Berberine Decreased Inducible Nitric Oxide Synthase mRNA Stability through Negative Regulation of Human Antigen R in Lipopolysaccharide-Induced Macrophages

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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, we investigated the inhibitory effects of berberine and the molecular mechanisms involved in lipopolysaccharide (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 NO synthase (iNOS) protein expression, but it 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 and 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 resulting from inhibition of the cytoplasmic translocation of HuR.

Introduction

Berberine, 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 potential in clinical applications because of its diverse pharmacologic properties, such as antimicrobial, antidiabetic, antihyperlipidemic, anti-inflammatory, antioxidant, neuronal protective, and anticancer effects (Fan et al., 2015). Berberine has been reported to have diverse and complex mechanisms for its pharmacologic actions. Some pharmacologic activities of berberine relate to stabilization of mRNAs, such as low-density lipoprotein receptor, tissue factor, and retinoblastoma protein (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 proinflammatory mediators, acute-phase proteins, and infiltration of inflammatory cells. It was shown that the anti-inflammatory activities of berberine were strongly linked to its negative regulatory activity on proinflammatory gene expression via transcription factor inactivation, such as nuclear factor-κB (NF-κB) and activator protein 1 (Li et al., 2014). Recent data, however, suggest that dysregulated post-transcriptional regulation of proinflammatory 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 proinflammatory 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 mediated largely 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, antibacterial, and antitumoral mechanisms, and, if uncontrolled, it can also be harmful to the host (Iadecola et al., 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 pathophysiology 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 (also named hnRNP D) (Kuo et al., 2004), and T cell–restricted intracellular antigen-related protein (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 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 physiologic and pathophysiologic processes, such as cell growth, differentiation, and inflammation. The stable overexpression of HuR in human colon adenocarcinoma DLD-1 cells resulted 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 lipopolysaccharide (LPS)-induced iNOS protein expression by reducing its mRNA stability in an HuR-dependent manner and identified the key mechanism responsible for its anti-inflammatory effects.

Materials and Methods

Chemicals. Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin, and streptomycin were obtained from Life Technologies Inc. (Carlsbad, CA). The antibodies against iNOS, HuR, protein kinase C δ (PKCδ), α-tubulin, and poly(ADP ribose)polymerase were purchased from Santa Cruz Biotechnology Inc. The RNA extraction kit was purchased from Intron Biotechnology (Gyeonggi-do, Korea). Magn RIP RNA-Binding Protein Immunoprecipitation Kit was purchased from Millipore (Billerica, MA). iNOS and β-actin oligonucleotide primers were purchased from Bioneer (Santa Fe, NM). Berberine chloride, actinomycin D, phenolmethylsulphonic acid (PMSF), iodoacetamide, N-[2-iminoethyl]lysine (L-NIL), actin, and GAPDH were purchased from Sigma Chemical Co (St. Louis, MO).

p-HuR Antibody Production. The phospho-specific rabbit polyclonal antibodies were raised against a synthetic peptide [sequence: C-RRpSPMGVDMHMSG] coupled to KLH (Pierce Biotechnology, Rockford, IL); subsequently, the coupled peptides were injected into rabbits four times. Peptide-KLH conjugate (1 mg) was dissolved in 500 μl phosphate-buffered saline (PBS) emulsified with 500 μl of complete (first injection) or incomplete (subsequent injections) Freund’s adjuvant. Female rabbits were injected subcutaneously at 4 weeks, 2 weeks, and 2-week intervals for 3 months. One week after the last immunization, 50 ml of blood was taken, and the centrifuged serum was stored at 20°C until use. Specificity of peptide antibodies was analyzed using indirect enzyme-linked immunosorbent assay methods. The antibodies were first affinity-purified on a column containing SulfaLink beads (Pierce Biotechnology) cross-linked to the phosphorylated peptides, eluted with 1 M glycine-HCl, pH 2.7, and immediately neutralized with an equal volume of 5% NH4O3. Bovine serum albumin (BSA, 1%) was then added, and the protein mixture was dialyzed overnight against PBS. Finally, the diazoyed antibody sample was run through a column containing SulfaLink beads (Pierce) cross-linked to the equivalent nonphosphorylated peptides, and the unbound fraction was 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 (Seoul, Korea). Cells were grown in RPMI and Dulbecco’s modified Eagle’s medium at 37°C containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO2. Human leukemia (THP-1) cells were differentiated to macrophages by treating with PMA (100 nM) for 48 hours, as previously described (Daigneault et al., 2010). Cells were incubated with berberine at various concentrations (1, 5, or 10 μM) or with positive-control chemicals and stimulated with LPS (1 or 10 μg/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 μl) was mixed with 100 μl 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 minutes, 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 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 × 106 cells/ml), treated with berberine (1, 5, and 10 μM), stimulated with LPS for 1 hour, washed once with PBS, resuspended in a cold culture medium, was measured as an indicator of NO production using the Griess reagent method. The culture supernatant (100 μl) was mixed with 100 μl 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 minutes, 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 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 × 106 cells/ml), treated with berberine (1, 5, and 10 μM), stimulated with LPS for 1 hour, washed once with PBS, resuspended in 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 MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 μg/ml aprotinin) and incubated on ice for 15 minutes. Cells were then lysed by adding 0.1% Nonidet P-40 and vortexed vigorously for 10 seconds. The nuclei were pelleted by centrifugation at 12,000 × g for 1 minute at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM diethiothreitol, 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 minutes. 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. After separation with 8%–12% SDS-PAGE, cellular protein from treated and untreated cell extracts was electroblotted onto a polyvinylidene fluoride (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 and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX) for 1 hour at room temperature. Blots were again washed three times with Tris-buffered saline and then developed by enhanced chemiluminescence (Amersham Life Sciences, Little Chalfont, UK).
RNA Preparation and Real-Time Polymerase Chain Reaction. 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 using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT12-18) 0.5 μg/μl. Polymerase chain reaction (PCR) amplification was performed with 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 CCA AGA AGT CTC ACT C-3’ and the suitable size of synthesized cDNA was 220 bp. Sense and antisense primers for murine β-actin were respectively: 5’-ATC ACT ATT GGC AAC GAG CG-3’ and 5’-TCA GGA ATG CCT GGG TAC AT-3’; and the suitable size of synthesized cDNA was 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 μM) for 1 hour and then treated with LPS (10 or 1 μg/ml) in presence or absence of berberine, and then fixed with 100% methanol for 30 minutes. The cells were then incubated with 0.1% Triton X-100 for 30 minute 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 (DAP). 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 the presence of iNOS mRNA (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. (Gyeyonggi, Korea). The animals were housed five per 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 regrouped and treated with sample.

Septic Shock in Mice. The C57BL/6 mice were injected i.p. with PBS or LPS (25 mg/kg). Berberine (0.5 or 1 mg/kg, dissolved in DMSO) was injected i.p. 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 H&E 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, Burlingame, CA) diluted 1:250, and subsequently streptavadin -conjugated with horseradish peroxidase (Vector Laboratories, diluted 1:250) was applied. DAB peroxidase substrate (Vector Laboratories) was used for visualization, and the specimens were counterstained with hemotoxylin (Sigma Chemical).

Statistical Analysis. Results are expressed as the mean ± SD or 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 μM) 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 (Fig. 1, A 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 (Supplemental Fig. 1). In addition, we confirmed that 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 μM (Supplemental 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 μM) 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 runoff assay (Supplemental 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
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% ± 6 ± 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 (Fig. 3, A and B). 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 μM) and then induced with LPS for 6 hours. Pretreatment with berberine attenuated LPS-induced cytoplasmic HuR translocation significantly by Western blotting and immunocytochemistry (Fig. 4, A and B). As changes in HuR translocation have been attributed to the phosphorylation of HuR by 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 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 μM) 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 PKCδ.** 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 PKCδ on HuR regulation in macrophages, cells were treated with Rottlerin, a PKCδ inhibitor. As shown in Fig. 5, A and B, 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 (Supplemental Fig. 4). Accordingly, we evaluated whether PKCδ 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 PKCδ accumulation (Fig. 5C), indicating that nuclear PKCδ 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 (Supplemental Fig. 5). Furthermore, berberine inhibited iNOS protein expression in the liver during sepsis by Western blot analysis and immunohistochemical staining (Fig. 6, A and B) 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 berberine suppressed cytosolic translocation of HuR induced by LPS (Fig. 6D). Lastly, whereas LPS injection resulted in 80% mortality at 36 hours postinjection, 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 4 and initiates a signal transduction cascade, which, in part, leads to transcription of proinflammatory 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 pathologic...
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 or without the indicated concentrations of berberine for 1 hour and then stimulated with LPS (10 or 1 μg/ml) for 6 hours. Nuclear (N) and cytoplasmic (C) extracts were isolated, and the levels of HuR in each fraction were determined by Western blotting. Poly(ADP ribose)polymerase and α-tubulin were used as internal controls. (B) Macrophages were preincubated with berberine (10 μM) for 1 hour and then LPS-treated (10 or 1 μg/ml) for 6 hours. The translocation of HuR from nucleus to cytoplasm was determined by colocalization of red dye bound to HuR with blue DAPI stained nuclei. (C) Lysates were prepared from controls, LPS-treated cells for 4 hours (10 or 1 μg/ml) alone, or berberine (1, 5, or 10 μM) 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 μM) of berberine for 1 hour and then incubated with LPS (10 or 1 μg/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 quantified.
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-oxidase-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 (Supplemental 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 trans-activation of iNOS promoter through transcription factors, such as NF-κB and activator protein 1, and by post-transcriptional 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 runoff 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-κB-dependent luciferase activity, p65 translocation, and IκBα degradation (Supplemental 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 injury by nitrative protein modification (nitrative stress) (Xia and Zweier, 1997; Roberts et al., 2009).

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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 injury by nitrative protein modification (nitrative stress) (Xia and Zweier, 1997; Roberts et al., 2009).
the LPS-induced iNOS protein expression, treatment with or without berberine did not affect cycloheximide-reduced iNOS protein expression in LPS-treated macrophages (Supplemental 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 the regulation of iNOS protein expression and NO production. This posttranscriptional mechanism involves interactions between iNOS mRNA 3′-UTR (containing AU-rich element) and RNA-BPs, such as HuR, KSRP, TTP, ARE/poly-(U)-binding/degradation factor-1, 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 downregulated in bone marrow–derived macrophages from myeloid-specific HuR knockout mice (Elavl1Mø KO), indicating that 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 an 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, interleukin (IL)-6, and cyclooxygenase-2, are maintained their mRNA stability by HuR (Liao et al., 2011), we tested the effects of berberine on LPS-induced mRNA stability of tumor necrosis factor-α, interleukin-6, and cyclooxygenase 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., 2015). 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 spanning residues 205–237 and located in the hinge region between RNA recognition motifs 2 and 3 (RRM2 and 3) (Fan and Steitz, 1998). Importantly, like RRM, the hinge region 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 PKCδ or PKCα (Abdelmohsen et al., 2007), phosphorylation of HuR at Ser 202 or Ser 242 by cyclin-dependent kinase 1 causes HuR to reside in the nucleus and be prevented from recruitment of its target mRNA encoding antipapoptotic proteins and enhancing cell survival to the translation machinery (Doller et al., 2008; Kim et al., 2008a,b). Moreover, a single phosphorylation of the distal RRM3 at Ser 318 by PKCδ 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 PKCδ-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 α2-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 α2-adrenergic receptor (Kang et al., 2002). On the other hand, berberine protected against LPS-induced intestinal or lung injury via α2-adrenergic receptor-independent mechanisms (Zhang et al., 2008; Li et al., 2011). Hence, the role of the α2-adrenoceptor or GRP 40 in berberine-induced suppression of PKCδ-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 post-transcriptional 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, Lee.

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

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

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

**References**


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Figure S1. Effect of berberine on iNOS enzyme activity in macrophages. RAW 264.7 macrophages were treated with LPS for 12 h and then changed fresh media. Cells were further incubated for 12 h in the absence or presence of berberine (1, 5, or 10 μM). iNOS enzyme activities were determined by measuring nitrite levels in culture media using Griess reaction. L-NIL (20 μM) was used as a positive control for iNOS enzyme activity. The level of iNOS enzyme activity in control cells was arbitrarily expressed as 100 %. Relative percent was calculated from 100 % control value. Experiments were repeated three times and similar results were obtained. Values are means ± SDs of three independent experiments. *** p< 0.001 versus LPS-treated cells.
Supplemental figure 2

Figure S2. Effect of berberine on cell viability in macrophages (A and B) THP-1 and RAW 264.7 macrophages were incubated with different concentrations of berberine for 24 h in presence of LPS, and cell viabilities were determined by MTT assay.
Supplemental figure 3

Figure S3. Effect of berberine on LPS-induced transcriptional activation of iNOS gene in RAW 264.7 macrophages (A) Cells were transfected with a pGL3-iNOS promoter vector; phRL-TK vector was used as an internal control. Cells were treated with/without the indicated concentrations of berberine for 1 h and then stimulated with LPS (1 μg/ml) for 12 h. Luciferase activity levels were determined using the Promega luciferase assay. Controls were not treated with LPS or berberine. (B) Nuclei were isolated from cells treated with LPS (1 μg/ml) for 12 h. Subsequently, the isolated nuclei were applied to in vitro nuclear run-off transcription reaction in the presence or absence of berberine. Total RNAs were sequentially isolated and analyzed by qRT-PCR using specific primers. Values are means ± SDs of three independent experiments. #p < 0.05 versus the control group; the significances of differences were determined using the Kruskal-Wallis test followed by Dunn’s test.
**Figure S4.** Involvement of PKCδ in LPS-induced iNOS expression in RAW 264.7 macrophages (A) Cells were transfected with PKCδ or Con siRNA (100 μM, 48 h). Total proteins were prepared and analyzed by Western blotting using specific antibodies. (B) Transfected cells were treated with LPS for 24 h. Total RNA was prepared for the real-time PCR analysis of iNOS from transfected macrophages treated with LPS (1 μg/ml) for 12 h. The mRNA levels of iNOS were determined using gene specific primers. Data were presented as means ±SDs of three independent experiments. #p < 0.001 vs. con siRNA-transfected cells. The experiment was repeated three times and similar results were obtained.
Figure S5. Effects of berberine on serum NO levels on mice with LPS-induced septic shock
(A) Different groups (n=10) of C57BL/6 mice were treated with vehicle (DMSO), vehicle
plus LPS (25 mg/kg, i.p.), or LPS plus berberine (1 mg/kg at 1 h before LPS injection).
Serum was collected 6 h after LPS injection, NO levels were quantified using a Griess
reaction assay. The experiments were repeated three times and similar results were obtained.
Values are means ± SDs (n=10). # p < 0.05 versus untreated controls; the significances of
differences were determined using the Kruskal-Wallis test followed by Dunn’s test.
Supplemental figure 6

Figure S6. Berberine inhibited LPS-induced formation of superoxide and 3-nitrotyrosine in RAW 264.7 macrophages. (A) Cells were pretreated with berberine (1, 5, or 10 μM) for 1 h, then with LPS for 24 h. Intracellular levels of superoxide were determined by the nitroblue tetrazolium (NBT) assay. The level of superoxide anions in control cells was arbitrarily expressed as 100%. Relative percent was calculated from 100% control value. # p < 0.05 vs. the control group; ** p < 0.01, *** p < 0.001 vs. LPS-stimulated cells. (B) Lysate was prepared from cells treated with LPS for 24 h in presence or absence of berberine (1, 5, or 10 μM). 3-nitrotyrosine (3-NT)-modified proteins were detected using specific antibodies. β-actin was used as an internal control.
Figure S7. Effects of berberine on NF-κB signaling in RAW 264.7 cells activated with LPS. (A) Cells were transfected with a pNF-κB-luc reporter vector; phRL-TK vector was used as an internal control. Cells were treated with/w ithout the indicated concentrations of berberine for 1 h and then stimulated with LPS (1 μg/ml) for 12 h. Luciferase activity levels were determined using the Promega luciferase assay. Controls were not treated with LPS or berberine. Values are means ± SDs of three independent experiments. #p < 0.05 versus the control group; the significances of differences were determined using the Kruskal-Wallis test followed by Dunn’s test. (B) Cells were treated with LPS in the presence or absence of berberine for 1 or 4 h. Nuclear (N) extracts were isolated, and levels of p65 were determined by Western blotting. PARP-1 was used as internal control. (C) Total proteins were isolated from cells treated with LPS in the presence or absence of berberine for indicated time. The levels of pIκBα and IκBα were determined by Western blotting using specific antibody.
Supplemental figure 8

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Figure S8. Effects of berberine on iNOS protein stability in RAW 264.7 macrophages

RAW264.7 macrophages were pretreated with LPS (1 μg/ml) for 24 h then stimulated with berberine (10 μM) for 1 h, and incubated with/without cycloheximide (1 μg/ml) for 8, 12 or 24 h. Total cellular proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and detected with iNOS antibodies. The experiments were repeated three times and similar results were obtained.