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# Berberine decreased iNOS mRNA stability through negative regulation of HuR in LPS-induced macrophages

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AMPK, AMP-activated protein kinase; AREs, AU-rich elements; ELAV, embryonic lethal abnormal vision; hnRNP A1, heteronuclear ribonucleoprotein A1; HNS, nucleocytoplasmic shuttling sequence; HR, hinge region; HuR, human antigen R; iNOS, inducible nitric oxide; LPS, lipopolysaccharide; KSRP, KH-type splicing regulatory protein; NF-κB, nuclear factor-

kappa B; NO, nitric oxide; RRM, RNA recognition motifs; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; TTP, tristetraprolin; 3´-UTR, 3´-untranslated region

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## Abstract

Berberine, a major isoquinoline alkaloid found in medicinal herbs, has been reported to possess anti-inflammatory effects. However, the underlying mechanisms responsible for its actions are poorly understood. In the present study, the authors investigated the inhibitory effects of berberine and the molecular mechanisms involved in LPS-treated RAW 264.7 and THP-1 macrophages and its effects in LPS-induced septic shock in mice. In both macrophage cell types, berberine inhibited the LPS-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) protein expression, but had no effect on *iNOS* mRNA transcription. Suppression of LPS-induced iNOS protein expression by berberine occurred via a human antigen R (HuR)-mediated reduction of *iNOS* mRNA stability. Molecular data revealed that the suppression on the LPS-induced HuR binding to *iNOS* mRNA by berberine was accompanied by a reduction in nucleocytoplasmic HuR shuttling. Pretreatment with berberine reduced LPS-induced iNOS protein expression, the cytoplasmic translocation of HuR in liver tissues and increased the survival rate of mice with LPS-induced endotoxemia. These results show that the suppression of iNOS protein expression by berberine under LPS-induced inflammatory conditions is associated with a reduction in *iNOS* mRNA stability due to inhibition of the cytoplasmic translocation of HuR.

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## Introduction

Berberine, which is an isoquinoline alkaloid, has been separated from the medicinal herbs such as *Hydrastis canadensis* (goldenseal), *Cortex phellodendri* (huangbai), and *Rhizoma coptidis* (huanglian) (Schmeller et al., 1997). It has a potential in clinical applications because of its diverse pharmacological properties, such as antimicrobial, antidiabetic, antihyperlipidemic, anti-inflammatory, antioxidant, neuronal protective, and anti-cancer effects (Fan et al., 2015). Berberine has been reported to have diverse and complex mechanisms for its pharmacological actions. Some pharmacological activities of berberine relate to stabilization of mRNAs, such as low-density lipoprotein receptor (LDLR), tissue factor (TF), and retinoblastoma protein (Rb) (Holy et al., 2009; Li et al., 2009; Chai et al., 2014). The anti-inflammatory activity of berberine was observed both *in vitro* and *in vivo* and was noted by a reduction of pro-inflammatory mediators, acute phase proteins, and infiltration of inflammatory cells. It was shown that the anti-inflammatory activities of berberine was strongly linked to its negative regulatory activity on pro-inflammatory gene expression via transcription factor inactivation, such as nuclear factor-kappaB (NF-κB) and activator protein 1 (AP-1) (Li et al., 2014). Recent data, however, suggest that dysregulated post-transcriptional regulation of pro-inflammatory gene expression plays a central role in the onset and maintenance of chronic inflammatory diseases (Bollmann et al., 2014). In contrast to transcriptional control by berberine, its effects on post-transcriptional regulation of pro-inflammatory mediators and underlying molecular mechanisms remain largely unknown.

Gene expression is controlled by both transcriptional and post-transcriptional mechanisms, and importantly, the post-transcriptional modulation of gene expression is largely mediated by the regulation of mRNA stability (Colton et al., 2006). Tight control of mRNA stability is an effective means of regulation of target protein production by the

modulation of mRNA expression. Increasing the expression of inducible nitric oxide synthase (iNOS) is a critical initial step in the production of nitric oxide (NO) in immune cells including macrophages (Chan and Riches, 2001). NO is an active component in many immunomodulatory, anti-bacterial, and anti-tumoral mechanisms, and if uncontrolled, it can also be harmful to the host (Iadecola et al., 1997). NO can combine with superoxide at a very fast rate to form peroxynitrite, a toxic reactive nitrogen species (RNS) (Xia and Zweier, 1997). Peroxynitrite is a highly reactive intermediate that can nitrate the tyrosine residues on proteins and also cause significant cellular oxidative damage (Coppey et al., 2001). Therefore, the dysfunctional induction of iNOS expression seems to be involved in the pathophysiologies of several human diseases, such as asthma, arthritis, multiple sclerosis, colitis, psoriasis, and neurodegenerative diseases (Adams and Hamilton, 1984).

During the last few years, several RNA-binding proteins (RNA-BPs) have been shown to be involved in regulation of iNOS mRNA stability. These include the embryonic lethal abnormal vision (ELAV) protein family members (the most important of which is ELAV-1, also named human antigen R (HuR)), KH-type splicing regulatory protein (KSRP), tristetraprolin (TTP) (Kim et al., 2007), heteronuclear ribonucleoprotein A1 (hnRNP A1) (Lee et al., 2007), ARE/poly-(U)-binding/degradation factor-1 (AUF-1, also named hnRNP D) (Kuo et al., 2004), and T cell-restricted intracellular antigen-related protein (TIAR) (Rodriguez-Pascual et al., 2000). Among these, HuR has been reported to stabilize many inherently unstable mRNAs (Hu et al., 2008) by binding to AU-rich elements (AREs) in the 3′-untranslated region (3′-UTR). Unlike its neuron-specific ELAV relatives (HuB, HuC, and HuD), HuR is ubiquitously expressed, and has been implicated in a large variety of physiological and pathophysiological processes, such as cell growth, differentiation, and inflammation. The stable overexpression of HuR in human colon adenocarcinoma DLD-1

cells was found to result in the upregulation of cytokine-induced iNOS expression. Conversely, the downregulation of HuR reduced cytokine-induced iNOS expression (Rodriguez-Pascual et al., 2000). Here, we demonstrated for the first time that berberine inhibited LPS-induced iNOS protein expression by reducing its mRNA stability in a HuR-dependent manner, and identified the key mechanism responsible for its anti-inflammatory effects.

## Materials and Methods

**Chemicals.** Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. The antibodies against iNOS, HuR, protein kinase C $\delta$ (PKC$\delta$), α-tubulin, poly(ADP ribose)polymerase (PARP) were purchased from Santa Cruz Biotechnology Inc. The RNA extraction kit was purchased from Intron Biotechnology. Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit was purchased from Millipore. iNOS and β-actin oligonucleotide primers were purchased from Bioneer. Berberine chloride, actinomycin D, phorbolmyristate acetate (PMA), phenylmethyl sulfonylfluoride (PMSF), dithiothreitol, L-$N^6$-(1-iminoethyl)lysine (L-NIL), protein A-sepharose from *Staphylococcus aureus*, triton X-100, *E. coli* LPS, and all other chemicals were purchased from Sigma Chemical Co.

**p-HuR Antibody Production.** The phospho-specific rabbit polyclonal antibodies were raised against a synthetic peptide (sequence: C-RFp(S)PMGVDHMSG) coupled to KLH (Pierce) and subsequently, the coupled peptides were injected into rabbits four times. Peptide-KLH conjugate (1 mg) was dissolved in 500 $\mu$l phosphate-buffered saline (PBS) emulsified with 500 $\mu$l of complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. Female rabbits were injected subcutaneously at 4 weeks, 2 weeks, and 2 weeks intervals for 3

months. One week after the last immunization, 50 ml blood was taken and the centrifuged serum was stored at -20°C until use. Specificity of peptide antibodies was analyzed using indirect ELISA methods. The antibodies were first affinity-purified on a column containing SulfaLink beads (Pierce) cross-linked to the phosphorylated peptides, eluted with 1 M glycine-HCl, pH 2.7, and immediately neutralized with an equal volume of 5% $NHCO_3$. Bovine serum albumin (BSA, 1%) was then added, and the protein mixture was dialyzed overnight against PBS. Finally, the dialyzed antibody sample was run through a column containing SulfaLink beads (Pierce) cross-linked to the equivalent nonphosphorylated peptides and the unbound fraction collected and stored at 4°C with azide. The specificity of antibody was confirmed by protein dephosphorylation using alkaline phosphatase.

**Cell Culture and Differentiation.** The THP-1 human monocyte cell line and RAW 264.7 murine macrophage cell line were obtained from the Korean Cell Line Bank. Cells were grown in RPMI and DMEM medium at 37°C containing 10% FBS, penicillin (100 units/ml) and streptomycin sulfate (100 $\mu$g/ml) in a humidified atmosphere of 5% $CO_2$. THP-1 cells were differentiated to macrophages by treating with PMA (100 nM) for 48 h as previously described (Daigneault et al., 2010). Cells were incubated with berberine at various concentrations (1, 5, or 10 $\mu$M) or with positive-control chemicals and stimulated with LPS (1 or 10 $\mu$g/ml).

**Nitrite Determination.** The nitrite, which accumulated in the culture medium, was measured as an indicator of NO production using the Griess reagent method. The culture supernatant (100 $\mu$l) was mixed with 100 $\mu$l of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine-HCl] for 10 min, and then the absorbance at 540 nm was measured with a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was

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determined with reference to a sodium nitrite standard curve.

**Nuclear and Whole Cell Extraction plus Western blot Analysis.** For nuclear extraction, the macrophages were plated in 100-mm dishes ($1 \times 10^6$ cells/ml), and treated with berberine (1, 5, and 10 $\mu$M), stimulated with LPS for 1 hour, washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously (Kim et al., 2008c). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 μg/ml aprotinin) and incubated on ice for 15 min. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 s. The nuclei were pelleted by centrifugation at 12,000 × g for 1 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). For whole cells, the macrophages were collected by centrifugation and washed once with PBS. The washed cell pellets were resuspended in extraction lysis buffer PRO-PREP (Intron Biotechnology) and incubated at 4°C for 30 min. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration of the lysates was determined using the Bio-Rad protein assay reagent and according to the manufacturer's instructions. Following separation with 8-12% SDS-polyacrylamide gel electrophoresis, cellular protein from treated and untreated cell extracts was electroblotted onto a PVDF membrane. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for overnight with a primary antibody. Blots were washed four times with Tween 20/Tris-buffered saline (T-TBS) and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. Blots were again washed three times with T-TBS, and then developed by enhanced chemiluminescence

(Amersham Life Science).

**RNA Preparation and Real-time PCR.** Total cellular RNA was isolated using Easy Blue kits (Intron Biotechnology) according to the manufacturer's instructions. From each sample, 100 ng of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT12-18) 0.5 $\mu$g/μl. PCR amplification was performed using the incorporation of SYBR green. Sense and antisense primers for murine *iNOS* were respectively: 5′- ATG GCA ACA TAG GCG GCC ATC ACT -3′ and 5′-GCT GTG TGT CAC AGA AGT CTC ACT C-3′ and the suitable size of synthesized cDNA were 220 bp. Sense and antisense primers for murine *β-actin* were respectively: 5′- ATC ACT ATT GGC AAC GAG CG-3′ and 5′- TCA GCA ATG CCT GGG TAC AT-3′ and the suitable size of synthesized cDNA were 200 bp. Steady-state mRNA levels of *iNOS* and *β-actin* were determined by real-time PCR using the Light Cycler1.5 (TAKARA). Dissociation curve analysis for *iNOS* and *β-actin* oligos showed a single peak for each. Mean Ct values of genes of interest were calculated from triplicate measurements and normalized versus the mean Ct of *β-actin*.

**Immunocytochemistry.** Both macrophages were pretreated without or with berberine (1, 5, or 10 $\mu$M) for 1 hour and then treated with LPS (10 or 1 $\mu$g/ml) in presence or absence of berberine, and then fixed with 100% methanol for 30 min. The cells were then incubated with 0.1% Triton X-100 for 30 min and blocked with 5% normal goat serum (NGS) for 1 hour. Cells were probed with mouse anti-HuR antibody (Santa Cruz, diluted 1:100) overnight at 4°C, followed by goat anti-mouse 594® Alexa conjugated secondary antibody (Invitrogen, diluted 1:100) 1 hour at 24°C, washed with PBS three times and then mounted with mounting solution containing 4',6-diamidino-2-phenylindole (DAPI). HuR subunit was observed with a microscope.

**HuR–RNA Immunoprecipitation Assay.** To determine whether HuR binds directly to iNOS

mRNA in THP-1 and RAW 264.7 cells, we used the RNA-Binding Immunoprecipitation Magma RIP Kit (Millipore). Experiments were performed following exactly the manufacturer's protocol. Briefly, both macrophage cell types were lysed in lysis buffer. The HuR–RNA complexes present in cell extracts were then immunoprecipitated using either the HuR antibody or the negative control normal mouse IgG with protein A/G magnetic beads. The magnetic beads bound complexes were then immobilized using a magnet followed by serial washings to wash out unbound material. Immunoprecipitated RNAs were then extracted and analyzed by real-time PCR for *iNOS* mRNA presence (indicating effective HuR–*iNOS* mRNA binding) again according exactly to the manufacturer's protocol, using the SYBR green kit.

**Animals.** Experiments were conducted under university guidelines from the Committee for Animal Care and Use of Laboratory Animals, College of Pharmacy, Kyung Hee University and according to an approved animal protocol (KHP-2010-10-3). C57BL/6 male mice weighing 20-25 g were purchased from the Orient Bio Inc. The animals were housed 5/cage under constant conditions (temperature: 20 ± 2°C, humidity: 40-60%, light/dark cycle: 12 hours) and were offered normal feed (Labdiet L79), beta chip contact bedding, and purified clean water for the duration of the experimental period. After adaptation for 1 week, mice were randomly re-grouped and treated with sample.

**Septic Shock in Mice.** The C57BL/6 mice were injected intraperitoneally with PBS or LPS (25 mg/kg). Berberine (0.5 or 1 mg/kg, dissolved in DMSO) was injected intraperitoneally for 1 hour before LPS injection. Survival was monitored for 48 hours. Blood was collected 6 hours after LPS injection and allowed to clot at room temperature. Serum was separated by centrifugation, and stored at -80°C until analysis. Liver samples were obtained 6 hours after LPS challenge and fixed for hematoxylin and eosin staining. Immunohistochemical detection

of iNOS was carried out using the avidin-biotin-DAB complex method on paraffin sections. Briefly, after an overnight incubation at 4°C with primary monoclonal antibodies against iNOS (Santa Cruz Biotechnology, diluted 1:50), a biotin-conjugated goat anti-rabbit secondary antibody (Vector Laboratories, diluted 1:250), and subsequently streptavidin conjugated with horseradish peroxidase (Vector Laboratories, diluted 1:250) was applied. DAB peroxidase substrate (Vector Laboratories) was utilized for visualization, and the specimens were counterstained with hemotoxylin (Sigma Chemical).

**Statistical Analysis.** Results are expressed as the mean ± standard deviations (SDs) of triplicate experiments. Statistically significant values were compared using a nonparametric multiple comparisons test (Kruskal-Wallis test) followed by Dunn's test. Statistical significance was set at $p< 0.05$.

## Results

**Berberine Inhibited LPS-induced NO Production and iNOS Expression in Macrophages.** Because berberine was previously found to inhibit LPS-induced NO production and iNOS protein expression in RAW 264.7 macrophages (Kim et al., 2007), we initially investigated whether the inhibitory effects of berberine were due to the suppression of the enzyme activity or expression of iNOS in THP-1 and RAW 264.7 macrophages. The cells were pretreated with berberine (1, 5, or 10 $\mu$M) for 1 hour and then treated with LPS (10 μg/ml for THP-1 or 1 μg/ml for RAW 264.7 cells) for 24 hours. As previously demonstrated (Kim et al., 2007), we observed that berberine significantly inhibited LPS-induced NO production and iNOS protein expression in a concentration- and time-dependent manner (Figs. 1A and 1B). Next, we investigated whether the enzyme activity of iNOS was affected by berberine. The enzyme-based iNOS-catalytic activity assay showed that berberine did not reduce the

production of NO (Suppl. Fig. 1). In addition, we confirmed the inhibitory effects of berberine were not caused by a nonspecific cytotoxic effect as berberine had no effect on cell viability in an MTT assay with concentrations from 1 to 10 $\mu$M (Suppl. Fig. 2).

**Berberine Decreased iNOS mRNA Stability in LPS-treated Macrophages.** Real-time PCR was used to investigate the effect of berberine on LPS-induced iNOS mRNA levels at four different time points (6, 12, 18, and 24 hours after LPS stimulation). Although berberine (10 $\mu$M) had no obvious effect on LPS-induced *iNOS* mRNA levels at 6 hours, LPS-induced iNOS mRNA levels were significantly reduced at 12, 18, and 24 hours by berberine treatment in THP-1 and RAW 264.7 macrophages (Fig. 2A). Furthermore, berberine had no effect on the transcriptional activities of iNOS gene, as determined by a promoter reporter gene assay and a nuclear run-off assay (Suppl. Fig. 3). Based on these results, we hypothesized that berberine might decrease iNOS expression through decreasing *iNOS* mRNA stability.

To determine whether berberine reduces LPS-induced *iNOS* mRNA stability, both macrophage cell types were pretreated with LPS for 12 hours, stimulated with berberine for 1 hour, and then incubated with actinomycin D (an inhibitor of gene transcription). Cells were then incubated further for 0, 1, 2, 4, 6, or 12 hours and total mRNA was extracted. Real-time PCR analysis showed that when transcription was blocked by actinomycin D, levels of *iNOS* mRNA decreased more rapidly in berberine plus LPS-treated cells than in LPS-treated cells (Fig. 2B). More specifically, the half-lives of *iNOS* mRNA reduced from 6 hours and >12 hours to 2.2 hours and 6 hours, respectively, when both macrophage cell types were treated with berberine.

**HuR Knockdown Abrogated Berberine-induced iNOS mRNA Instability in LPS-treated Macrophages.** It has been reported that cytokine mixture treatment enhanced binding between HuR and human iNOS mRNA, and stabilized iNOS mRNA (Linker et al., 2005). Therefore, we analyzed the role of HuR in berberine-induced iNOS mRNA instability in macrophages. To determine whether HuR participates in the regulation of iNOS mRNA stability by berberine, we undertook the siRNA-mediated knockdown of HuR in THP-1 and RAW 264.7 macrophages. Transfection of HuR-specific siRNAs into both macrophage cell types substantially downregulated HuR protein levels (by 50% ± 10.5% and 45% ± 9.3%, respectively, Fig. 3A). Furthermore, the suppressive effects of berberine on LPS-induced iNOS protein expression and NO production were significantly diminished by HuR knockdown (Figs. 3A and 3B). As shown in Fig. 3C, iNOS mRNA stability was significantly reduced by berberine in LPS-treated control siRNA-transfected macrophages, whereas berberine had little effect on *iNOS* mRNA stability in LPS-treated HuR knockdown macrophages. These results demonstrate that HuR plays an essential role in reducing *iNOS* mRNA stability by berberine in LPS-treated macrophages in both cases.

**Berberine Inhibited the Cytoplasmic Translocation and Phosphorylation of HuR in LPS-treated Macrophages.** Since the cytoplasmic localization of HuR is associated with the nuclear export and stabilization of its mRNA targets (Brennan and Steitz, 2001), we first investigated the subcellular localization of HuR under conditions of iNOS induction. Cells were pretreated with berberine (1, 5, or 10 $\mu$M) and then induced with LPS for 6 hours. Pretreatment with berberine was found to attenuate LPS-induced cytoplasmic HuR translocation significantly by Western blotting and immunocytochemistry (Figs. 4A and 4B). As changes in HuR translocation have been attributed to the phosphorylation of HuR by a

various kinases (Kim and Gorospe, 2008), we investigated whether the LPS-induced phosphorylation of HuR (Ser 221) was inhibited by berberine. The phosphorylation of HuR (Ser 221) was found to be concentration-dependently inhibited by berberine, but Western blot analysis using whole cell lysates revealed that the total amount of HuR was unchanged by LPS or LPS plus berberine (Fig. 4C). In addition, since HuR has been shown to bind and stabilize *iNOS* mRNA (Rodriguez-Pascual et al., 2000), we examined the effect of berberine (1, 5, or 10 $\mu$M) on the binding of *iNOS* mRNA and HuR in LPS-treated in both macrophage cell types. Treatment with berberine concentration-dependently inhibited LPS-induced binding between HuR and *iNOS* mRNA in both macrophage cell types (Fig. 4D).

**Berberine Inhibited the LPS-induced Nuclear Translocation of PKC$\delta$.** It has previously been shown that PKCδ mediates the nucleocytoplasmic shuttling and binding of HuR to target ARE-mRNAs via direct tandem phosphorylation of HuR at Ser 221 and Ser 318 in renal mesangial cells, and thus, increasing the stability and translation of those mRNAs (Doller et al., 2010). To confirm the role of PKC$\delta$ on HuR regulation in macrophages, cells were treated with Rottlerin, a PKC$\delta$ inhibitor. As shown in Figs. 5A and 5B, Rottlerin significantly inhibited LPS-induced cytoplasmic translocation and phosphorylation of HuR. We investigated the impact of knockdown of PKCδ in iNOS expression using siRNA strategy. The efficiency in suppressing expression of PKCδ was confirmed by Western blot analysis. The siRNA-mediated knockdown of PKCδ in macrophages decreased LPS-induced *iNOS* mRNA expression (Suppl. Fig. 4). Accordingly, we evaluated whether PKC$\delta$ was involved in berberine-induced HuR shuttling in LPS-treated macrophages. Stimulation of LPS-treated for both macrophage cell types with berberine resulted in strong reductions in nuclear PKCδ

levels and concomitant marked cytoplasmic PKC$\delta$ accumulation (Fig. 5C), indicating that nuclear PKC$\delta$ reduction by berberine regulates the mRNA binding and shuttling of HuR.

**Berberine Protected Mice from Endotoxemic Shock.** In view of the ability of berberine to attenuate LPS-induced iNOS protein expression and NO production by inhibiting the cytoplasmic translocation of HuR in macrophages, we examined the effect of berberine in a mouse model of sepsis. LPS injection (25 mg/kg, i.p.) markedly increased the serum level of NO, but pretreatment with berberine (1 mg/kg, i.p.) significantly decreased LPS-induced NO production (Suppl. Fig. 5). Furthermore, berberine inhibited iNOS protein expression in liver during sepsis by Western blot analysis and IHC staining (Figs. 6A and 6B) and berberine consistently prevented the translocation of HuR from nuclei to cytosol in the livers of endotoxemic mice (Fig. 6C). In peritoneal macrophages from endotoxemia mice, it was found that berberine suppressed cytosolic translocation of HuR induced by LPS (Fig. 6D). Lastly, whereas LPS injection resulted in 80% mortality at 36 hours post injection, pretreatment with berberine reduced this to 30% and 10% at doses of 0.5 and 1 mg/kg at 48 hours, respectively (Fig. 6E).

## Discussion

Mediators of inflammation induced by macrophages are critical for a variety of human inflammatory disorders and autoimmune diseases. LPS binds to toll-like receptor (TLR) 4 and initiates a signal transduction cascade, which in part, leads to transcription of pro-inflammatory genes, such as iNOS, and subsequently produces NO (Palsson-McDermott and O'Neill, 2004). In addition, it was demonstrated that iNOS was capable of producing superoxide and iNOS-mediated formation of superoxide/peroxynitrite contributes to

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pathologic injury by nitrative protein modification (nitrative stress) (Xia and Zweier, 1997; Roberts et al., 2009).

The anti-inflammatory properties of berberine are well known in various cell types and animal models. In LPS-induced macrophages, it was reported that berberine inhibited iNOS-mediated NO and nicotinamide adenine dinucleotide phosphate-oxidases (NOXes)-mediated superoxide production (Kim et al., 2007). Consistent with previous findings, we found that berberine significantly inhibited LPS-induced iNOS expression and NO production in THP-1 and RAW 264.7 macrophages. To evaluate the protective effects of berberine on LPS-induced oxidative and nitrative stress, we analyzed LPS-induced formation of superoxide and of 3-nitrotyrosine-modified proteins, a marker for peroxynitrite. Berberine decreased LPS-induced superoxide production and 3-nitrotyrosine formation in RAW 264.7 macrophages (Suppl. Fig. 6), suggesting that the anti-inflammatory capability of berberine is partly mediated through its ability to downregulate iNOS-related NO and/or peroxynitrite production.

Induction of the *iNOS* gene is regulated by the transactivation of *iNOS* promoter through transcription factors, such as NF-κB and AP-1, and by posttranscriptional mechanisms, such as the stabilization of *iNOS* mRNA (Kleinert et al., 2004). A notable finding in this study was that berberine suppressed LPS-induced iNOS protein expression by destabilizing *iNOS* mRNA. This conclusion was supported by transcription experiments using *iNOS* promoter-luciferase constructs and nuclear run-off assay, in which berberine did not inhibit the LPS-induced *iNOS* transcriptional activation. Since berberine had no effect on the transcription of iNOS mRNA, the acceleration of mRNA degradation was the other reasonable explanation for the lowered *iNOS* mRNA levels in the cells treated by berberine. Also, berberine had no effect on LPS-induced NF-*κ*B-dependent luciferase activity, p65

translocation, and I$\kappa$B$\alpha$ degradation (Suppl. Fig. 7), indicating that berberine did not affect the activity of *iNOS* promoter transactivation through NF-κB.

To assess the possibility that berberine-induced iNOS protein suppression was related to iNOS translational stability, we examined the effect of berberine on iNOS protein stability in cycloheximide (a protein synthesis inhibitor)-treated RAW 264.7 macrophages. Although cycloheximide treatment reduced the LPS-induced iNOS protein expression, treatment with/without berberine did not affect cycloheximide-reduced iNOS protein expression in LPS-treated macrophages (Suppl. Fig. 8), indicating that the reduction of iNOS protein expression by berberine was not mediated by iNOS protein stability.

The regulation of *iNOS* mRNA stability is of general interest as it seems to be a significant mechanism in regulation of iNOS protein expression and NO production. This posttranscriptional mechanism involves interactions between *iNOS* mRNA 3'-UTR (containing AREs) and RNA-BPs, such as HuR, KSRP, TTP, AUF1, and hnRNP I, that result in the modulation of *iNOS* mRNA stability (Rodriguez-Pascual et al., 2000; Kleinert et al., 2004). KSRP is a key negative regulator of iNOS expression by binding to its 3´-UTR (Linker et al., 2005). Another RNA-BP, TTP is involved in the cytokine-induced stabilization of *iNOS* mRNA. TTP does not directly bind to the *iNOS* 3´-UTR; rather, TTP expression is enhanced by cytokine treatment and the TTP-KSRP interactions induce dislodgment of KSRP from the *iNOS* 3'-UTR. Since KSRP and HuR compete for the same binding site in the *iNOS* 3'-UTR, these responses enhance HuR binding to *iNOS* mRNA and markedly stabilize *iNOS* mRNA (Fechir et al., 2005; Linker et al., 2005; Lisi et al., 2011).

It has also been reported that rapamycin (an mTOR kinase inhibitor) decreases *iNOS* mRNA stability by upregulating TTP expression in astrocytes (Lisi et al., 2011). In contrast, in the present study, LPS enhanced TTP protein expression, but berberine pretreatment did

not affect this LPS-induced expression in RAW 264.7 macrophages (data not shown), which suggests that TTP might not mediate the berberine-induced destabilization of *iNOS* mRNA.

Recently, it was reported that iNOS expression was down-regulated in bone marrow derived macrophages from myeloid-specific HuR knock-out mice (*Elavl1*Mø KO), indicating HuR stabilizes *iNOS* mRNA in macrophages *in vivo* (Chang et al., 2013). Accordingly, we focused on HuR as a target of berberine for the destabilization of iNOS mRNA and by using a RNA interference strategy, sought to determine whether berberine could control iNOS mRNA stability via HuR regulation. As expected, HuR gene silencing resulted in significant reduction in LPS-induced iNOS protein expression and NO production, and also in induction of *iNOS* mRNA destabilization. Furthermore, in this study we found that berberine did not affect LPS-induced iNOS mRNA stability, iNOS protein expression, or NO production in HuR gene-silenced macrophages, which suggests that HuR is an essential regulator of *iNOS* mRNA stability by berberine in LPS-treated macrophages. Because it has been demonstrated that numerous inflammation-related genes, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and cyclooxygenase-2 (COX-2), are maintained their mRNA stability by HuR (Liao et al., 2011), we tested the effects of berberine on LPS-induced mRNA stability of TNF-α, IL-6, and COX-2 in macrophages. Interestingly, berberine did not affect their mRNA stability (data not shown).

HuR binds to the two distal AUUUA-elements of the 3'-UTR of human *iNOS* mRNA and is generally localized to the nucleus, but it can translocate to the cytoplasm after cell stimulation and induce mRNA stabilization (Lowenstein and Padalko, 2004; Doller et al., 2014). In the present study, berberine inhibited the LPS-induced cytoplasmic translocation of HuR, and thus reduced its physical interaction with *iNOS* mRNA. Structurally, the nucleocytoplasmic shuttling of HuR relies on a nucleocytoplasmic shuttling sequence (HNS)

spanning residues 205 to 237 and located in the hinge region (HR) between RNA recognition motifs 2 and 3 (RRM2 and 3) (Fan and Steitz, 1998). Importantly, like RRMs, the HR of HuR is a target of different posttranslational modifications (Doller et al., 2010). In contrast to the inducible effects on HuR shuttling observed after the phosphorylation of Ser 221 by PKC$\delta$ or PKC$\alpha$ (Abdelmohsen et al., 2007), phosphorylation of HuR at Ser 202 or Ser 242 by cyclin-dependent kinase 1 (Cdk1) causes HuR to reside in the nucleus, and being prevented from recruitment of its target mRNA encoding anti-apoptotic proteins and enhancing cell survival to the translation machinery (Doller et al., 2008; Kim et al., 2008a; Kim et al., 2008b). Moreover, a single phosphorylation of the distal RRM3 at Ser 318 by PKC$\delta$ modulates angiotensin II-induced HuR binding to ARE-mRNA. Therefore, PKCδ is one of the most significant positive regulators of HuR in terms of increasing cytoplasmic translocation and binding to target mRNA via phosphorylation of Ser 221 and Ser 318 in HuR. We provided that berberine reduced the LPS-induced phosphorylation of HuR at Ser 221 in macrophages. Our studies suggest that the suppression of PKC$\delta$-dependent HuR phosphorylation by berberine is an important mechanism for inhibiting HuR translocation to cytosol and its subsequent binding to iNOS mRNA. These results show that the reduced cytoplasmic translocation of HuR might represent a novel pattern of response to berberine in LPS-treated macrophages and prompted our examination of the signaling pathways involved.

Berberine was reported to function as an antagonist for a number of G-protein coupled receptors, such as $\alpha$2-adrenergic (Hui et al., 1991) and GRP 40 receptor (Rayasam et al., 2010). In support of this, it was reported that berberine induced IL-12 p40 production in macrophages, at least in part, through the $\alpha$2-adrenergic receptor (Kang et al., 2002). On the other hand, berberine protected against LPS-induced intestinal- or lung injury via $\alpha$2-adrenergic receptor-independent mechanisms (Zhang et al., 2008; Li et al., 2011). Hence, the

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role of the $\alpha$2-adrenoceptor or GRP 40 in berberine-induced suppression of PKC$\delta$-HuR activation needs to be examined.

To confirm that berberine also inhibits inflammatory responses *in vivo*, we evaluated the effects of berberine in a model of LPS-induced sepsis. In mice with established endotoxemia induced by LPS, berberine reduced serum levels of NO and increased survival rates, which suggests that the suppressive effect of berberine on HuR-regulated *iNOS* mRNA stability in macrophages had an anti-inflammatory effect in our model of sepsis. Furthermore, we found that berberine inhibited iNOS protein expression, and the cytoplasmic translocation of HuR in livers and macrophages of LPS-induced endotoxemic mice.

In summary, the results presented here show for the first time that berberine-induced reductions in *iNOS* expression and NO production are controlled by posttranscriptional mechanisms involving the modulation of *iNOS* mRNA stability, caused by the reduced cytoplasmic HuR translocation.

## Authorship Contributions

Participated in research design: H.E. Choi, Shin, J.H. Choi, Baek, and Lee

Conducted experiments: H.E. Choi, Shin and Seo

Contributed new reagents or analytic tools:

Performed data analysis: H.E. Choi, and Shin

Wrote or contributed to the writing of the manuscript: H.E. Choi, Shin, and Lee

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## Footnotes

1. J.S. Shin and H. E. Choi contributed equally to this work as co-first authors.

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## Figure Legends

**Fig. 1.** Effects of berberine on LPS-induced NO production and iNOS protein expression in THP-1 and RAW 264.7 macrophages (A) Both macrophage types were pretreated with different concentrations (1, 5, or 10 $\mu$M) of berberine for 1 hour, then with LPS (10 or 1 $\mu$g/ml), and incubated for 24 hours. NO production in culture media was quantified using a Griess reaction assay. Lysates were prepared from control, LPS alone, or berberine (1, 5, or 10 $\mu$M) plus LPS treated cells for 24 hours. Total cellular proteins were separated by SDS-PAGE, transferred to PVDF membranes, and detected with iNOS antibodies. (B) Both macrophage types were pretreated with berberine (10 $\mu$M) for 1 hour, then with LPS (10 or 1 $\mu$g/ml), and incubated for 12, 18 or 24 hours. NO production in culture media was quantified using a Griess reaction assay. Lysates were prepared from control, 12, 18, or 24 hours LPS alone, or berberine (10 $\mu$M) plus LPS-treated cells. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with iNOS antibodies. Experiments were repeated three times and similar results were obtained. Values are means ± SDs; $n$=3. $^{\#}$ $p < 0.05$ versus untreated controls. $^{**}$ $p < 0.01$, $^{***}$ $p< 0.001$ versus LPS-treated cells

**Fig. 2.** Effects of berberine on LPS-induced iNOS mRNA levels and mRNA stability in THP-1 and RAW 264.7 macrophages (A) Both macrophage types were pretreated with berberine (10 $\mu$M) for 1 hour, then with LPS (10 or 1 $\mu$g/ml), and incubated for 6, 12, 18 or 24 hours. iNOS mRNA levels at 24 hours of LPS was set to 100% iNOS expression, and relative percentages were calculated with respect to 100% LPS control group at 24 hours. (B) For the real-time PCR analysis of iNOS, total RNA was prepared from both macrophage cell types, pretreated with LPS for 12 hours, then stimulated with berberine (10 $\mu$M) for 1 hour, and

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incubated with actinomycin D (1 $\mu$g/ml) for 0, 1, 2, 4, 6, or 12 hours. iNOS mRNA value for only LPS treated group without actinomycin D (0 hour) was set as 100 % iNOS mRNA, and the relative percentages were calculated with respect to the 100% LPS control at other time points. The experiments were repeated three times and similar results were obtained. Values are means ± SDs; $n$=3; $^{\#}$ $p < 0.05$ versus untreated controls. $^{**}$ $p < 0.01$, $^{***}$ $p< 0.001$ versus LPS-treated cells

**Fig. 3.** Effects of HuR siRNA on the berberine-induced reduction of iNOS protein expression, NO production, and iNOS mRNA stability in LPS-treated THP-1 and RAW 264.7 macrophages (A) Both macrophage types were transfected with HuR or Con siRNA (1 $\mu$M, 24 hours). Lysates were prepared from control, 24 hours LPS (10 or 1 $\mu$g/ml) alone or berberine (10 $\mu$M) plus LPS treated cells. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with anti-iNOS antibodies. (B) Transfected or control macrophages were pretreated with berberine (10 $\mu$M) for 1 hour and then with LPS (10 or 1 $\mu$g/ml) for 24 hours. NO levels in culture media were quantified using a Griess reaction assay. (C) Total RNA was prepared for the real-time PCR analysis of iNOS from transfected or control macrophages pretreated with LPS (10 or 1 $\mu$g/ml) for 12 hours and then stimulated with berberine (10 $\mu$M) for 1 hour, and incubated with actinomycin D (1 $\mu$g/ml) for 0, 1, 2, 4, 6, or 12 hours. iNOS mRNA value of only LPS treated group without actinomycin D (0 hour) was set as100 % iNOS mRNA, and the relative percentages were calculated with respect to the 100% LPS control at other time points. The experiments were repeated three times and similar results were obtained. Values are the means ± SDs; $n$=3. $^{\#}$ $p < 0.05$ versus untreated controls. $^{***}$ $p< 0.001$ versus LPS-treated Con siRNA cells

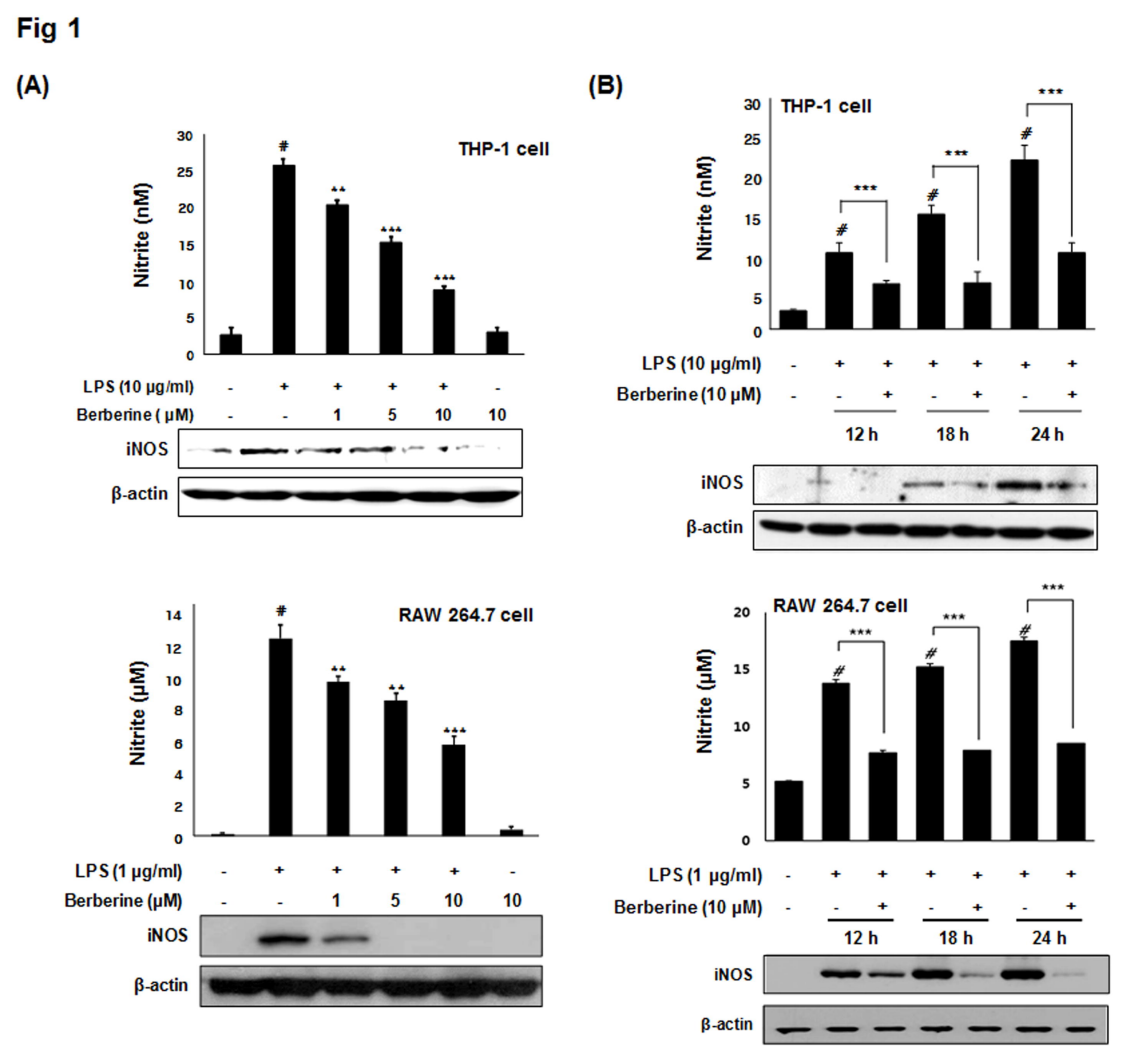
**Fig. 4.** Effects of berberine on LPS-induced HuR translocation, its phosphorylation, and binding to iNOS mRNA in THP-1 and RAW 264.7 macrophages (A) Both macrophage types were pretreated with/without the indicated concentrations of berberine for 1 hour and then stimulated with LPS (10 or 1 $\mu$g/ml) for 6 hours. Nuclear (N) and cytoplasmic (C) extracts were isolated, and levels of HuR in each fraction were determined by Western blotting. PARP-1 and $\alpha$-tubulin were used as internal controls. (B) Macrophages were pre-incubated with berberine (10 $\mu$M) for 1 hour and then LPS treated (10 or 1 $\mu$g/ml) for 6 hours. The translocation of HuR from nucleus to cytoplasm was determined by co-localization of red dye bound to HuR with blue DAPI stained nuclei. (C) Lysates were prepared from control, 4 h LPS (10 or 1 $\mu$g/ml) alone or berberine (1, 5, or 10 $\mu$M) plus LPS treated cells. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with specific antibodies. (D) Macrophages were pretreated with different concentrations (1, 5, or 10 $\mu$M) of berberine for 1 hour and then incubated with LPS (10 or 1 $\mu$g/ml) for 4 hours. Whole cell extracts were prepared for the Western blotting of HuR using specific anti-HuR monoclonal antibodies. HuR binding to iNOS mRNA was determined as described in the *Materials and Methods* section. mRNA value of LPS-treated group was set to represent 100% iNOS mRNA/HuR protein binding, and the relative percentages was calculated from the 100% LPS control group. The experiments were repeated three times and similar results were obtained. Values are the means ± SDs; $n$=3. $^{\#}p< 0.05$ versus untreated controls; $^{**}p< 0.01$, $^{***}p< 0.001$ versus LPS-treated cells

**Fig. 5.** Effects of berberine on LPS-induced nuclear translocation of PKC$\delta$ in THP-1 and RAW 264.7 macrophages. Both macrophage types were pretreated with berberine (1, 5, or 10 $\mu$M) for 1 hour, then with LPS and incubated for 1 hour. Nuclear (N) and cytosolic C.

extracts were isolated, and levels of PKC$\delta$ in each fraction were determined by Western blotting. PARP-1 and $\alpha$-tubulin were used as internal controls. The experiments were repeated three times and similar results were obtained.

**Fig. 6.** Effects of berberine on iNOS protein expression, HuR shuttling, and the survival rates in LPS-induced septic shock mice. Different groups of mice ($n$=10) were treated with vehicle (DMSO), vehicle plus LPS (25 mg/kg, i.p.), LPS plus berberine (0.5 or 1 mg/kg at 1 hour before LPS injection), or berberine-only (1 mg/kg). (A and B) Liver lysates were analyzed by Western blotting using antibodies against iNOS. Liver samples were obtained 6 hours after LPS challenge and fixed for hematoxylin and eosin staining. Immunohistochemical detection of iNOS was carried out using the avidin-biotin-DAB complex method on paraffin sections. (C and D) Extracts were prepared from the liver (C) or peritoneal macrophages (D) for the Western blotting of HuR using specific anti-HuR monoclonal antibodies. PARP and α-tubulin were used as internal controls. (E) Survival rates were measured every 12 hours throughout this experiment. The experiments were repeated three times and similar results were obtained. Values are means ± SDs ($n$=10). $^{\#}$ $p < 0.05$ versus untreated controls.

Fig 1


(A)
THP-1 cell
Nitrite (nM)
LPS (10 μg/ml)
Berberine ( μM)
iNOS
β-actin
RAW 264.7 cell
Nitrite (μM)
LPS (1 μg/ml)
Berberine (μM)
(B)
THP-1 cell
Nitrite (nM)
LPS (10 μg/ml)
Berberine (10 μM)
12 h
18 h
24 h
iNOS
β-actin
RAW 264.7 cell
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12 h
18 h
24 h
iNOS
β-actin

Fig 2

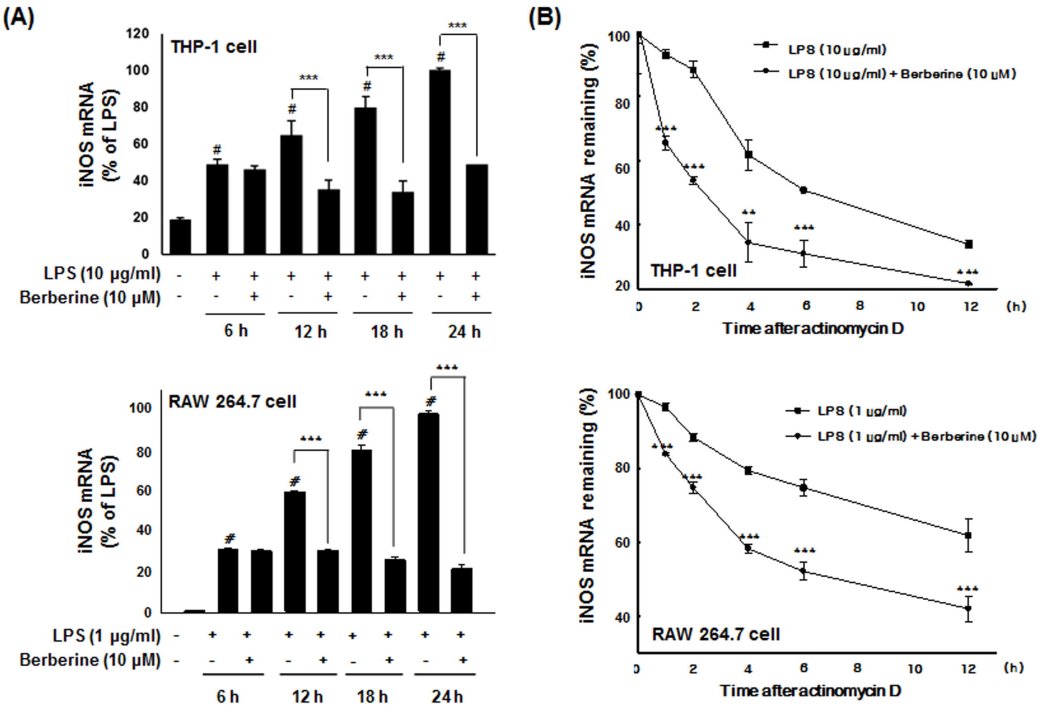

(A)
THP-1 cell
iNOS mRNA (% of LPS)
LPS (10 μg/ml)
Berberine (10 μM)
6 h
12 h
18 h
24 h
RAW 264.7 cell
LPS (1 μg/ml)
(B)
LPS (10 μg/ml)
LPS (10 μg/ml) + Berberine (10 μM)
iNOS mRNA remaining (%)
Time after actinomycin D
LPS (1 μg/ml)
LPS (1 μg/ml) + Berberine (10 μM)

Fig 3

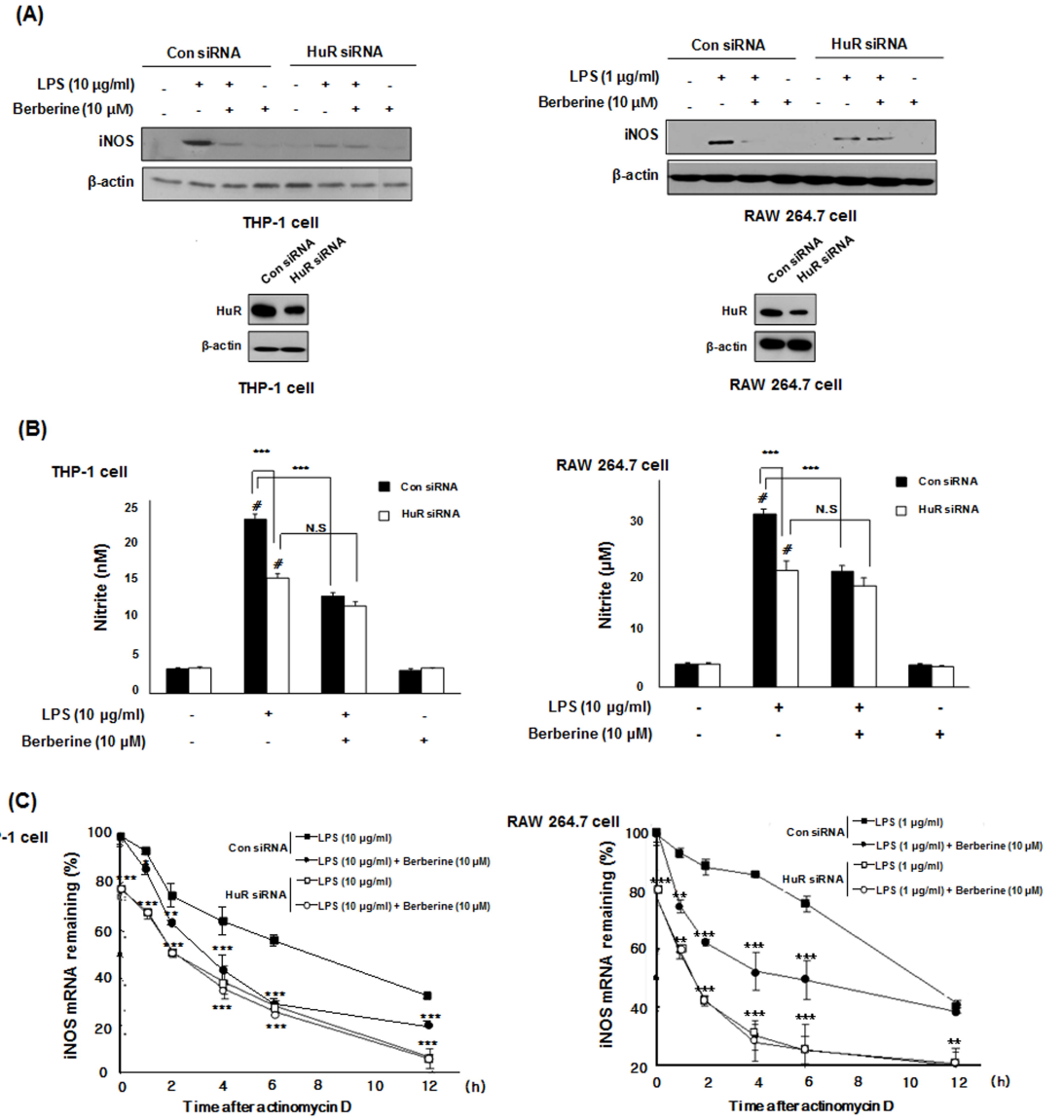

(A)
Con siRNA
HuR siRNA
LPS (10 μg/ml)
Berberine (10 μM)
iNOS
β-actin
THP-1 cell
LPS (1 μg/ml)
RAW 264.7 cell
HuR
(B)
Nitrite (nM)
Nitrite (μM)
N.S
(C)
iNOS mRNA remaining (%)
Time after actinomycin D
(h)
LPS (10 μg/ml)
LPS (10 μg/ml) + Berberine (10 μM)
LPS (1 μg/ml)
LPS (1 μg/ml) + Berberine (10 μM)

Fig 4

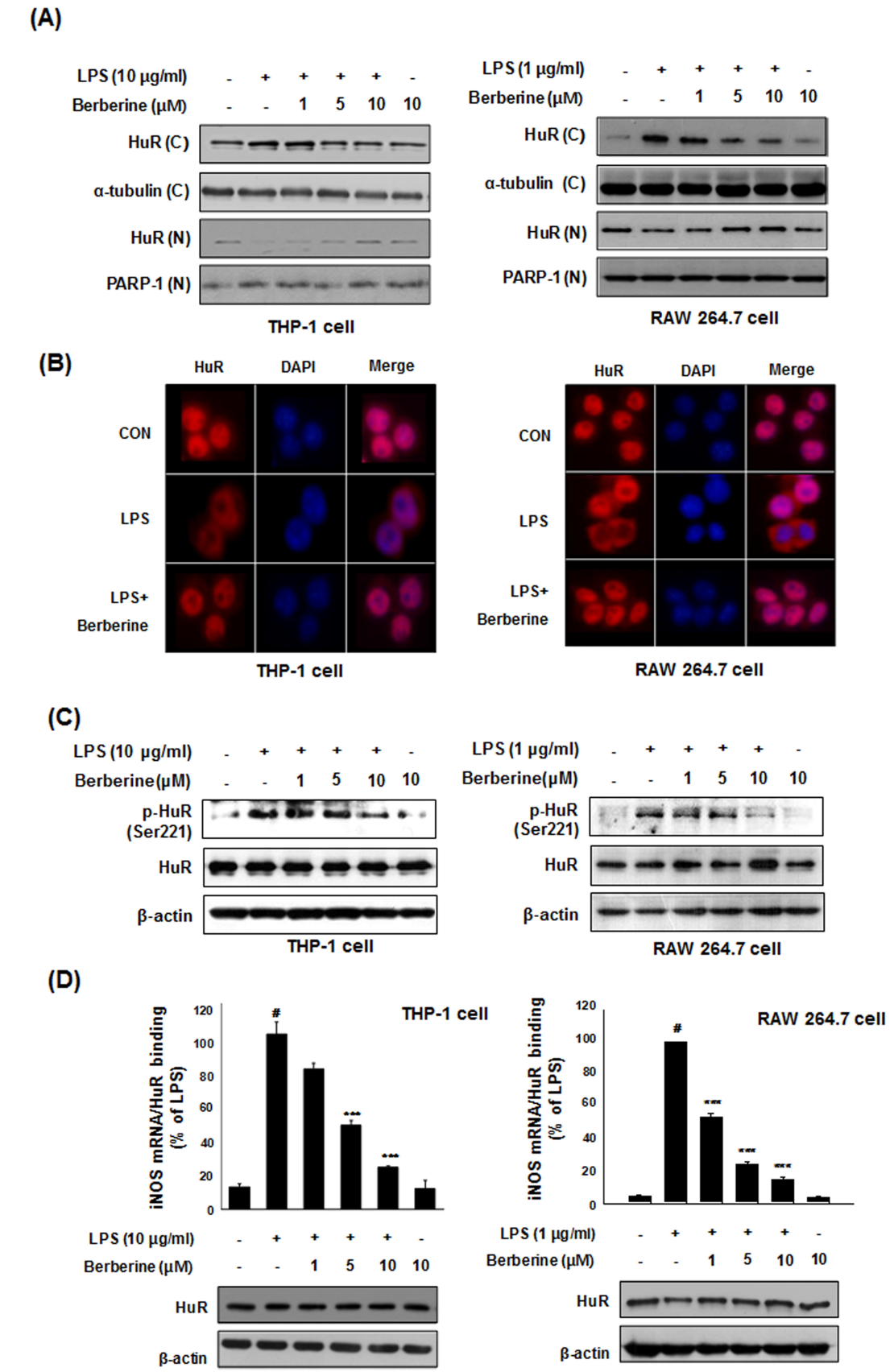

(A)
LPS (10 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
HuR (C)
α-tubulin (C)
HuR (N)
PARP-1 (N)
THP-1 cell
LPS (1 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
HuR (C)
α-tubulin (C)
HuR (N)
PARP-1 (N)
RAW 264.7 cell
(B)
HuR
DAPI
Merge
CON
LPS
LPS+ Berberine
THP-1 cell
HuR
DAPI
Merge
CON
LPS
LPS+ Berberine
RAW 264.7 cell
(C)
LPS (10 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
p-HuR (Ser221)
HuR
β-actin
THP-1 cell
LPS (1 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
p-HuR (Ser221)
HuR
β-actin
RAW 264.7 cell
(D)
iNOS mRNA/HuR binding (% of LPS)
THP-1 cell
RAW 264.7 cell
LPS (10 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
HuR
β-actin
LPS (1 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
HuR
β-actin

Fig 5

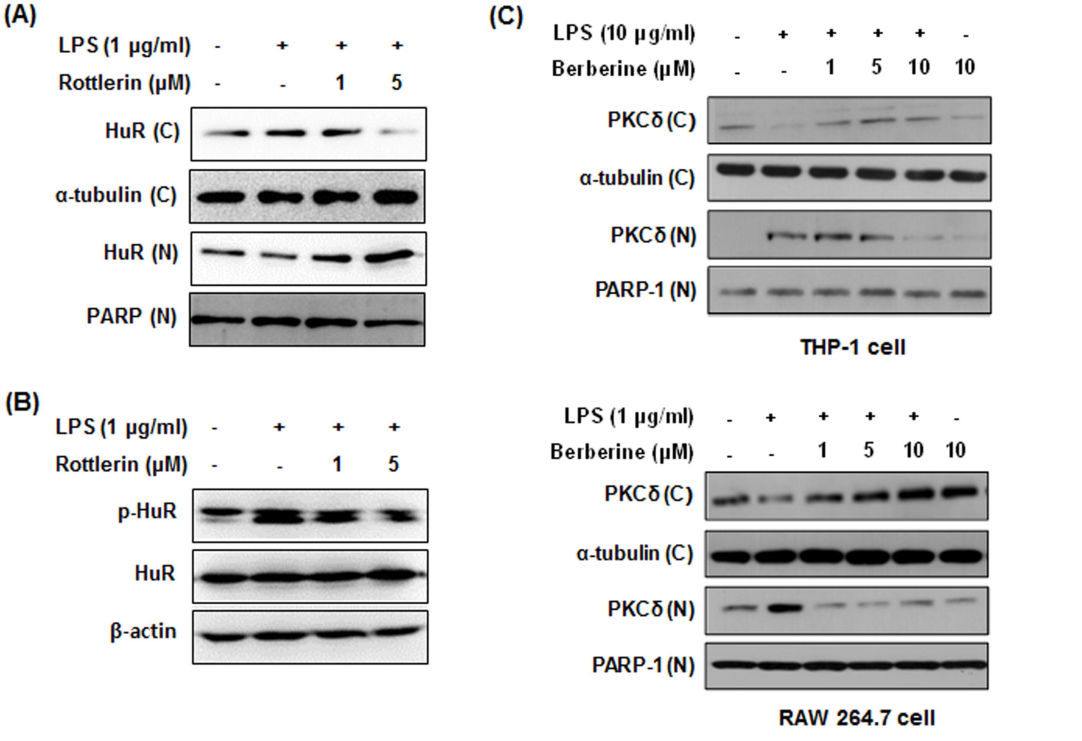
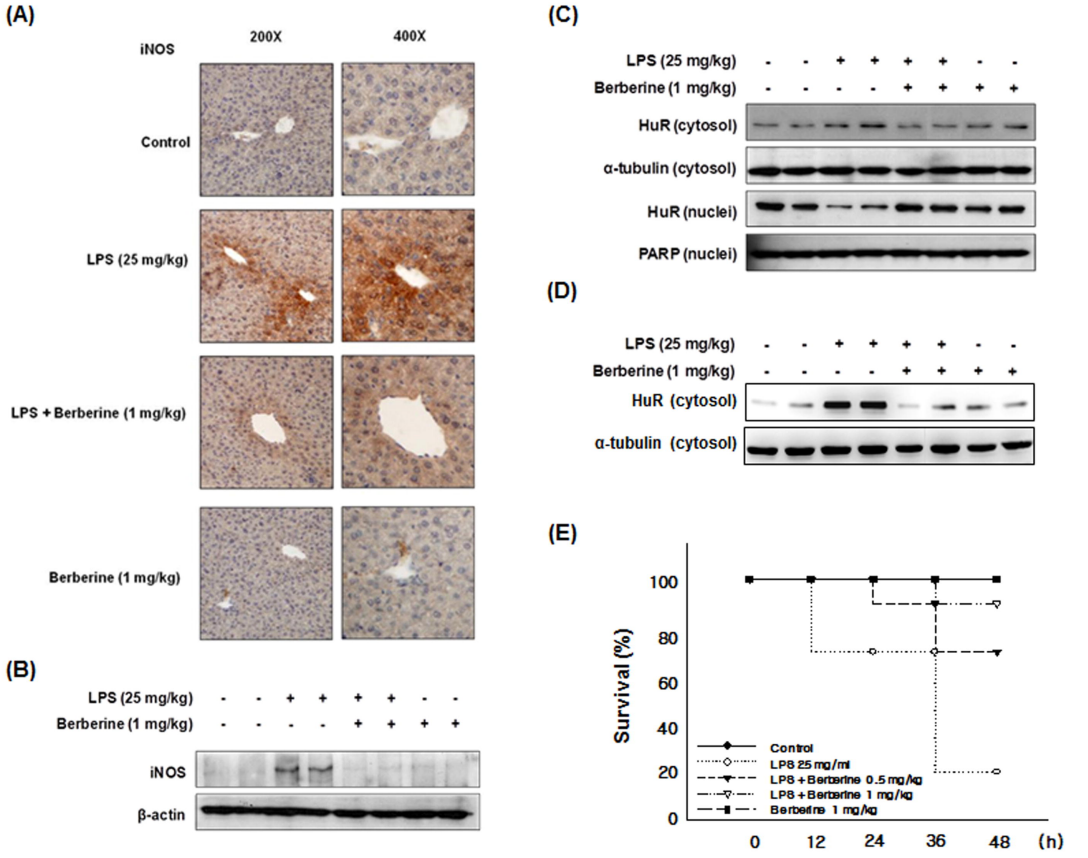

(A)
LPS (1 μg/ml) - + + +
Rottlerin (μM) - - 1 5
HuR (C)
α-tubulin (C)
HuR (N)
PARP (N)
(B)
LPS (1 μg/ml) - + + +
Rottlerin (μM) - - 1 5
p-HuR
HuR
β-actin
(C)
LPS (10 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
PKCδ (C)
α-tubulin (C)
PKCδ (N)
PARP-1 (N)
THP-1 cell
LPS (1 μg/ml) - + + + + -
Berberine (μM) - - 1 5 10 10
PKCδ (C)
α-tubulin (C)
PKCδ (N)
PARP-1 (N)
RAW 264.7 cell

Fig 6


(A)
iNOS
200X
400X
Control
LPS (25 mg/kg)
LPS + Berberine (1 mg/kg)
Berberine (1 mg/kg)
(B)
LPS (25 mg/kg) - - + + + + - -
Berberine (1 mg/kg) - - - - + + + +
iNOS
β-actin
(C)
LPS (25 mg/kg) - - + + + + - -
Berberine (1 mg/kg) - - - - + + + +
HuR (cytosol)
α-tubulin (cytosol)
HuR (nuclei)
PARP (nuclei)
(D)
LPS (25 mg/kg) - - + + + + - -
Berberine (1 mg/kg) - - - - + + + +
HuR (cytosol)
α-tubulin (cytosol)
(E)
Survival (%)
100
80
60
40
20
0
0 12 24 36 48 (h)
Control
LPS 25 mg/ml
LPS + Berberine 0.5 mg/kg
LPS + Berberine 1 mg/kg
Berberine 1 mg/kg