Research article

HMGB1-binding heptamer suppresses the synergistic effect of HMGB1 and LPS by interacting directly with HMGB1

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HIGHLIGHTS

• HBHP is a heptamer peptide selected as a HMGB1 binding peptide using a phage display approach.  
• HBHP suppresses the synergistic NO induction by LPS (5 ng/ml) and NCM (HMGB1).  
• HBHP suppresses the synergistic activation of primary microglia cultures by HMGB1 and LPS (5 ng/ml).  
• Direct inhibition of LPS–HMGB1 interaction by HBHP might underlie the mechanism.

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ABSTRACT

High mobility group box 1 (HMGB1) is an endogenous danger signal molecule. In the postischemic brain, HMGB1 is massively released during acute damaging process and triggers inflammatory processes. Moreover, it has been reported HMGB1 augments the proinflammatory effect of LPS by direct interaction. In previous studies, the authors showed intranasally delivered a HMGB1 binding heptamer peptide (HBHP; HMSKPVQ) has robust neuroprotective effects in the ischemic brain after middle cerebral artery occlusion and that it exerts an anti-inflammatory effect. In the present study, the authors investigated whether HBHP suppresses the augmentation of the proinflammatory effect of LPS by HMGB1. In primary microglial cultures, low doses of LPS (5 ng/ml) and recombinant HMGB1 (rHMGB1, 20 ng/ml) synergistically activated microglial cells, and HMGB1–LPS binding was detected. In addition, synergistic NO accumulation along with direct HMGB1–LPS binding was also observed when primary microglial cultures were treated with LPS (5 ng/ml) and HMGB1 accumulated in NMDA-conditioned medium (NCM). Co-treatment of microglial cells with HBHP and LPS or rHMGB1 (NCM), or treatment with rHMGB1 or NCM and LPS after pre-incubating rHMGB1 (or NCM) with HBHP markedly suppressed their synergistic activation. Furthermore, interactions between rHMGB1 and LPS or between HMGB1 in NCM and LPS were suppressed dose-dependently by HBHP, indicating that HBHP suppressed the synergism between HMGB1 and LPS and the underlying mechanism involved inhibition of HMGB1–LPS binding. Together these results show HBHP has anti-inflammatory effects, and that it inhibits synergism caused by the binding of HMGB1 and LPS.

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1. Introduction

High mobility group box 1 (HMGB1) is an endogenous danger signal molecule and an inducer of inflammation, and is released by necrotic cells or actively secreted by macrophages and monocytes into the extracellular milieu [1,2]. When released extracellularly, HMGB1 upregulates inflammatory cytokines, such as, IL-1β, IL-6, and TNF-α, which lead to the activations of various immune-related cells, including microglia in brain [2–4]. In accordance with these observations, high plasma levels of HMGB1 have been reported to be correlated with disease severity in various pathological conditions, including sepsis [5], pancreatitis [6], and stroke [4].

It has been reported HMGB1 interacts with various molecules, including LPS [7,8], IL-1β [9,10], ssDNA [11], CpG-ODN [12], and peptidoglycan [7] and that the resulting complexes augment inflammatory response by binding with appropriate receptors. For example, complexes containing HMGB1 and IL-1β induce the productions of proinflammatory cytokines, such as, TNFα, IL-6, and...
IL-8, and the production of MMP-3 via IL-1R in synovial fibroblasts [10]. Furthermore, it has been reported that LPS and HMGB1 synergistically induce the production of NO in mouse peritoneal macrophages [7] and HMGB1 transfers LPS to CD14 and activates NF-κB signaling through TLR4 and RAGE ligand [7,8].

A number of heptamer peptides that bind to HMGB1 A box were identified in a ligand screening study using a phage-displayed heptapeptide library [13]. In a previous study, we found that a HMGB1-binding heptamer peptide (HBHP) with the sequence HMSKPVDQ directly bound to HMGB1 under cell free conditions and in N-Methyl-D-aspartate (NMDA)-conditioned media (NCM), and exerted a neuroprotective effect in the postischemic rat brain, that is, it suppressed infarct formation in the rat brain after middle cerebral artery occlusion (MCAO) and ameliorated neurological deficits [14]. Subsequently, HBHP was found to confer anti-inflammatory effects in primary microglia cultures [15]. In the present study, we investigated whether HBHP suppresses the synergism shown by HMGB1 and LPS and what is the mechanism involved.

2. Materials and methods

2.1. Primary cortical neuron culture

Neuron and astrocyte mixed cortical cells were prepared from embryonic day 15.5 (E15.5) mouse cortices and cultured as described previously [4]. Dissociated cortical cells were plated at a density of five hemispheres per 24-well plate (4 × 10^5 cells per well), plates were coated beforehand with poly-D-lysine (100 μg/ml) and laminin (100 μg/ml). Cultures were maintained without antibiotics in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and 5% horse serum. At 6 days in vitro (DIV), when astrocytes had reached confluence underneath neurons, cytosine arabinofuranoside (ara-C) was added to a final concentration of 10 μM and cultures were maintained for a further 2 days to halt microglial growth. FBS and glutamine were not supplemented from DIV 6 and media were changed twice weekly after DIV 8. Cultures were used at DIV 13–15.

2.2. NMDA-conditioned media (NCM) preparation

Primary cortical cells were treated with serum-free MEM containing 150 μM of NMDA (Sigma, St. Louis, MO) for 30 min and cultured in fresh medium for 24 h. Medium was then collected and concentrated to 40 μl using a Centricron 10 (Pall Life Sciences, Port Washington, NY). This concentration is referred to as NMDA-conditioned medium (NCM, x1).

2.3. Primary microglia culture

Cells dissociated from the cerebral hemispheres of 1 day old postnatal rat brains (Sprague–Dawley strain) were seeded at a density of 1.2 × 10^6 cells/ml in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA) containing 10% FBS (HyClone, Logan, UT) and 1% penicillin-streptomycin (Gibco) in a T-75 flask (SPL Life Sciences, Pocheon, Korea). Two weeks later, microglia were detached by mild shaking and filtered through a cell strainer (BD Falcon, Bedford, MA) to remove astrocytes. After centrifugation (1000 × g) for 5 min, cells were resuspended in fresh DMEM containing 10% FBS and 1% penicillin-streptomycin and plated at a final density of 1.5 × 10^5 cells/well on a 24 multi-well culture plate. After 2 h, the medium was changed for DMEM containing 5% FBS and 500 μM B27 supplement (Gibco).

2.4. NO assay

Primary microglia cells (1.5 × 10^5) were seeded in 24-well plates and 1 day later treated with LPS, recombinant HMGB1 (HMGB1), or NCM. To measure the amount of NO produced, 50 μl of culture medium was mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 0.05% N-naphthylene-diamine-H-chloride, and 2.5% H3PO4) and incubated for 5 min at room temperature. Absorbances of mixture were measured at 550 nm using a microplate reader. NaNO2 standards were used to calculate NO2− concentrations.

2.5. siRNA Transfection

Cells were seeded at a density of 1.5 × 10^5 cells per well in 24-well plates. All transfections were performed using oligofectamine (Invitrogen, Carlsbad, CA) as a carrier. SMART pool siRNA, specifically targeting HMGB1 (ON-TARGET plus SMART pool siRNA L-114889, accession no. NM_001109373; Dharmacon, Lafayette, CO), and non-specific siRNA (on-TARGET plus Non-targeting pool D-001810, Dharmacon) were used. Transient transfections were carried out according to the manufacturer’s instructions. siRNA and lipid complexes were added to the wells of 96-well culture plates to final concentration of 40 pM siRNA and 1.8 μl oligofectamine.

2.6. Immunoblot analysis

Proteins (20 μg) were separated in 12% sodium dodecyl sulfate-polyacrylamide gel, and after blocking the membranes so obtained
**Fig. 2.** Synergistic NO induction in primary microglial cultures by LPS and HMGB1 in NCM.

(A) NMDA-conditioned media (NCM) was collected from primary cortical cultures at 24 h after NMDA treatment (150 μM, 30 min). Primary microglia cultures were treated with NCM for 24 h in the presence of 1, 2, or 5 ng/ml LPS. (B) Primary microglia cultures were incubated with NCM for 24 h in the presence of 5 ng/ml of LPS, which had been pre-incubated with HMGB1 A box, HMGB1 B box, or anti-HMGB1 antibody for 6 h. (C) HMGB1 levels were determined in whole cell lysates and in culture media of primary cortical cultures 24 h after transfection with HMGB1 siRNA (HMGB1<sup>−</sup>) or nonspecific (NS<sup>−</sup>) siRNA (40 pM) by immunoblotting. (D) NCM was collected from primary cortical cultures transfected with HMGB1 siRNA or non-specific siRNA and added to primary microglial cultures for 24 h in the presence of 2 ng/ml LPS. (A,B,D) NO production was measured using a Griess assay. Data are presented as means ± SEMs. **p < 0.01.

with 5% non-fat milk for 1 h, membranes were incubated with primary antibodies diluted 1:1000 for anti-HMGB1 (Abcam, Cambridge, UK), anti-α-tubulin (Cell Signaling, Danvers, MA), anti-CD14 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-LPS (Abcam) overnight at 4 °C. The next day, membranes were detected using a chemiluminescence kit (Roche, Basel, Switzerland) using anti-rabbit HRP-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology).

2.7. Immunoprecipitation-linked immunoblotting

Total lysates containing 500–1000 μg of protein or conditioned culture medium were immunoprecipitated with 1 μg of anti-HMGB1 polyclonal antibody (Abcam, Cambridge, UK) at 4 °C overnight. Pre-equilibrated protein G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were then added and incubated for 2 h at 4 °C on a rotating wheel. Bead-bound complexes were pelleted, washed several times with lysis buffer, boiled at 99 °C in SDS sample buffer for 5 min, subjected to SDS-PAGE, and Western blotted.

2.8. Biotinylated pull-down assay

To investigate LPS to HMGB1 binding, a pull-down assay was performed using 20 μl of streptavidin agarose beads (50% slurry) (Pierce, Rockford, IL) and pull-down complexes were separated by 14% SDS-PAGE and analyzed by Western blotting. Biotin-tagged LPS (1 or 5 μg/ml) was incubated with recombinant HMGB1 (HMG-biotech, Milano, Italy) in the presence or absence of HBHP (1 or 5 μg/ml) at 4 °C for 6 h with rotation, and the biotin complexes obtained were incubated with streptavidin beads for 1 h at 4 °C with rotation, centrifuged at 8000 rpm for 1 min, and Western blotted.

3. Results

3.1. HBHP inhibited synergistic NO induction by rHMGB1 and LPS in primary microglial cultures

To examine the synergism between LPS and rHMGB1, primary microglia cultures were treated with low dose LPS (1, 2, or 5 ng/ml) which had been pre-incubated with 10 or 20 ng/ml of rHMGB1 for 16 h at 4 °C. NO induction was negligible when primary microglia cultures were treated with 10 or 20 ng/ml of rHMGB1 (Fig. 1A). However, when they were co-treated with LPS, NO induction was enhanced in a rHMGB1 dose-dependent manner (Fig. 1A). When HBHP (10, 50, or 100 ng/ml) was co-treated with LPS (5 ng/ml) and rHMGB1 (10 or 20 ng/ml), NO induction by rHMGB1/LPS was significantly suppressed by 10 ng/ml of HBHP and to be further suppressed by 100 ng/ml of HBHP (Fig. 1B). However, it was not suppressed by HBHP-sc (PMQSKHV) containing a scrambled sequence of the amino acids in HBHP (Fig. 1B). Furthermore, HBHP has no effect on LPS-only- or rHMGB1-only-induced NO induction (Fig. 1C). These results demonstrate that HBHP suppressed the synergistic activation of primary microglia cultures by rHMGB1 and LPS.

3.2. Synergistic NO induction by HMGB1 in NMDA-conditioned media (NCM) and LPS in primary microglial cultures

To confirm that endogenous HMGB1, released after NMDA-induced neuronal death [14,16], is capable of synergistically enhancing LPS-induced inflammation, we examined NO production after treating primary microglia cultures with LPS and NMDA-conditioned medium (NCM). NCM-alone was unable to induce NO production, but NO production was markedly increased by adding LPS (Fig. 2A). In particular, with 2 ng/ml of LPS, NO production was increased to 1.7 fold by co-treating NCM (Fig. 2A). Moreover, when concentrated NCM collected from 2 plates (x2)
was used, NO production increased to 2.4 fold (Fig. 2A). However, synergistic NO induction by NCM plus LPS was abrogated by treating NCM after pre-incubating it with HBHP A box or anti-HMGB1 antibody (Fig. 2B), indicating that the HMGB1 in NCM participated in the synergistic NO induction by LPS and NCM. In addition, this synergism was not detected when NCM collected from HMGB1 siRNA-transfected primary cortical cultures was used but was detected when NCM from non-specific siRNA-transfected cultures was used (Figs. 2C and D). These results show that HMGB1, released from neurons after excitotoxic damage, synergistically enhanced LPS-mediated microglial activation.

HBHP suppressed synergistic NO induction by HMGB1 in NCM and LPS in primary microglial cultures via inhibiting HMGB1–LPS binding

Synergistic activation of microglia by LPS and HMGB1 (in NCM) was also significantly suppressed by pre-incubating cells with HBHP (100 or 200 ng/ml) to 80.4 ± 5.8% and 66.4 ± 12.2 (n = 3, p < 0.01), respectively, however, HBHP-sc failed to suppress NO induction (Fig. 3A). Furthermore, HMGB1 peptide 1 or 6 (Hpep1 or -6), which have been shown to bind LPS [17], suppressed this synergistic induction of NO (Fig. 3B). To determine whether HBHP inhibits binding between HMGB1 and LPS, pull-down assay was conducted by using biotin-tagged LPS (bt-LPS). Immunoblotting using anti-HMGB1 antibody showed that HMGB1/LPS bindings were significantly inhibited dose-dependently by HBHP but not by HBHP-sc (Fig. 3C). These findings indicate that HBHP inhibits the synergistic effects of HMGB1 and LPS on microglia activation and inhibition of LPS–HMGB1 binding might be an underlying mechanism.

3.3. HBHP inhibited HMGB1–LPS binding in LPS-treated primary microglia cultures

To further confirm the inhibition of HMGB1–LPS binding by HBHP in more physiological context, we examined HMGB1–LPS interaction in the culture media of LPS-treated primary microglia. In a previous study, we showed that HMGB1 was accumulated in the culture media of LPS-treated primary microglia [18]. We found that HMGB1 secreted in LPS-treated culture media made a complex with LPS remained in culture media (Fig. 4A). Binding between HMGB1 and LPS, as evidenced by co-immunoprecipitation, was detected as early as 6 h after LPS (100 ng/ml) treatment and gradually increased to peak at 24 h after LPS treatment (Fig. 4A), which parallels with the levels of HMGB1 secreted and accumulated in culture media (Fig. 4B). HMGB1 levels in LPS-treated microglia culture media gradually increased up to 24 h but HMGB1 levels in cell lysates gradually decreased (Fig. 4B). As was expected, the HMGB1 to LPS binding was suppressed by co-treating LPS with HBHP but not with HBHP-sc (Fig. 4C). These results further confirm that HBHP exerts its anti-inflammatory effects by inhibiting HMGB1/LPS binding.

4. Discussion

It has been reported that HMGB1 has two LPS-binding motifs. One is BBXB (B: any basic aa, X: any hydrophobic aa) which are located in two HMG domains, A box (sequences 7–10) and B box (sequences 86–90). The B box domain harbors another LPS-binding motif...
motif (B2B motif; B: any basic aa, Z: aromatic ring-containing aa) at sequences 86–90 [17]. Youn et al. (2011) [17] synthesized 12 peptides (Hpeps) from the HMGB1 and showed that Hpep1 and Hpep6 bind to LPS and thus inhibit HMGB1 to LPS binding. In a previous study, we showed that HBHP interacts with HMGB1, especially HMGB1 A box [15]. Here, we demonstrate that HBHP inhibits HMGB1 to LPS binding and occluding the LPS binding site on HMGB1 A box might be an underlying mechanism. Although further study is needed to elucidate the exact contact point between HMGB1 and HBHP, it might be localized near Hpepi [17], which harbors amino acids 3–15 in HMGB1 A box. However, we do not exclude the possibility that different sites are also involved in HBHP-HMGB1 binding or that specific interactions depend on cell and/or activating stimulus type. Furthermore, since relationship between differential modifications of HMGB1 and its functions have been reported [19,20], these modifications might affect the interaction between HMGB1 and HBHP.

In the present study, we showed that rHMGB1 and HMGB1 in NCM binds to LPS and that these complexes augmented LPS-induced microglia activation in primary microglia cultures. Furthermore we also demonstrated that LPS interacts with HMGB1 secreted in LPS-treated microglia culture media. Together all these observations support the relevance of the HMGB1–LPS interaction in various pathological conditions. It is interesting to note here that a massive HMGB1 release (secretion) and subsequent infection can be observed in various disease conditions. For example, in the postischemic brain, HMGB1 is massively released by acute damage, and it then exacerbates neuronal damage by triggering delayed inflammatory processes [4]. HBHP not only confers a robust neuroprotective effect in the postischemic brain as it had been reported [14] but it might also be able to ameliorate subsequent damage in the case of post-stroke infection by suppressing HMGB1–LPS binding. Post-traumatic, post-epileptic, and even chronic inflammation represents similar conditions, and thus, a HBHP-mediated LPS-neutralization strategy could be possibly used to treat these pathological conditions.

Considering that HMGB1 is known to interact with numerous proinflammatory ligands, such as, CpG-ODN, peptidoglycan (PGN), Pam3CSK4, Beclin 1, and IL-1β, it is possible HBHP also affects interactions between HMGB1 and these molecules. These interactions could occur in extracellular space as for PGN [7], or in cytoplasm in the cases of CpG-ODN [12] and Beclin 1 [21]. Although HBHP might interact mainly with extracellular HMGB1, it is possible that HBHP penetrates the cell membrane and interacts with intracellular HMGB1. However, different modifications of HMGB1 in different subcellular localizations might affect its interactions with HBHP. All these possibilities need further study.

In conclusion, HBHP was found to exert its anti-inflammatory effects by inhibiting binding between HMGB1 and LPS. Furthermore, HBHP also conferred neuroprotective effects by direct binding to extracellular HMGB1, which is released from damaged neuron and augments LPS-induced microglia activation. Further studies are needed to identify the HBHP binding site of HMGB1.

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