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Moesin is Involved in Migration and Phagocytosis Activities of Primary Microglia

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Moesin is a member of the ezrin, radixin and moesin proteins that are involved in the formation and/or maintenance of cortical actin organization through their cross-linking activity between actin filaments and proteins located on the plasma membranes as well as through regulation of small GTPase activities. Microglia are immune cells in the central nervous system. They show dynamic reorganization of the actin cytoskeleton in their process elongation and retraction as well as phagocytosis and migration. They also secrete proinflammatory cytokines such as tumor necrosis factor−α (TNF−α). Moesin is the predominant ezrin, radixin and moesin family protein in microglia. Recently, we found that moesin is involved in microglial activation accompanying morphological changes and reorganization of actin cytoskeleton by using moesin knockout mice *in vivo* and *ex vivo*. Here we studied the effects of a small molecule inhibitor specific for ezrin and moesin, NSC305787, on the moesin phosphorylation, phagocytosis, migration, and TNF−α secretion of the primary microglia. NSC305787 at the concentration of 10 μM inhibited the moesin phosphorylation, UDP-induced phagocytosis, ADP-induced migration and lipopolysaccharide-induced TNF−α secretion without effect on cell viability. These results in combination with the previous results using moesin knockout mice suggest the functional importance of moesin in microglial activities.

Key words microglia, moesin, actin cytoskeleton, phagocytosis, migration

INTRODUCTION

Microglia, immune cells resident in the central nervous system, contain numerous long and branched processes and continuously survey their environment to identify abnormalities in surrounding cells under normal or resting conditions. In responding to injury, they retract their processes, induce hypertrophy of their cell bodies, and rapidly migrate toward the site of injury. They also secrete a number of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), and cytotoxic molecules such as nitric oxide (NO) and reactive oxygen species as well as neuroprotective molecules such as interleukin-10.[1] They are also engaged in the clearance of dead and dying neurons and neuronal debris by phagocytosis, which is crucial to the maintenance of brain functions.[2] Such process elongation and retraction and phagocytosis are driven by dynamic reorganization of the actin cytoskeleton.[3]

Moesin is a member of ezrin, radixin and moesin (ERM) proteins, which are involved in the formation and/or maintenance of cortical actin organization through their cross-linking activity between actin filaments and proteins and/or phospholipids located on the plasma membranes.[4] ERM proteins also regulate the function of small GTPases including Rac, Rho, and Cdc42.[4,5] Consequently, ERM proteins in general play important roles in the formation of microvilli, filopodia, uropods, and ruffling membranes where actin filaments are associated with plasma membranes.[6]

Moesin is the predominant ERM protein in microglia. Microglia exhibit dynamic reorganization of the actin cytoskeleton as mentioned above. Recently we reported that moesin is involved in microglial activation accompanying morphological changes and reorganization of actin cytoskeleton by using moesin knockout (Msn-KO) mice *in vivo* and *ex vivo*.[7] Here, we studied the roles of moesin in phagocytosis, migration, TNF−α secretion of primary cultured microglia by using a small molecule NSC305787, which directly interacts with ERM proteins, especially ezrin and moesin, and inhibits their function.[8] We newly found that the phagocytosis and migration as well as TNF−α secretion activities were inhibited by NSC305787.

MATERIALS AND METHODS

Animals All works with animals in this study were performed with approval of the Animal Ethics Committees of Ritsumeikan University (BKC2017-055). All of the experiments followed the guidelines of the Committees.

Antibodies and Reagents Anti-phospho–Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) (48G2) rabbit monoclonal antibody (#3726), anti-ERM antibody (rabbit) (#3142), and anti-moesin antibody (rabbit) (Q480) were purchased from Cell Signaling Technologies (Danvers, MA, USA). Anti-ionized calcium-binding adapter molecule (Iba1) antibody (rabbit) (019-19741) was from FUJIFILM Wako Pure Chemical (Osaka,

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Japan). Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (A11008) was from Thermo Fischer Scientific (Waltham, MA, USA). NSC305787 (CAS NO. 785718-37-8) was purchased form COSMO Bio. (Tokyo, Japan). Fluoresbrite Carboxylate Microspheres fluorescent beads (6 μm) were purchased from Polysciences Inc. (Warrington, PA, USA). CellTiter-Blue Cell Viability Assay was purchased from Promega (Madison, WI, USA).

Preparation of Primary Cultured Microglia and Immunofluorescence Analysis Primary cultured microglia were prepared from newborn mice as described previously.[7,9] Microglia cultured on cover slips were fixed in 4% paraformaldehyde at room temperature for 15 min and washed three times with phosphate-buffered saline (PBS) containing 10 mM glycine. Cells were permeabilized in a permeabilization buffer (PBS containing 0.1% Triton X-100) at room temperature for 5 min. The coverslip was blocked by pre-incubating in PBS containing 3% BSA at room temperature for 10 min. All antibody incubations were carried out using Can Get Signal Immunostain Solution A (Toyobo, Osaka, Japan). The coverslip was incubated overnight at 4°C with anti-Iba1 (1:100 dilution) antibody, followed by wash three times with PBS containing 0.1% BSA three times. Alexa Fluor 488 goat anti-rabbit IgG (H+L) (A11008, 1:100 dilution) was incubated with the cells on the coverslip for 30 min at room temperature. After a final PBS wash, the coverslips were mounted with fluorescent mounting medium (Funakoshi, H-1000, Tokyo, Japan) and examined using a confocal laser scanning microscope (FV-10i, Olympus, Tokyo, Japan). Usually, cultures were found to contain 90% microglia judging from the immunofluorescence analysis with an antibody against Iba1.

Cell Viablity Primary microglia (5 x 10^4 cells/well) were seeded on 96 well plate. After overnight culture, NSC305787 was added to the plate. After 24 h incubation, the cell viability was validated by using CellTiter-Blue Cell Viability Assay (Promega) based on the reduction of resazurin to resorufin by viable cells following the manufacturer's instruction.

Phagocytosis Assay Phagocytosis was examined with Fluoresbrite Carboxylate Microspheres beads as described previously.[7] Microglial cells (2 x 10^5 cells) were cultured in DMEM containing 10% FBS on poly-D-lysine-coated 24-well plates overnight and incubated with serum-free DMEM in the presence or absence of NSC305787 for 1 h. The cells were then incubated with the FBS-coated Fluoresbrite Carboxylate Microspheres fluorescent beads (6 μm diameter) in serum-free DMEM at 37°C for 45 min with or without 10 μM UDP. After the cells were fixed and permeabilized, they were stained with an anti-Iba1 antibody. The number of fluorescent microspheres incorporated in microglia was counted under the fluorescent microscope and the confocal laser scanning microscope.

Migration Migration was examined in Boyden chambers as described previously.[7] In short, primary microglia (5 x 10^4 cells/well) suspended in serum-free DMEM in the presence or absence of NSC305787 were plated on fibronectin-coated Costar Transwell® plates (6.5 mm diameter insert, 8.0 μm pore size, polycarbonate membrane filter; Corning Inc.), and allowed to migrate to the bottom chamber containing either 0 or 10 μM ADP for 2 h at 37°C. Following migration, cells that migrated across the filter were fixed, and stained with Giemsa solution. The number of cells was counted using a phase contrast microscopy.

Western Blotting of Cell Lysate Primary microglia (5 x 10^4 cells/well) in 96 well plates were cultured in DMEM containing 10% FBS for 1 h, followed by incubation with serum-free DMEM in the presence or absence of NSC305787 for 2 h. The cells were washed with PBS, homogenized with the lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% Nonidet-P40 with protease inhibitor cocktail) and centrifuged at 16,000 x g for 20 min, and the supernatant was used as cell lysate. SDS-PAGE and Western blotting were performed as described previously.[7]

ELISA of TNF−α Primary microglia (5 x 10^4 cells/well) suspended in DMEM containing 10% FBS were seeded in 96 well plates, and incubated with 1 μg/mL LPS for 24 h at 37°C. Supernatants were collected and assayed for TNF−α production using the Mouse TNF−α Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

Statistical Analysis Statistical analysis was performed by Student t-test. The confidence limit of $p < 0.05$ was considered to be statistically significant. The results are expressed as the means ± SEM.

RESULTS

NSC305787 did not affect the cell viability of primary microglia up to 10 μM (data not shown) as reported in the experiments using HUVEC and osteosarcoma K7M2 cells.[8] Therefore, we studied the inhibitory effects of NSC305787 on phosphorylation of moesin, phagocytosis, migration, and TNF−α secretion at concentrations up to 10 μM.

Phosphorylation of ERM Proteins The primary microglia abundantly express moesin whereas they express very small amounts of ezrin and radixin as shown in the previous study.[7] At first we studied the effect of NSC305787 on the phosphorylation of moesin by Western blotting. ERM proteins in general are activated by the phosphorylation of threonine residue (Thr^{558} in moesin) located on their carboxy-terminal domain. As shown in Fig. 1, moesin was phosphorylated spontaneously even in the absence of

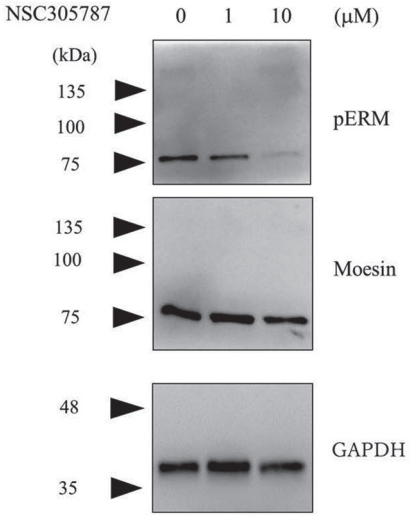

Fig. 1. Phosphorylation of Moesin was Inhibited by NSC305787

Microglia was incubated with 1 and 10 μM NSC305787 in FBS-free DMEM at 37°C for 2 h, and the cell lysate (10 μg) was blotted with an anti-phospho–Ezrin/Radixin/Moesin antibody (upper lane), anti-moesin (middle lane), and anti-GAPDH antibody (bottom), respectively.

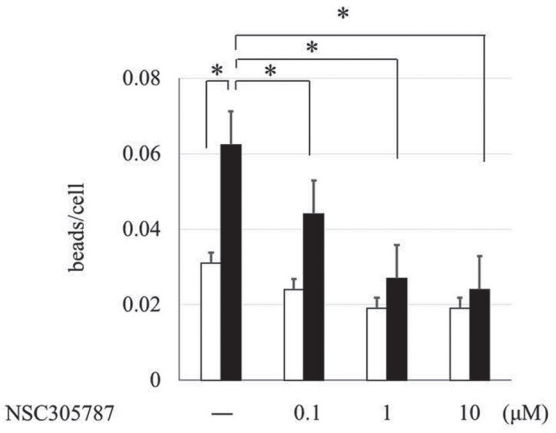

Fig. 2. Microglial Phagocytosis was Inhibited by NSC305787

Primary microglia (2 x 10^5 cells/well) cultured overnight were incubated with serum-free DMEM in the presence or absence of 0.1, 1 and 10 μM NSC305787 at 37°C for 1 h, and the phagocytosis of fluorescent beads was measured following the treatment with or without 10 μM UDP for 45 min. The number of fluorescent beads per microglia was counted in the presence and absence of 10 μM UDP, and shown by white (without UDP) and black columns (with UDP), respectively. 150-200 cells were studied in each experiment. Results are shown as mean ± SEM. *P < 0.05 vs. control (in the absence of NSC305787).

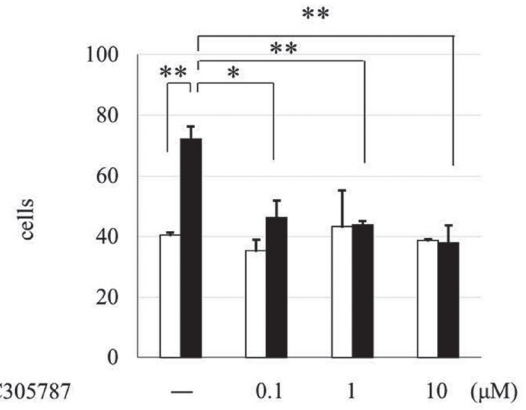

Fig. 3. Microglial Migration Toward ADP was Inhibited by NSC305787

Primary microglia were incubated with 0.1, 1 and 10 μM NSC305787 at 37°C for 2 h, and allowed to migrate towards 0 (control medium) or 10 μM ADP for 2 h in the Boyden chamber, and the number of migrated cells was counted. The numbers of migrated microglia are shown by white (without ADP) and black columns (with ADP), respectively. Results are shown as mean ± SEM. (N=3). *P < 0.05 vs. control, **P < 0.01 vs. control.

stimulating agonists. This finding is in good agreements with the report that primary microglia are activated to a certain extent during the procedures involved in preparing them for culture.[10] NSC305787 at the concentrations of 1, 10 μM partly inhibited the phosphorylation of moesin.

Phagocytosis Assay Phagocytosis is one of the most important physiological functions of microglia and is the process that mediates the uptake of amyloid−β (Aβ) fibrils by actin-based mechanisms.[1,11] The number of fluorescent microbeads taken up by primary microglia was significantly increased in the presence of 10 μM UDP. In order to study the role of moesin in this process, we compared the phagocytotic activity in the presence and absence of 0.1, 1 and 10 μM NSC305787. NSC305787 at 10 μM slightly inhibited basal phagocytotic activity in the absence of UDP (Fig. 2). This inhibitor at the concentrations of 0.1 μM partly inhibited the UDP-stimulated phagocytotic activity. The phagocytotic activity was almost completely inhibited by 1 and 10 μM of this compound (Fig. 2). These results suggest that moesin is involved in microglial phagocytotic activity.

Migration Extracellular ADP induces cell migration behavior consistent with chemo-attraction.[12] Figure 3 shows the numbers of microglia that migrated toward serum-free DMEM (control) and ADP in the Boyden chamber in the presence or absence of 0.1, 1 and 10 μM NSC305787. The number of the cells migrated toward serum-free DMEM was unchanged in the presence or absence of 0.1, 1 and 10 μM NSC305787. The number of migrated cells increased in the presence of ADP as reported previously.[7] The number of migrated cells was partly decreased in the presence of 0.1 μM NSC305787, and almost completely inhibited by 1 and 10 μM of this compound. These results suggest that moesin is also important for the regulation of migration.

TNF−α Secretion TNF−α secretion was measured in the presence and absence of 1 μg/mL LPS. The secretion which was increased in the presence of LPS was partly inhibited (70% inhibition) in the presence of 10 μM NSC305787 (Fig. 4). These results suggest that moesin is important for the TNF−α secretion. However, the inhibitory effects of this compound on TNF−α secretion seems to be weaker than the effects on phagocytosis and migration.

DISCUSSION

Microglia show dynamic reorganization of their actin cytoskeleton from the resting ramified to the activated ameboid morphology. Cultured primary microglia have quite different properties compared with microglia in brain. For example, primary microglia *in vitro* do not have the ramified structure typically seen in the normal brain. However, many of the phenotypes associated with brain microglia can be observed in the primary microglia.[13] They migrate

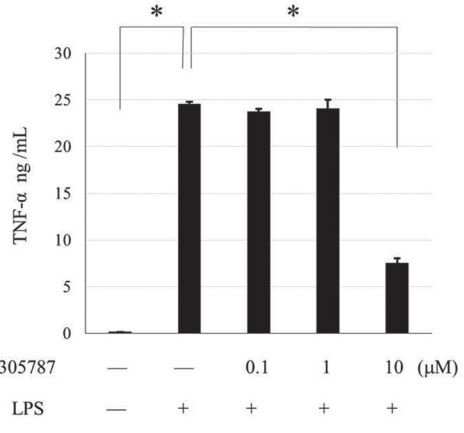

Fig. 4. TNF-α Secretion Up-regulated by Treatment with LPS was Inhibited by NSC305787

Primary microglia were incubated with or without 1 μg/mL LPS in the presence or absence of 0.1, 1 and 10 μM NSC305787 at 37°C for 24 h, and TNF-α released was measured by ELISA. TNF-α concentration is shown by white (without LPS) and black columns (with LPS), respectively. Results are shown as mean ± SEM. (N=3). *P < 0.01 vs. control (in the absence of NSC305787).

toward ADP and ATP, which are signaling factors ("find-us" signal) released from damaged or dead cells. They phagocytose particles in the presence of UDP, which is also released from damaged or dead cells as the "eat-us" signal.[14] They also secrete TNF−α and NO by the treatment with LPS.[13]

Moesin is a member of the ERM protein family whose members are reported to be involved in the formation and/or maintenance of cortical actin organization.[4,5] They are activated by phosphorylation by Rho kinase and also involved in neurite and axonal outgrowth, morphological rearrangement, and motility of primary neurons.[15-18] Among the ERM proteins, moesin is involved in polarization and chemotaxis in neutrophils through the interactions with small G proteins, Rac, Rho, and Cdc-42.[19] In resting neutrophils, moesin is activated and prevents cell polarization by inhibiting the small GTPases whereas it is deactivated by attractant-sensitive myosin phosphatase to break symmetry and establish polarity. Moesin is the predominant ERM protein in microglia which express very low levels of ezrin and radixin as shown in our previous study.[7] Recently, we found that moesin is involved in their activation accompanying morphological changes and reorganization of actin cytoskeleton by using Msn-KO mice.[7] In our previous report, microglial activation after systemic LPS application *in vivo* is partly inhibited in Msn-KO mice compared with the wild-type mice. Furthermore, the Msn-KO primary microglia showed higher phagocytotic activity in the absence of UDP, which was not further increased by the treatment with UDP. They also exhibited decreased ADP-stimulated migration activities compared with the wild-type microglia.[7] To further confirm the roles of moesin in microglial activation, here we studied the effects of a small molecule inhibitor of ezrin and moesin, NSC305787 on the phenotypes accompanying their morphological changes and reorganization of the actin cytoskeleton from the different point of view.

Primary microglia phagocytose particles in response to treatment with 10 μM UDP. In our previous study, we showed that the phagocytosis activity was not induced by UDP in the Msn-KO microglia.[7] Here, we newly found that the phagocytosis activity stimulated by 10 μM UDP was inhibited by 1 μM NSC305787. Primary microglia also migrate toward ATP and ADP. Previously, we showed that the migration activity of Msn-KO microglia was significantly lower compared with the wild-type microglia.[7] Here, we newly found that the migration activity stimulated by ADP was inhibited by 1 μM NSC305787.

In this study, we examined the effect of NSC305787 on the TNF−α secretion activity. Low concentration of NSC305787 did not affect the TNF−α secretion whereas high concentration (10 μM) of this drug partly inhibited the secretion. It should be noted that Msn-KO mice retained TNF−α secretion activity.[7] At present, it is difficult to reconcile these two results. The possibility that high concentration (10 μM) of NSC305787 may have non-specific activity other than the inhibitory effect on moesin. The precise molecular mechanisms through which NSC305787 exerts these effects will be explored more extensively in a future study.

Conclusions In combination with our previous results using Msn-KO microglia, the present results suggest that moesin is important for UDP-induced phagocytosis and ADP-induced migration of primary microglia accompanying actin cytoskeleton reorganization.

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Conflict of interest The authors declare no conflict of interest.

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