dimethyl sulfoxide (DMSO; control) or piperine (1 or 5 mg/kg). One h after piperine administration, control and piperine-administered mice were injected i.p. with 37.5 mg/kg of LPS. Survival was monitored for 120 h. Animal use and relevant experimental procedures were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, and experiments were approved by the Animal Care Committee of Wonkwang University.

2.3. In vivo preparation of blood samples

Piperine (1 or 5 mg/kg of body weight) was administered i.p. to mice ($n=6$ per group). Serum samples obtained from each mouse 3 h after challenge with 37.5 mg/kg of LPS were stored at −70 °C until use.

2.4. Peritoneal macrophage culture

TG-elicited macrophages were harvested 4 days after i.p. injection of 2.5 ml of TG (Narumi et al., 1990). Peritoneal lavage was performed using 8 ml of Hanks' balanced salt solution containing 10 U/ml of heparin. The cells were distributed in RPMI supplemented with 10% heat-inactivated FBS in 12-well tissue culture plates (1×10^6 cells/well). After incubation for 3 h, non-adherent cells were removed and adherent cells were treated with LPS, poly (I:C), or CpG-ODN in the presence or absence of piperine.

2.5. Western blot

Peritoneal macrophages (5×10^6 cells/well) were stimulated with 500 ng/ml of LPS. Nuclear extracts were prepared from peritoneal macrophages using cytosolic and nuclear extract kits (Promega, Madison, WI, USA). Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris–HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 20% glycerol, and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then blocked with 5% skim milk in PBS-Tween-20 (PBST) for 2 h at room temperature, and incubated with antibodies to phosphorylated ERK1/2, phosphorylated p38, phosphorylated JNK, phosphorylated IRF-3, phosphorylated STAT-1, phosphorylated Iκ-Bα, and Iκ-Bα overnight. After washing three times in PBST, each blot was incubated with secondary Ab for 1 h and the Ab-specific proteins were visualized using an enhanced chemiluminesence detection system (Amersham, Piscataway, NJ, USA) according to the manufacturer's recommended protocol.

2.6. ELISA

Mouse peritoneal macrophages were stimulated with 500 ng/ml of LPS, 50 μg/ml poly (I:C), or 10 μg/ml CpG-ODN and/or in the presence of various concentrations of piperine for 24 h. Culture supernatants were collected and stored at −70 °C until use. Cytokine levels in the supernatants were determined using a commercial system (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The ELISA was devised by coating 96-well plates with mAb specific for TNF-α, IL-1β, and IL-6. The coated plates were washed with PBS containing 0.05% Tween-20. All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant TNF-α, IL-1β, and IL-6 were diluted and used as a standard. Serial dilutions starting from 20 ng/ml were used to establish the standard curve. Assay plates were exposed sequentially to biotinylated mouse TNF-α, IL-1β, and IL-6 avidin peroxidase, and a substrate solution of 2,2′-azino-bis-[3-

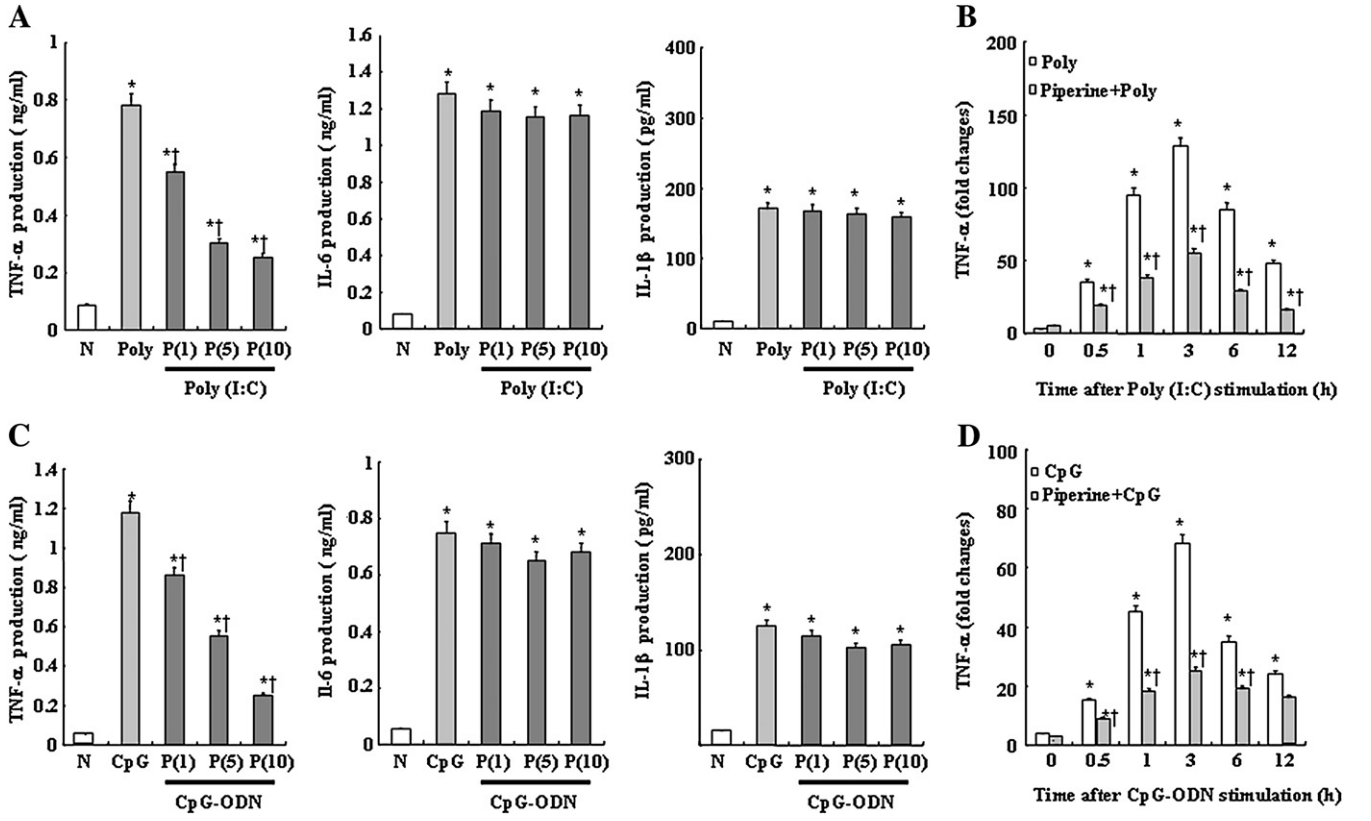

Fig. 2. Effect of piperine on poly (I:C)- and CpG-ODN-induced cytokine. The cells were pretreated with 1, 5, or 10 μM piperine for 30 min prior to stimulation with A. poly (I:C; 50 μg/ml) or C. CpG-ODN (10 μg/ml) for 24 h. Culture supernatants were harvested for detection of TNF-α, IL-1β, and IL-6. The kinetics of B. poly (I:C)- or D. CpG-ODN-dependent TNF-α transcription were examined by real time RT-PCR. To examine the effect of piperine on LPS-induced TNF-α mRNA expression, cells were pretreated with 10 μM piperine for 30 min prior to stimulation with poly (I:C) or CpG-ODN for the indicated time points. *, $P<0.05$ vs. DMSO treatment; †, $P<0.05$ vs. LPS treatment alone. Data represent the mean ± S.E.M. of three independent experiments.

ethylbenzthiazoline-6-sulfonic acid] (ABTS) containing 30% hydrogen peroxide. The plates were read at 405 nm.

2.7. Detection of TLR-4/MD2 complex by flow cytometry

TG-elicited macrophages were treated with DMSO/piperine (10 μM) for 1 h, and cells were immediately fixed with 4% paraformaldehyde for 15 min at 37 °C. Cells were then washed, and stained with anti-TLR-4/MD-2 complex Ab or isotype control for 30 min and analyzed by flow cytometry.

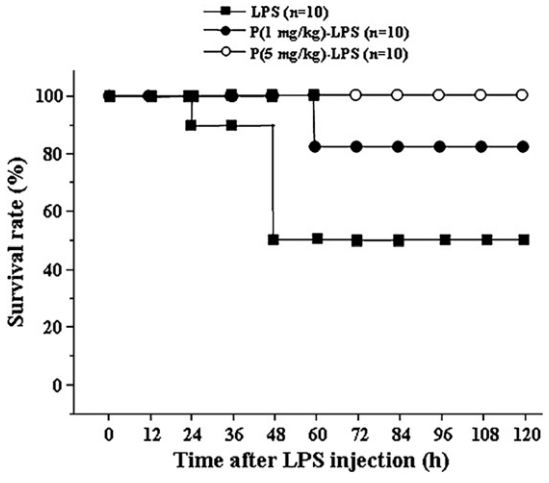

Fig. 3. Effect of piperine LPS-induced endotoxin shock in TNF-α KO mice. Endotoxin shock was induced in female, 6–8-week-old TNF-α KO mice by i.p. injection of bacterial endotoxin (37.5 mg/kg LPS from *E. coli* serotype O55:B5). The three groups of mice ($n=10$ per group) were administered DMSO (control) and piperine (1 or 5 mg/kg). Three h later the mice received 37.5 mg/kg of LPS. Survival was monitored for 120 h.

2.8. RNA quantification

Total RNA was extracted from harvested cells for quantitative real-time RT-PCR with the SV Total RNA Isolation System (Promega). The RNA isolation protocol included a DNase I treatment. We quantified RNA and reverse transcribed cDNAs from 2 μg of total RNA per 20 μl RT reaction with oligo (dT)12–8 primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). RT-PCR was conducted in a 25 μl solution containing 67.7 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 200 nM each of dATP, dCTP, dGTP, and 400 nM dUTP, 4.5 mM $MgCl_2$, 300 nM of each primer, 200 nM probe, 2 U Taq DNA polymerase, and 1/10 (by volume) of the cDNA synthesis reaction. Thermal cycling conditions consisted of 4 min at 95 °C followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The fluorescence generated by probe hydrolysis owing to the 5′-′ exonuclease activity of the DNA polymerase was measured with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Threshold cycle (Ct) values were calculated with the sequence detection SDS 1.7 software (Applied Biosystems) and normalization of the reporter signal to the quencher. Each sample was amplified in duplicate ($Ct<32$) or triplicate ($Ct>32$) with standard deviations of Ct values not exceeding 0.5 (for $Ct<33$). The Ct values were translated into a quantitative result by constructing a standard curve. Thus, a cDNA standard was prepared under the same conditions as the experimental samples. This was serially diluted to cover the range of Ct values that included the amount of target mRNA expected in the experimental samples, and analyzed for the gene of

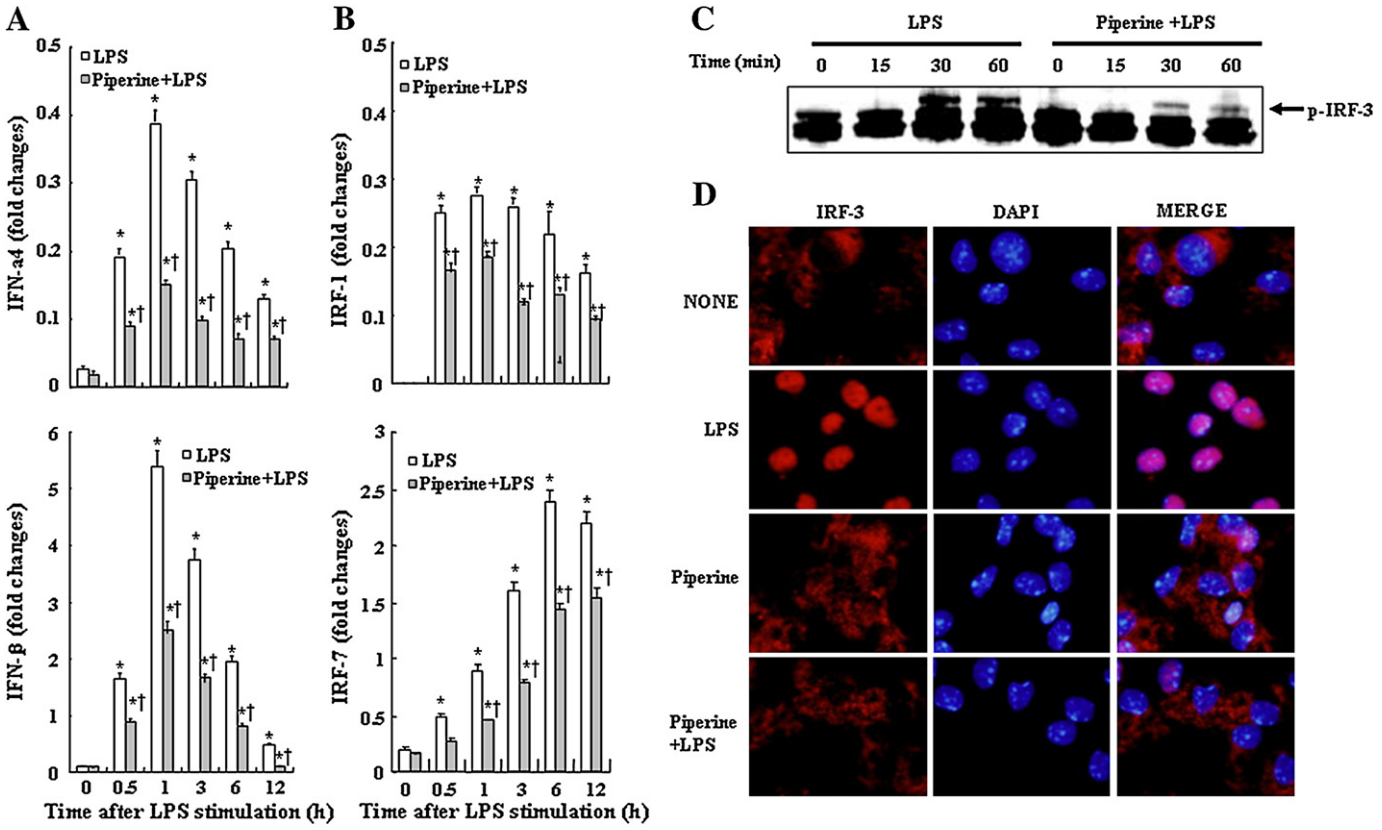

Fig. 4. Effect of piperine on LPS-induced type-1 IFN production. To examine the effect of piperine on LPS-induced A. type 1 IFN and B. IRF mRNA expression, the cells were pretreated with or without 10 μM piperine for 30 min prior to incubation with 500 ng/ml of LPS for the indicated time points. C. Phosphorylation of IRF-3 was also examined. The cells were pretreated with 10 μM piperine for 30 min and then stimulated with 500 ng/ml of LPS or LPS alone for the indicated times. Twenty μg of protein from each cell lysate was resolved on 10% SDS-PAGE. Western blot analysis was performed to detect the activation of IRF-3. D. Nuclear translocation of IRF-3 was examined using immunostaining. The cells were pretreated with 10 μM piperine for 30 min and then stimulated with 500 ng/ml of LPS or LPS alone for 30 min. *, $P<0.05$ vs. DMSO treatment; †, $P<0.05$ vs. LPS treatment alone. Data represent the mean ± S.E.M. of three independent experiments.

interest and endogenous control. The resultant Ct values were plotted against the logarithm of initial RNA mass to generate linear regressions ($y = mx + b$). The amount of experimental RNAs was calculated from the standard curve (correlation coefficient $R^2 > 0.990$). The mRNA level, given as the n-fold change in transcription of the gene of interest, was determined by normalization to the RNA mass value determined from the endogenous control and calibration to the wild-type expression before LPS stimulus. Alternatively, the amplitude of mRNA transcription (dynamic range) was calculated by calibration to the basal mRNA expression of the respective genotype. In the case of IFN-α4, the non-stimulated samples did not show basal expression, thus the first time point showing transcriptional response in wild-type cells was arbitrarily applied as the calibrator.

2.9. Primers and probes

Forward (f) and reverse (r) primers (Invitrogen), TaqMan probes (MWG-Biotech AG, Ebersberg, Germany), and TaqMan minor groove binder (MGB) probe (Applied Biosystems) were designed using Primer Express 1.5 software (Applied Biosystems). Real-time PCR was performed with the following primers: TTG CTC GAG ATG TfCA TGA AGG A (mHPRT-f); TGA GAG ATC ATC TCC ACC AAT AAC T (mHPRT-r); CCG AAG ACC TTA TGA AGC TCT TTG (mIRF-1-f); GCA AGT ATC CCT TGC CAT CG (mIRF-1-r); CTG GAG CCA TGG GTA TGC A (mIRF-7-f); AAG CAC AAG CCG AGA CTG CT (mIRF-7-r); CCT GTG TGA TGC AGG AAC C (mIFN-α4-f); TCA CCT CCC AGG CAC TGA (mIFN-α4-r); ATG AGT GGT GGT TGC AGG C (mIFN-β-f); TGA CCT TTC AAA TGC AGT AGA TTC A (mIFN-β-r); TCT CTT CAA GGG ACA AGG CTG (mTNF-α-f); and ATA GCA AAT CGG CTG ACG GT (mTNF-α-r). TaqMan probes consisted of an oligonucleotide labeled at the 5′ end with the reporter dye 6-carboxyfluorescein (FAM) and at the 3′ end with the quencher dye 6-carboxytetramethylrhodamine (TAMRA). In the TaqMan MGB probe format, a non-fluorescent quencher (NFQ) was used to enhance spectral performance. Probe sequences were FAM-TGG GAG GCC ATC ACA TTG TGG CTA MRA (mHPRT); FAM-CAG TCT GAG TGG CAG CGG ACA CAC A-TAMRA (mIRF-1); FAM-CTG GAG GGC GTG CAG CGT GA-TAMRA (mIRF-7); FAM-AGA CTC CCT GCT GGC TGT GAG GAC A-MGB-NFQ (mIFN-α4); FAM-AAG CAT CAG AGG CGG ACT CTG GGA-TAMRA (mIFN-β); and FAM-CCC GAC TAC GTG CTC CTC ACC CA-TAMRA (mTNF-α).

2.10. Immunostaining

The peritoneal macrophage cells were plated in a chamber slide and incubated with 500 ng/ml of LPS for 6 h at 37 °C. The cells were fixed in 4% paraformaldehyde for 30 min at 48 °C and washed 3 times with PBS. The cells were treated with 0.1% TritonX-100 for 15 min at room temperature. After washing, the cells were reacted with a blocking serum for 1 h and incubated overnight with a 1:100 dilution after primary IRF3 antibody (Invitrogen, Carlsbad, CA, USA). The cells were then washed and incubated with a 1:50 dilution of Alexa-fluor®568 goat anti-rabbit IgG (Invitrogen) for 4 h in a darkened room. For nuclear staining, the cells were incubated with a 1:1000 dilution of DAPI for 30 min. The slide was finally washed and mounted for microscopic examination. Staining with IRF antibody exhibits a red fluorescence, which can be detected by fluorescence microscopy.

2.11. Leukocyte accumulation counting

Three h after LPS injection, the peritoneal fluids were collected using repetitive (2 times) instillation and withdrawal of 2 and 5 ml respectively of sterile saline solution using a syringe with a 22 G needle. The peritoneal lavage sample was placed on ice, immediately processed for centrifugation at 4 °C, and the supernatant and the cell pellet were collected separately. The cell pellet was used for total and differential cell counts. Differential cell counts were determined on cytospin preparations of peritoneal lavage stained with Wright-stain (LeukoStat). The total number of peritoneal leukocytes was determined by coulter counter (Roche). Additionally, the presence of neutrophils, macrophage and lymphocyte in peritoneal lavage was confirmed by immunohistochemical staining of cytospin preparation.

2.12. Cecal ligation and puncture

The cesum was ligated by silk 4–0 and punctured it twice with a 21-gauge needle, gently squeezed it to express a small amount of fecal material and then returned it to the central abdominal cavity. In sham-operated mice, we located the cecum but neither ligated nor punctured it. We closed the abdominal incision in two layers with 6–0 nylon sutures. After surgery, we gave 1 ml per 30 g body weight of pre-warmed normal saline.

2.13. Statistical analysis

The experiments shown are a summary of the data from at least three experiments and are presented as the mean ± S.E.M. Independent *t*-test or one-way ANOVA were used to analyze a statistical significance of the results between or among groups, and if statistically significant, *post hoc* analysis using the Duncan method was followed as a multiple comparison among groups. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. Influence of piperine on LPS-induced endotoxin shock and inflammatory response

To examine the effect of piperine on LPS-induced endotoxin shock, we compared the DMSO controls and piperine treatment group for susceptibility. Injection of 37.5 mg/kg of LPS was lethal for mice, with death usually occurring within 2 days (Fig. 1A). Administration of piperine (1 or 5 mg/kg body weight) before LPS challenge increased survival rate (Fig. 1A). It has been reported that LPS could cause several subsets of leukocytes, then we examined the leukocyte accumulation in peritoneal cavity (Crockett et al., 2004). Piperine was administrated i.p. to mice ($n=6$ per group), then the peritoneal lavages were harvested 3 h after LPS challenge. As shown in Fig. 1B, the pre-treatment of piperine inhibited LPS-induced leukocyte accumulation. LPS induces several kinds of cytokines, including TNF-α, IL-1β, and IL-6, thus mediating the development of various inflammatory reactions (Dinarello, 2000). To examine the effect of piperine on LPS-induced production of TNF-α, IL-1β, and IL-6, we examined its effect on the production of these cytokines. Piperine (1 or 5 mg/kg of body weight) was administered i.p. to mice ($n=6$ per group) and serum samples were obtained 3 h after challenge with 37.5 mg/kg of LPS. Consistent with the lethality curve, injection of LPS induced the appearance of high levels of TNF-α, IL-1β, and IL-6 in the serum. However, piperine inhibited LPS-induced production of TNF-α,

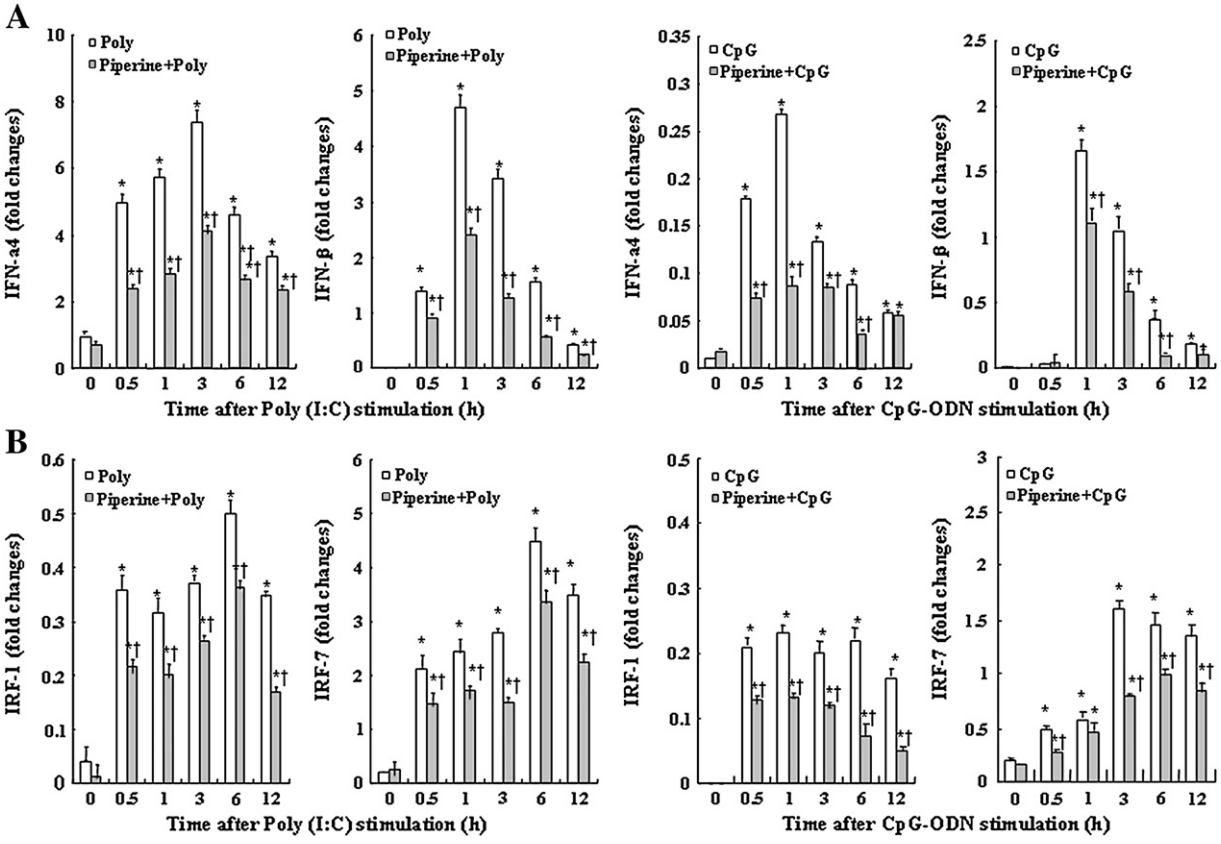

Fig. 5. Effect of piperine on poly (I:C)- and CpG-ODN-induced type 1 IFN induction. To examine the effect of piperine on (A) poly (I:C) or CpG-ODN-induced type 1 IFN and (B) IRF-1 and IRF-7 mRNA expression, the cells were pretreated with or without 10 μM piperine for 30 min prior to incubation with poly (I:C) or CpG-ODN. *, $P<0.05$ vs. DMSO treatment; †, $P<0.05$ vs. LPS treatment alone. Data represent the mean ± S.E.M. of three independent experiments.

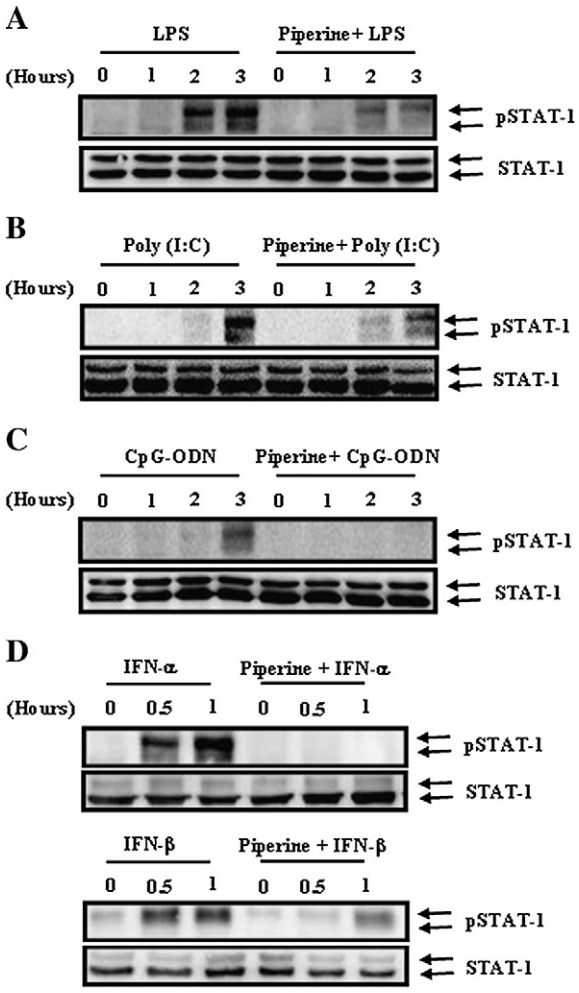

Fig. 6. Piperine inhibits STAT-1 phosphorylation. To examine the effect of piperine on inhibition of STAT-1 activation, the cells were pretreated with 10 μM piperine for 30 min prior to stimulation with A. LPS, B. poly (I:C) and C. CpG-ODN for the indicated time points. To examine the effect of piperine on IFN-α/β-induced STAT-1 activation, the cells were pretreated with piperine for 30 min prior to stimulation with IFN-α (250 U/ml)/β (100 U/ml) for the indicated time points. Twenty μg of protein from each cell lysate was resolved on 10% SDS-PAGE. Western blot analysis was performed to detect the activation of STAT-1. Similar results were obtained in three independent experiments.

but not IL-1β and IL-6 (Fig. 1C). Dysregulation of macrophage activity during the LPS response can result in endotoxin shock in mice.

In peritoneal macrophages, we examined inflammatory mediators, including TNF-α, IL-1β, and IL-6. The cells were pretreated with 1, 5, or 10 μM piperine for 30 min and then stimulated with 500 ng/ml of LPS for 24 h. In accordance with the *in vivo* results, piperine inhibited the production of TNF-α in a dose-dependent manner, but had no effect on IL-1β and IL-6 production (Fig. 1D). When the kinetics of LPS-dependent TNF-α transcription were examined by real-time RT-PCR, consistent with the level of cytokine, piperine inhibited LPS-induced expression of TNF-α mRNA at the indicated time points (Fig. 1E). To rule out the possibility that piperine reduced the expression of TLR-4/MD-2 complex, we examined the effect of piperine on receptor expression. As shown in Fig. 1F, piperine did not inhibit the expression of TLR-4/MD-2 complex in peritoneal macrophages. LPS activates either the MyD88-dependent or the MyD88-independent signaling pathway. Therefore, we used poly (I:C) (for MyD88-independent responses) and CpG-ODN (for MyD88-dependent responses) to examine the inhibitory effect of piperine on TNF-α production. Piperine also inhibited both poly (I:C)- and CpG-ODN-induced production of TNF-α, but not of IL-1β and IL-6 (Fig. 2A and C). The level of TNF-α mRNA was also inhibited for the indicated time (Fig. 2B and D).

Taken together, these data demonstrate that piperine improved mortality in LPS-induced endotoxin shock.

3.2. Influence of piperine on LPS-induced endotoxin shock in TNF-α KO mice

As shown in Figs. 1 and 2, piperine partially inhibited LPS-induced endotoxin shock and TNF-α production in serum and peritoneal macrophages. TNF is a crucial effector of LPS *in vivo* toxicity (Ito et al., 2006). To test whether this inhibitory effect of piperine on LPS-induced endotoxin shock could be dependent on TNF-α, TNF-α KO mice were used. After injection of 37.5 mg/kg LPS, the survival rate was 50% within 2 days. However, administration of piperine (1 or 5 mg/kg body weight) before LPS challenge increased survival by 80% and 100%, respectively (Fig. 3). In sum, we showed that piperine inhibited TNF-α production *in vitro* and *in vivo*, but further showed that piperine improved mortality in TNF-α KO mice in the endotoxin shock model. These data suggest that the effect of piperine on improving mortality might not be due to the effects on the TNF pathway.

3.3. Influence of piperine on LPS-induced production of the type 1 IFN in peritoneal macrophages

Type 1 IFN is an essential effector in LPS-induced lethality (Karaghiosoff et al., 2003). Therefore, we investigated the effect of piperine on type 1 IFN by real-time RT-PCR. In agreement with the above observations, IFN-α4 and IFN-β expression was induced in LPS-stimulated macrophages. Piperine inhibited LPS-induced expression of IFN-α4 and IFN-β (Fig. 4A). During viral infection, cells defective for both IRF-3 and IRF-7 completely fail to induce the IFN-α/β genes (Honda et al., 2005). It is possible that the activation or induction of IRF-1, IRF-3, and IRF-7 may be essential for the maximum production of type 1 IFN by LPS challenge. Therefore, we examined induction or activation of IRF. Piperine inhibited LPS-induced expression of IRF-1 and IRF-7 mRNA, and also phosphorylation and nuclear translocation of IRF-3 (Fig. 4B, C, and D). Furthermore, we examined the effects of piperine on the poly (I:C) and CpG-ODN-induced production of type 1 IFN. Type 1 IFN production and induction of IRF-1 and -7 were inhibited by piperine (Fig. 5A and B). As a whole, these data demonstrated that piperine inhibited type 1 IFN through inhibition of IRF-1 and IRF-7 induction and IRF-3 activation.

3.4. Influence of piperine on STAT-1 activation in peritoneal macrophages

In addition to analyzing the production of type 1 IFN after LPS, poly (I:C) and CpG-ODN, we also examined the intracellular signaling pathway downstream of type 1 IFN in peritoneal macrophages. It was reported that type 1 IFN resulted in the activation of STAT-1 (Levy et al., 2003). Piperine also inhibited the production of type 1 IFN (Figs. 4A and 5A). Therefore, we examined STAT-1 activation. In agreement with a previous report (Toshchakov et al., 2002), STAT-1 was activated 2–3 h after LPS treatment in mouse peritoneal macrophages. Poly (I:C)/CpG-ODN also induced STAT-1 activation (Fig. 6A, B, and C). Consistent with real-time RT-PCR results (Figs. 4A and 5A), piperine inhibited activation of STAT-1 (Fig. 6). Furthermore, we showed that piperine inhibited the activation of STAT-1 in IFN-α/β-treated cells. Taken together, these data suggested that the effect of piperine on reduced STAT-1 activation might be associated with reduction of type 1 IFN production.

4. Discussion

Plant alkaloids, such as berberine, tetrandrine, vinblastin, paclitaxel, and poperine are recognized as chemopreventive agents, and are believed to be pharmacologically harmless (Henderson et al., 2003; Sikorska et al., 2004; Hwang et al., 2006). Piperine has been experimentally evaluated for its multiple biological activities, including anti-inflammation and immunomodulation (Srinivasan, 2007; Pradeep and Kuttan, 2003). The anti-inflammatory activity of piperine has been reported in rats using experimental models, such as carrageenan-induced rat paw edema, cotton pellet granuloma, and cotton oil-induced granuloma pouch (Mujumdar et al., 1999). In these models, inflammation was attributed to pro-inflammatory cytokines in macrophages. We have demonstrated that piperine inhibits LPS/poly (I:C)/CpG-ODN-induced production of TNF-α and type 1 IFN, but not IL-1β and IL-6; piperine also attenuates LPS-induced endotoxin shock, at least in part, via inhibition of type 1 IFN production.

Resistance to LPS challenge is conferred by targeted disruption of primary LPS signaling, e.g., TLR4 (Yamamoto et al., 2002; Hoshino et al., 1999), CD14 (Haziot et al., 1996), MD-2 (Nagai et al., 2002), MyD88 (Kawai et al., 1999), and IRAK-4 (Suzuki et al., 2002). Therefore, to rule out the effect of piperine on the level of expression of the TLR-4/MD-2 complex, we analyzed the TLR-4/MD-2 complex in piperine-treated peritoneal macrophages. The level of the TLR-4/MD-2 complex was not altered by piperine (data not shown). The immediate-early production of NO and pro-inflammatory cytokines, such as IL-1, IL-6, and TNF are involved in endotoxin shock (Kawai et al., 1999). Administration of piperine inhibited LPS-induced production of TNF-α, but not of IL-1β and IL-6 (Fig. 1). Excessive production of TNF-α causes tissue injury, septic shock, and autoimmune diseases, such as inflammatory bowel disease (Pasparakis et al., 1996; Pfeffer et al., 1993; Kontoyiannis et al., 1999; La Sala et al., 2005). A previous study has documented that a long-term (4 day) treatment with piperine is capable of inhibiting TNF-α production in LPS- and Con-A-stimulated Balb/c mice (Pradeep and Kuttan, 2004). To determine whether an increase in survival could be dependent on TNF-α production, TNF-α KO mice were used. TNF-α KO mice were more resistant to LPS-induced endotoxin shock compared to control mice (data not shown). Interestingly, administration of piperine inhibited LPS-induced endotoxin shock in TNF-α KO mice (Fig. 3), indicating that the effects of piperine on improving mortality may be not due to the effects on the TNF pathway.

The pathogenesis of sepsis involves a progressive and dynamic expansion of a systemic inflammatory response to bacterial infection (Glauser, 1996). LPS, one of the components of the outer membrane of Gram-negative bacteria, induces a variety of responses to severe infection, such as local inflammation, antibody production, and septic shock (Li et al., 2006). The inflammatory mediators, such as TNF, IL-1, NO, and IFN can induce endotoxin shock. However, these inflammatory mediators may have dual effects. Both abnormally low and high levels of these mediators may contribute to the pathogenesis of inflammatory diseases. Thus, potential inhibitors of endotoxin-induced IFN production may be effective therapeutically in preventing inflammatory reactions and diseases, such as systemic lupus erythematosus, an autoimmune disease in which type 1 IFN is thought to be pathogenic (Baccala et al., 2007). We also examined that piperine could show a therapeutic effect on sepsis. Although the pre-treatment of piperine have showed decreased mortality, the post-treatment of piperine didn't inhibited the LPS-induced sepsis (data not shown). Also in cecal ligation and puncture (CLP) model, piperine didn't show the any effects on sepsis-induced death (data not shown). This result showed that the effect of piperine may be limited in prophylactic effects on LPS-induced inflammation.

Type 1 IFN was originally described as a potent antiviral cytokine produced upon infection of animals with virus (Bogdan, 2000; Le Page et al., 2000). Virus-induced IFN-β sensitizes the host response to LPS (Yaegashi et al., 1995). Recent studies with TLR4 signaling, however, emphasize the involvement of IFN-β during the immune response to LPS, even in the absence of viral infection. LPS induces type 1 IFN in a MyD88-independent manner. Maximal type 1 IFN production upon microbial infection depends mainly on IRF-3 and IRF-7, and involves a positive-feedback mechanism (Sato et al., 2000). Our results also showed that piperine inhibited LPS-induced induction of IRF-1 and IRF-7 (Fig. 4A and B), indicating that piperine inhibited type 1 IFN production owing to a reduced availability of IRF-1 and IRF-7. Inhibition of the NF-κB pathway or IRF-3 activity equally inhibits IFN-β gene expression (Sato et al., 2000; Jiang et al., 2004). Piperine reduced Iκ-Bα phosphorylation and degradation and inhibited nuclear translocation of IRF-3 (data not shown, Fig. 4D). These results suggest that piperine inhibits type 1 IFN production through inhibition of IRF-1 and IRF-7 gene expression, and of nuclear translocation of IRF-3, and that piperine, at least in part, might be involved in the NF-κB pathway associated inhibition of type 1IFN production.

IFN-β-deficient mice are resistant to challenge with a high level of LPS (Karaghiosoff et al., 2003). Piperine inhibited LPS-induced type 1 IFN production (Fig. 4A). Furthermore, both MyD-88-dependent and -independent type 1 IFN production were inhibited (Fig. 5A). These results suggest that reduced IFN-β production by piperine may be involved in inhibition of endotoxin shock.

IFN-β is expressed at an earlier time point than IFN-γ so that it may activate STAT-1 before IFN-γ can do so. Our data showed that piperine inhibited LPS/poly (I:C)/CpG-ODN-induced STAT-1 activation (Fig. 6A, B and C). Interaction of IFN with the receptors causes tyrosine phosphorylation of the cytoplasmic STAT-1 protein, leading to its migration to the nucleus and transcription of a number of IFN-responsive genes. STAT-1 also contributes to the development of endotoxin shock (Durbin et al., 1996). Piperine inhibited activation of STAT-1 in IFN-α/β-treated cells (Fig. 6D). These results demonstrated that reduced STAT-1 activation in piperine-treated cells might be a consequence of reduced type 1 IFN production.

In conclusion, the present results show that piperine inhibits LPS-induced endotoxin shock through inhibition of type 1 IFN production. Taken together, our results suggest that the effect of piperine on LPS-induced endotoxin shock may be associated with reduction of STAT-1 activation through inhibition of type 1 IFN production and that black pepper and piperine may be useful as a gastrointestinal anti-inflammatory strategy.

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