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Inhibitory effects of berberine on lipopolysaccharide-induced inducible nitric oxide synthase and the high-mobility group box 1 release in macrophages

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ABSTRACT

We investigated the molecular mechanism by which berberine reduces nitric oxide (NO) expression and high-mobility group box 1 (HMGB1) release in lipopolysaccharide (LPS)-induced macrophages. Berberine significantly inhibited the LPS-stimulated NO production and HMGB1 release in macrophages. In addition, berberine also induced heme oxygenase (HO)-1 in a dose-dependent manner, which was mediated through activation of p38 MAPK and NF-E2-related factor 2 (Nrf2) signaling cascade in macrophages. The inhibitory effect of berberine on LPS-stimulated NO and HMGB1 release was reversed by siRNA-Nrf2, SB203580 (p38 MAPK inhibitor) and zinc protoporphyrin (ZnPP; HO-1 inhibitor) within macrophages. Therefore, we conclude that berberine inhibits the proinflammatory response to LPS in macrophages by up-regulation of the HO-1 level, in which p38 MAPK and Nrf2 have an important role. These results suggest that berberine may be useful as a therapeutic agent for the treatment of inflammatory diseases.

1. Introduction

Severe sepsis and septic shock caused by invasive infection, represent systemic inflammation in response to invading pathogens [1]. The pathological sequence of sepsis is mediated by proinflammatory cytokines such as the tumor necrosis factor (TNF)-alpha, interleukin-1 (IL-1), and high-mobility group box 1 (HMGB1), that are released from innate immune cells [2]. Among proinflammatory cytokines, HMGB1 contributes to the high lethality of sepsis because of the late-acting downstream effectors. HMGB1 was first identified as a nuclear DNA binding protein and gene transcription and the activity of steroid hormone receptors [3–5]. Interestingly, administration of neutralizing antibodies to HMGB1 dramatically improved the survival of septic animals, thus suggesting it as a possibile therapeutic target for septic patients seen in an Emergency Department [6]. Recently, other studies have reported that HO-1 can be induced by stimulants such as cytokines, heat shock, heavy metals, and oxidants [7,8]. Interestingly, HO-1 can improve patient survival and decrease the circulating HMGB1 level in septic animals, which further suggests the beneficial effects of HO-1 for treating inflammatory disorders [9]. In oxidative injury and inflammation conditions, an increase in the synthesis of the HO-1 gene is linked to the transcription factor, NF-E2-related factor 2 (Nrf2)-ARE pathway [4,10]. It also suggests that Nrf2 nuclear translocation requires the activation of several signal transduction pathways, such as mitogen-activated protein kinases (MAPKs), Akt, and phosphatidylinositol 3-kinase (PI3K) [11]. It was recently reported that the HO-1 system provides a therapeutic effect in many experimental pathological conditions [12–14]. However, although effective therapeutic targets of inflammatory mediators may be ideal to treat sepsis, they have not yet been clinically approved.

Berberine, a natural isoquinoline alkaloid, has been used in Ayurvedic and Chinese Medicine for hundreds of years with a wide range of pharmacological and biochemical effects [15]. A growing number of studies have revealed that berberine has a wide variety of biological effects, including anti-tumor properties [16,17], cardiovascular-protective actions [18], antimicrobial effects [19], and antidiabetic properties [20]. More recently, berberine inhibited LPS-induced inflammation in microglia cells via regulation of HO-1 expression by modulating the Nrf2 signaling pathways [21]. Until now, the role of HMGB1 release and the related molecular mechanisms have not been completely clarified by berberine. Therefore, we demonstrate that berberine is able to induce HO-1 through

Abbreviations: iNOS, nitric oxide synthase; HMGB1, high-mobility group box 1; HO-1, heme oxygenase-1; ZnPP, zinc protopor-phyrin; LPS, lipopolysaccharide; Nrf2, NF-E2-related factor 2; TNF-α, tumor necrosis factor-alpha; MAPKs, mitogen-activated protein kinases; PI3K, phosphatidylinositol 3-kinase; ARE, antioxidant response element.

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activation of p38 MAPK and Nrf2 expression in macrophages and in turn to reduce HMGB1 under proinflammatory stimulus.

2. Materials and methods

2.1. Materials

Reagents used in this study were purchased from the following sources: berberine, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], LPS and zinc protopor-phyrin (ZnPP) from Sigma–Aldrich (St. Louis, MO, USA); SB203580, PD98059, and SP600125 were purchased from Calbiochem (La Jolla, CA, USA); RPMI-1640, fetal bovine serum and Trizol were supplied by Gibco BRL (Grand Island, NY, USA); antibodies for p38, phospho-p38 MAPK, and HRP-conjugated anti-mouse IgG from Cell Signaling Technology (Beverly, MA); antibodies for HMGB1, iNOS, Nrf2 and HO-1 from Calbiochem (La Jolla, CA, USA). All chemicals and reagents were of analytical grade.

2.2. Animals

Specific pathogen-free BALB/C mice (female, 7 weeks old) were obtained from Central Laboratory Animal Inc. (Seoul, South Korea). Animals were housed under normal laboratory conditions, i.e. at 21–24 °C and 40–60% relative humidity under a 12 h light/dark cycle with free access to standard rodent food and water. All animals were raised under specific pathogen-free conditions, and the protocol was reviewed and approved by the Animal Subjects Committee of Asan Medical Center (Seoul, South Korea).

2.3. Preparation of peritoneal macrophages and cell culture

Peritoneal macrophages were isolated from mice that had been injected intraperitoneally with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were plated on a flat-bottom culture plate and then incubated for 2 h at 37 °C in a 5% CO_2 humidified incubator.

2.4. Cell treatment and viability assay

Berberine was dissolved in dimethylsulfoxide (DMSO) and the stock solutions were added directly to the culture media. The control cells were treated with culture medium only. The final concentration of solvent was always <0.1%. The cells (5×10^3/well) in 10% FBS-RPMI-1640 were seeded into the 48-well plates. After incubation for 24 h, various concentrations of berberine and LPS were added to the well, and the plates were incubated at 37 °C for an additional 24 h. The cells were used for the MTT-based assay by measuring the according to the manufacturer's instructions. Relative cytotoxicity was quantified by absorption measurements at 550 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA). This wavelength was not found to interfere with berberine.

2.5. NO assay

NO was measured as its stable oxidative metabolite, nitrite (NO_x), as previously described [22]. At the end of incubation, 100 μl of the culture medium was mixed with the same volume of Griess solution (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% phosphoric acid). Light absorbance was measured at 550 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA, USA), and the nitrite concentration was determined using a curve calibrated on sodium nitrite standards.

2.6. HMGB1 release assay

The levels of HMGB1 in the culture medium were determined using commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN) and were performed according to the manufacturer's instructions.

2.7. RNA preparation and mRNA analysis by real-time quantitative PCR

The total RNA was isolated from cells using Trizol (GibcoBRL, Grand Island, NY, USA). Accumulated PCR products were directly detected by monitoring the increase in reporter dye (SYBR®). The expression levels of HO-1, iNOS and HMGB1 in the exposed cells were compared to those in the control cells at each time point using the comparative cycle threshold (*Ct*) method [24]. The following primer sequences were used: the HO-1 primers were sense 5′-CGCC TTCCTGCTCAACATT-3′ and antisense 5′-TGTGTTCCTCTGTCAGCATCAC-3′; the HMGB1 primers were sense 5′-TTGTGCAAACTTGCCGGGAGGA-3′ and antisense 5′-ACTTCTCCTTCAGCTTGGCAGC-3′; the iNOS primers were sense 5′-AACGGAGAACGTTGGATTTG and antisense 5′-CAGCACAAGGGGTTTTCTTC; Mouse ribosomal protein S18 (S18) sense 5′-GATGGGCGGGGGAAAAT-3′; and S18 antisense 5′-CTTGTACTGGCGTGGATTCTGC-3′. The quantity of each transcript was calculated as described in the instrument manual and was normalized to the amount of S18, a housekeeping gene.

2.8. Western immunoblot analysis

Cells were harvested and washed 3 times with cold phosphate-buffered saline (PBS). The cytoplasmic and nuclear protein fractions were extracted using NE-PER extraction reagents according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL). Cytoplasmic/nuclear protein extracts or whole protein extracts were used for Western blot analysis. Western blotting was performed using anti-HO-1, anti-iNOS, anti-HMGB1, anti-p38, anti-phospho-p38, and anti-actin antibodies. Protein samples were heated at 95 °C for 5 min and were analyzed using SDS–PAGE. Immunoblot signals were developed by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

2.9. siRNA knockdown

siNrf2 and scrambled siRNA were acquired from Santa Cruz Biotechnology. siRNA was transfected into macrophages according to the manufacturer's protocol and using the transfection reagent, Lipofectamine 2000© (Invitrogen, CA, USA). The cells were incubated with 100 nM of target siRNA or scramble siRNA for 4 h in serum- and antibiotic-free media. The cells were then incubated for 18°h in media containing antibiotics and FBS, and cells were washed, pretreated with or without berberine for 1 h, and treated with LPS.

2.10. Transient transfection and luciferase assay

Cells (3×10^5 cells/well) were seeded in 24-well plates, incubated overnight and transiently co-transfected with ARE-promoter-luciferase construct and pRL-SV40 plasmid (*Renilla* luciferase expression for normalization) (Promega, Madison, WI, USA) using LipofectAMINE™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). Relative luciferase activities were calculated by normalizing ARE-promoter-driven firefly luciferase activity to *Renilla* luciferase activity.