INTRODUCTION

High mobility group box 1 (HMGB1) is an endogenous danger signal molecule. In the postischemic brain, HMGB1 is massively released during NMDA-induced acute damage and triggers inflammatory processes. In a previous study, we demonstrated that intranasally delivered HMGB1 binding heptamer peptide (HBHP; HMSKPVQ) affords robust neuroprotective effects in the ischemic brain after middle cerebral artery occlusion (MCAO, 60 minutes). In the present study, we investigated HBHP-induced anti-inflammatory effects on microglia activation. In LPS-treated primary microglia culture, HMGB1 was rapidly released and accumulated in culture media. Furthermore, LPS-conditioned media collected from primary microglia cultures (LCM) activated naive microglia and markedly induced NO and proinflammatory cytokines. However, the suppression of HMGB1 by siRNA-HMGB1, HMGB1 A box, or anti-HMGB1 antibody significantly attenuated LCM-induced microglial activation, suggesting that HMGB1 plays a critical role in this process. A pull-down assay using biotin-labeled HBHP showed that HBHP binds directly to HMGB1 (more specifically to HMGB1 A box) in LCM. In addition, HBHP consistently inhibited LCM-induced microglial activation and suppressed the inductions of iNOS and proinflammatory cytokines. Together these results suggest that HBHP confers anti-inflammatory effects in activated microglia cultures by forming a complex with HMGB1.

Key words: HMGB1, HBHP, inflammation, microglia
microglial cells were treated with serum-free DMEM for 24 hrs. Centricon 10 (Millipore, Billerica, MA, USA). For control, primary microglia were detached from flasks by mild shaking and filtered through a cell strainer (BD Falcon, Bedford, MA, USA) to remove astrocytes. After centrifugation (1,000×g) for 5 min, cells were resuspended in fresh DMEM containing 10% FBS and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA). Two weeks later, microglia were detached from flasks by mild shaking and filtered through a cell strainer (BD Falcon, Bedford, MA, USA) to remove astrocytes. After centrifugation (1,000×g) for 5 min, cells were resuspended in fresh DMEM containing 10% FBS and 1% penicillin-streptomycin and plated at a final density of 1.5×10⁶ cells/ml in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) containing 10% FBS (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (Gibco, Carlsbad, CA, USA). Two weeks later, microglia were detached from flasks by mild shaking and filtered through a cell strainer (BD Falcon, Bedford, MA, USA) to remove astrocytes. After centrifugation (1,000×g) for 5 min, cells were resuspended in fresh DMEM containing 10% FBS and 1% penicillin-streptomycin and plated at a final density of 1.5×10⁶ cells/well on a 24 multiwell culture plate. After 2 hrs, the medium was changed for DMEM containing 5% FBS and 500 µM B27 supplement (Gibco, Carlsbad, CA, USA).

**LPS-Conditioned Media (LCM) preparation**

Primary microglial cells were treated with serum-free DMEM containing 100 ng/ml of LPS (Sigma, St. Louis, MO, USA) for 18 hrs. After washing with twice with DMEM, medium was replaced with fresh DMEM. LPS-conditioned media (LCM) was collected 24 hrs later and concentrated from 600 µl to 20 µl using a Centricon 10 (Millipore, Billerica, MA, USA). For control, primary microglial cells were treated with serum-free DMEM for 24 hrs.

**NO measurement**

Primary microglia cells (1.5×10⁶) were seeded in 24-well plates and 1 day later treated with LCM or LPS (100 ng/ml). To measure the amount of NO produced, 50 µl of conditioned 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 mixtures were measured at 550 nm using a microplate reader. NaNO₃ standards were used to calculate NO₂ concentrations.

**siRNA transfection**

Cells were seeded at a density of 1.5×10⁵ cells per well in 24-well plates. All transfections were performed using oligofectamine (Invitrogen, Carlsbad, CA, USA) as a carrier. SMART pool siRNA specifically targeting HMGB1 (ON-TARGET plus SMART pool siRNA L-114889, accession no.NM_001109373; Dharmacon, Lafayette, CO, USA) and non-specific siRNA (on-TARGET plus Non-targeting pool D-001810, Dharmacon, Lafayette, CO, USA) were used. Transient transfections were carried out according to the manufacturer’s instruction. siRNA and lipid complexes were added to the wells of 96-well culture plates to a final concentration of 40 pM siRNA and 1.8 µl oligofectamine.

**Real-time PCR**

Microglial cells were treated with LCM or LPS (100 ng/ml) and total RNA was purified with TRI reagent (Sigma-Aldrich, St Louis, MO) according to the manufacturer’s instructions. First-strand cDNA was synthesized using the Takara RNA PCR kit (Takara Bio, Otsu, Japan) in a total volume of 20 µl containing 1 µg of total RNA. Real-time PCR was performed in a final volume of 20 µl containing 10 µl of 2x SYBR Green supermix (Takara Bio, Otsu, Japan), 1 µl each of 5 pmol/µl forward and reverse primers, and 5 µl of cDNA (50 ng; 1/100 dilution) using the Mini-Opticon Real-Time PCR System Detector (Bio-Rad, Richmond, CA). PCR was performed using: 5 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C. The specificity of amplification was determined by DNA melting curve analysis using the built-in software. Differences in amplification fold were calculated based on the real-time PCR amplification of the target gene versus GAPDH using the built-in Gene Expression Analysis software in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Richmond, CA). The following primers sets were used: 5’-TCATTGAC CTCAACTACATGGT-3’ and 5’-CTAACGATGGTTGGTGCCAG-3’ for GAPDH. Real-Time PCR was performed in quadruplicate.
**Biotin pull-down Assay**

To analyze the binding of HBHP to HMGB1, a pull-down assay was performed using a 20 µl of streptavidin agarose beads (50% slurry) (Pierce, Rockford, IL, USA). Pull-down complexes were separated by 12% SDS-PAGE and analyzed by Western blotting. Biotin-labeled HBHP (HMSKPVQ; 1 or 5 µg/ml) or scrambled HBHP (PMQSKHV; 5 µg/ml) was incubated with LCM (containing HMGB1) at 4°C for 4 hrs with rotation. A competition assay was performed using 5 µg/ml of HBHP, which was preincubated with the A box domain (1, 5, or 10 µg/ml) or the B box domain (1, 5, or 10 µg/ml) of HMGB1 for 1 hr at 4°C with rotation. The biotin complexes obtained were incubated with streptavidin beads for 1 hr at 4°C with rotation, centrifuged at 8,000 rpm for 1 min, and analyzed by Western blotting.

**Immunoblotting**

Cells were washed twice with cold PBS and lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium-deoxycholate, 150 mM NaCl) containing 1 complete Mini protease inhibitor cocktail tablet (Roche diagnostics, Basel, Switzerland). For protein preparations from the supernatant, media were collected and concentrated as previously described and cells were treated with RIPA buffer. The lysates obtained were incubated in ice for 15 min and centrifuged at 12,000 rpm for 15 min at 4°C and supernatants were loaded onto 12% SDS-PAGE gels. The primary antibodies (diluted at 1:1,000) used were as follows: anti-HMGB1 (Abcam, Cambridge, UK) and anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Primary antibodies were detected using a chemiluminescence kit (Roche, Mannheim, Germany) using goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000, Millipore, Billerica, MA, USA).

**Statistical analysis**

Statistical analysis was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test. All data are presented as means±SEMs and statistical significance was accepted at the 5% level.

**RESULTS**

**LPS-conditioned primary microglia culture media contained HMGB1 and produced pro-inflammatory effects**

HMGB1 levels in primary microglia culture media were examined at after 1, 3, 6, 12, 18, or 24 hrs of LPS treatment (100 ng/ml). HMGB1 accumulation was detected after 3 hrs of LPS treatment and its levels gradually increased to peak at 18 hrs (Fig. 1A). On the other hand, HMGB1 levels in whole cell lysates decreased gradually from 3 hrs (Fig. 1B), whereas α-tubulin levels were similar at all time points (Fig. 1B).

**Fig. 1.** HMGB1 accumulation in LPS-treated primary microglial culture media (LCM). (A, B) Primary microglial cultures (1.5×10⁵ cells/well) were incubated with LPS (100 ng/ml) for 1, 3, 6, 12, 18, or 24 hrs and HMGB1 levels in culture media (A) and in whole cell lysates (B) were determined by immunoblotting. α-Tubulin was used as a control for whole cell lysates (B). (C) After treating cells with LPS (100 ng/ml) for 18 hrs, primary microglial culture medium was replaced with fresh LPS-free medium and LCM was collected at 12 or 24 hrs after media replacement. HMGB1 levels were determined by immunoblotting. (D) LCM was concentrated, and treated to fresh primary microglial cultures. Nitrite levels were measured 24 hrs later. Data are presented as means±SEMs (n=4), *p<0.05, **p<0.01.
To obtain LPS-free LCM, culture media were replaced with fresh medium after 18 hrs of LPS treatment and LCM was collected at 12 or 24 hrs later. Immunoblot analysis revealed HMGB1 accumulation in LCM-free LCM, and the level of HMGB1 at 24 hrs after medium replacement was slightly higher than that at 12 hrs (Fig. 1C). When fresh primary microglia cultures were incubated with LCM for 24 hrs after LCM treatment, culture media were replaced with fresh medium, and LCM was collected at 24 hrs after medium replacement. Naïve primary microglia were then cultured in this LCM for 24 hrs and nitrite levels were measured. (C) Nitrite levels were measured at 24 hrs after LCM treatment in the presence of HMGB1 A box domain (10, 50, 100 ng/ml) or anti-HMGB1 antibody (100 or 500 ng/ml). HMGB1 B box domain (50, 100 ng/ml) and anti-human IgG (500 ng/ml) were used as negative controls. Data are presented as means±SEMs (n=4). **p<0.01.

**HMGB1 accumulation in LCM was responsible for LCM-induced microglia activation**

To determine whether HMGB1 in LCM was responsible for microglial activation, the proinflammatory potency of LCM was examined after HMGB1 knockdown. HMGB1 levels in primary microglia decreased to 28.6±3.9% of the control level at 12 hrs after HMGB1-siRNA transfection (Fig. 2A). Furthermore, the LCM of HMGB1-siRNA transfected cells induced significantly less NO (59.9±0.9%) than LCM-treated control cells (Fig. 2B). However, NO levels were unchanged, when LCM from non-specific siRNA (NS)-transfected cells was used (Fig. 2B). These results suggest that HMGB1 plays an important role in LCM-induced microglial activation. LCM-induced NO production was also significantly suppressed by co-treating cells with HMGB1 A box (50 ng/ml) to 46.2±2.3% (n=4, p<0.01), a well-known antagonist of HMGB1 [20], or by co-treating anti-HMGB1 antibody (500 ng/ml) to 50.5±3.3% (n=4, p<0.01). These findings further support the critical role played by HMGB1 in LCM-induced microglia activation (Fig. 2C).

**HBHP suppressed LCM-induced microglial activation**

To determine whether HBHP affects LCM-induced microglial activation, NO levels were measured after co-treating cells with LCM and HBHP. NO levels were suppressed to 56.7±5.1%, 30.8±1.6%, and 32.5±10.8% of the control when HBHP was treated at 10, 50, or 100 ng/ml, respectively (Fig. 3A). Whereas scrambled HBHP (PMQSKHV; sc-HBHP, 50 or 100 ng/ml) did not produce such effects. In addition, proinflammatory cytokine productions in LCM-treated primary microglia cultures were also suppressed by HBHP treatment (10, 50, or 100 ng/ml), but not by scrambled HBHP (sc-HBHP, 50 or 100 ng/ml) (Fig. 3B), further supporting the notion that HBHP has anti-inflammatory effects in LCM-treated primary microglia cultures.

**HBHP directly bound to HMGB1 in LCM**

Next, we examined whether HBHP binds to the HMGB1 released by activated microglia to LCM. A biotin pull-down assay using biotin-labeled HBHP revealed the presence of binding between HBHP and HMGB1 in LCM in a HBHP-dose dependent manner, whereas, this binding was not detected when scrambled HBHP was used (Fig. 4A). Moreover, the formation of HBHP-HMGB1 was significantly suppressed when the LCM of HMGB1-ablated cells was used (Fig. 4B). When LCM was incubated with biotinylated HBHP (5 μg/ml) in the presence of HMGB1 A box (1, 5, or 10 μg/ml) or B box (5 or 10 μg/ml), HMGB1/HBHP binding...
was clearly inhibited dose dependently by HMGB1 A box but not by HMGB1 B box (Fig. 4C). Accordingly, these results suggest that HBHP binds directly and specifically with HMGB1 A box in LCM and that the suppression of LCM-induced microglial activation by HBHP is due to this binding.

DISCUSSION

The present study shows that HMGB1 rapidly accumulates in LPS-treated primary microglia culture media (LCM), and that LCM is capable of activating microglia, and thus, of inducing proinflammatory markers. Furthermore, the study provides evidence that HBHP suppresses LCM-induced microglial activation, and that this is achieved by its direct binding to HMGB1 A box. In a previous report, we found that intranasal delivered HBHP ameliorates neuronal damage in the postischemic rat brain, and that HBHP confers neuroprotection to NMDA- or Zn²⁺-treated primary cortical cultures, wherein HBHP interacts with accumulated HMGB1 in NMDA-conditioned media [19]. Under pathological conditions, including that of the postischemic brain, where HMGB1 might be released from neurons after an excitotoxic insult [3] or secreted from activated microglia (Figs. 1 and 4), the neuroprotective effect of HBHP might be associated with its direct targeting of HMGB1 from various cellular origins. In view of the findings that HBHP has anti-inflammatory (present study) and neuroprotective effects [19], the robust neuroprotective effect of HBHP in the postischemic brain [19] is due to the combination of its anti-excitotoxic and anti-inflammatory effects. HBHP suppressed LCM-induced nitrite production in HMGB1-ablated primary microglial cultures to 59.9±0.9% and suppressions of LCM-induced nitrite production in A box- or HMGB1 antibody-treated cells were 46.2±2.3% and 50.5±3.3%, respectively. These results indicate that although HMGB1 plays a crucial role in inflammation, factors localized in LCM other than HMGB1 might also play a role. Regarding binding region(s) on HMGB1, competition with recombinant HMGB1 A box in a pull-down experiment revealed that HBHP probably interacts with HMGB1 A box (Fig. 4C), which concurs with our previous result that was obtained under cell free conditions [19]. However, we do not exclude the possibility that different sites of HMGB1 are also involved in HBHP-HMGB1 binding and that site involvements depend on the cell and/or activating stimulus types, since relationship between differential modifications of HMGB1 and its specific function has been reported [21, 22] and these modifications might affect the interaction with HBHP.

Accumulating evidence indicate that HMGB1 forms complexes
with various exogenous or endogenous molecules. For example, HMGB1 binds to LPS, IL-1β, and ssDNA, and the resulting complexes act as inflammatory inducers or enhancers [23-25]. In addition, an interaction between HMGB1 and integrin αvβ3 and phosphatidylserine has been reported to be involved in the modulation of macrophage phagocytic activity [26, 27]. Interactions between HMGB1 and LPS, IL-1β, integrin αvβ3, and phosphatidylserine usually occur in the extracellular space, after passive HMGB1 release or active secretion. Furthermore, HMGB1 might bind to cytoplasmic proteins, such as, Beclin and src kinase [28, 29]. Here, we speculate that HBHP might interact with extracellular HMGB1 in LCM. (A) LCM was incubated with biotinylated-HBHP (Bt-HBHP, 1 or 5 μg/ml) or with biotinylated-scrambled peptide (Bt-HBHP-sc, 5 μg/ml) for 4 hrs and pull-down assays were performed using streptavidin agarose beads. Amounts of HMGB1 were determined by immunoblotting using anti-HMGB1 antibody. (B) LCM was collected from HMGB1 siRNA (HMGB1-; 40 pM)- or nonspecific siRNA (NS-; 40 pM)-transfected microglia cultures and pull-down assays were performed after incubating LCMs with biotinylated-HBHP. (C) Biotinylated-HBHP (5 μg/ml) was incubated with LCM in the presence of 1, 5, or 10 μg/ml of HMGB1 A box or 5 or 10 μg/ml of HMGB1 B box and pull-down assays were performed. Input controls before immunoprecipitations were presented by immunoblotting with anti-HMGB1. Results are representative of three independent experiments.

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