Mast Cell Tryptase Induces Microglia Activation via Protease-activated Receptor 2 Signaling

Shu Zhang^{1*}, Xiaoning Zeng^{1*}, Haiwei Yang¹, Gang Hu² and Shaoheng He¹

¹Clinical Research Center, the First Affiliated Hospital of Nanjing Medical University, Nanjing, ²Jiangsu Key Laboratory of Neurodegeneration, Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu, *Both authors contributed equally to this work

Key Words

Tryptase • Microglia activation • Protease-activated receptor 2 • MAPK • NF- κ B

Abstract

Background: Mast cell tryptase can stimulate peripheral mononuclear cells activation to cause widespread inflammation. However, the influence of tryptase on microglia, the resident immune cells in the brain, remains uninvestigated. Since microglia plays a pivotal role in immune surveillance of CNS, we studied the effect of tryptase on microglia activation. **Methods:** Induction of microglia activation by tryptase was examined with primary cultured microglia. TNF- α and IL-6 was measured with a commercial ELISA kit. Intracellular ROS was determined by dichlorodihydrofluorescein oxidation. Mitochondrial membrane potential was assessed with the MitoProbeTM JC-1 assay kit. And MAPK and NF- κ B phosphorylation were evaluated by Western blot. **Results:** We found that tryptase stimulated microglia activation and subsequently produced pro-inflammatory factors TNF- α , IL-6 and ROS. Inhibition of PAR-2 activation reduced tryptase-induced TNF- α , IL-6 and ROS production, and

mitochondrial membrane potential loss in microglia. Among the three members of MAPK pathway, ERK and p38, but not JNK mediated tryptase-induced microglia activation. Inhibition of PAR-2 suppressed tryptase-induced ERK and p38 MAPK pathway activation in microglia. Tryptase also activated NF- κ B within 30 min, and ammonium pyrrolidinedithiocarbamate, an inhibitor of NF- κ B, reduced tryptase-induced TNF- α and IL-6 release. **Conclusions:** Our results suggest that tryptase can induce microglia activation and pro-inflammatory mediator release via PAR-2-MAPK-NF- κ B signaling pathway, which will contribute to the development of microglia-mediated inflammation in brain.

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Introduction

Mast cells, best known for their role in allergic inflammation, are distributed in a variety of anatomical sites, including the central nervous system (CNS), where they are often found adjacent to blood vessels and nerves [1, 2]. In addition, mast cells are an important source of

several inflammatory mediators, including proteases and vasoactive amines such as histamine. Mast cell tryptase is a major secretory protein of human mast cells, which is elevated in the CSF of patients with multiple sclerosis (MS) [3]. It can stimulate peripheral mononuclear cells to secrete TNF- α and IL-6 [4], as well as activate PAR-2 to induce widespread inflammation [5].

PARs are a family of G protein-coupled receptors that are widely expressed on neurons and glial cells in the nervous system [6]. Among the four PARs identified to date, PAR-1, PAR-3, and PAR-4 can be activated by thrombin, whereas trypsin and mast cell tryptase can activate PAR-2 [7, 8]. The role of PAR-2, which is widely distributed throughout the nervous system, has been principally investigated in the peripheral nervous system, where it is known to play major roles in injury, inflammation, neuronal signaling, and nociception [9, 10]. McLarty reported that tryptase/ PAR 2 interactions induce selective mitogen-activated protein kinase signaling and collagen synthesis by cardiac fibroblasts [11]. PAR-2 is also known to be expressed on neurons, astrocytes and microglia in CNS, and several studies have implicated that it is involved in the pathogenesis of ischemia and neurodegeneration [12, 13]. In cultured rat primary microglia, activation of PAR-2 has been found to induce microglia activation [14].

Microglia, the resident immune cells in the brain, plays a pivotal role in immune surveillance of CNS. Reactive microglia has been implicated to contribute to the pathogenesis of a broad range of CNS disorders. These include not only infectious CNS diseases but also acute CNS injuries such as traumatic brain injury (TBI) [15], spinal cord injury [16], stroke, and brain ischemia [17]. Increasing evidence has demonstrated that microglia-mediated neuroinflammatory process is critically involved in the initiation and development of neurodegenerative disorders, such as PD [18], Alzheimer's disease (AD) [19] and MS [20]. When subjected to abnormal stimulation, such as neurotoxins, neuronal debris, or injury, microglia become gradually activated and produce a host of factors, including TNF- α , prostaglandin E₂ (PGE₂), IL-6, nitric oxide (NO), and reactive oxygen species (ROS). Thus, inhibition of microglia activation and subsequent inflammatory process may identify novel therapeutic strategies to eliminate microglia deleterious effects.

Yuan reported that mast cell activation had the capacity to promote the expression of P2X₄R and BDNF in microglia [21]. However, whether tryptase can activate microglia has not yet been studied. In the present study,

we investigate the possibility that tryptase could trigger microglia activation through PAR-2. To elucidate this, we examined whether (1) tryptase activates microglia and induces the production of TNF- α , IL-6 and ROS; (2) tryptase-induced microglial activation through PAR-2 involves the MAPK and NF- κ B pathways.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco-BRL (Grand Island, NY, USA). Tryptase, PD98059, SP600125, SB203580, PDTC and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). PAR-2 inhibitor FSLLRY-NH₂ were synthesized by CL Bio-Scientific Inc. (Xi An, China). Rat IL-6 Immunoassay Kit and Rat TNF- α Immunoassay Kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). LIVE green reactive oxygen species detection kit and MitoProbe™ JC-1 assay kit were purchased from Molecular Probes Invitrogen (Carlsbad, CA, USA). Specific OX-42 antibody (a marker for microglia) was purchased from AbD Serotec (Raleigh NC, USA). Specific monoclonal antibodies against p44/42 MAPK, Phospho-p44/42 MAPK, p38, Phospho-p38, SAPK/JNK, Phospho-SAPK/JNK and NF- κ B were obtained from Cell Signaling (Beverly, MA, USA).

Microglia-enriched cultures

Rat primary microglia was prepared according to previously described protocol with slight modification [22]. Briefly, tissues from whole brains of postnatal (P1–P2) Sprague-Dawley rats were triturated, and then cells were plated on poly-D-lysine precoated cell culture flasks in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air. After reaching a confluent monolayer of glial cells (10–14 days), microglia were separated from astrocytes by shaking off for 5 h at 100 r.p.m., and replated in 24-well culture plates at a density of 10⁵ cells/cm². The enriched microglia was >98% pure as determined by OX-42-IR.

Cell viability assay

Cell viability was measured by Thiazolyl blue method. Briefly, cells were collected and seeded in 96-well plates at a density of 10⁵ cells/cm². After incubation for 48 h, cells were exposed to fresh medium containing various concentrations of tryptase (0.001, 0.01, 0.1 and 1 μ g/ml) at 37°C. After incubation for up to 24 h, 20 μ l of MTT tetrazolium salt dissolved in Hank's balanced salt solution at a final concentration of 5 mg/ml was added to each well and incubated in CO₂ incubator for 4 h. Finally, the medium was aspirated from each well and 150 μ l of DMSO was added to dissolve the formazan crystals and the absorbance of each well was obtained using a Model 680 microplate reader (Bio Rad., USA) at test and reference wavelengths of 570 and 630 nm, respectively.

TNF-alpha and IL-6 assay

The amount of TNF-alpha and IL-6 in the culture medium was measured with commercial ELISA kits from R&D Systems, respectively.

Intracellular reactive oxygen species assay

The production of intracellular reactive oxygen species (ROS) was measured by DCFH oxidation. The dichlorodihydrofluorescein diacetate (DCFH-DA) reagent passively enters cell where it is de-acetylated by esterase to nonfluorescent DCFH. Inside the cell, DCFH reacts with ROS to form DCF, the fluorescent product. For this assay, 10 mM of DCFH-DA was dissolved in methanol, and was diluted 500-fold in HBSS to give 20 μ M of DCFH-DA. Enriched-microglia cultures seeded (5×10^4) in 96-well plates were then exposed to DCFH-DA for 1 h, followed by treatment with HBSS containing various concentrations of tryptase for 2 h. After incubation, the fluorescent signal from dichlorofluorescein (DCF; excitation 495 nm, emission 529 nm) was registered every 2 min for up to 20 min of incubation in a Perkin-Elmer LS-5B Luminescence Spectrometer (Perkin-Elmer, Oak Brook, IL, USA) [23]. To calculate the amount of intracellular ROS produced, the mean control treatment was subtracted from the mean treatment group. Four independent sets of samples were analyzed.

Measurement of microglia mitochondrial membrane potential

Microglia mitochondrial membrane potential ($\Delta\Psi$ m) was assessed with the MitoProbe™ JC-1 assay kit. JC-1, a cationic dye, exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration dependent formation of red fluorescent J-aggregates. Microglia, suspended in 1 ml PBS at approximately 1×10^6 cells/ml, was incubated with 2 μ M of JC-1 at 37°C for 15 min, and was analyzed on a flow cytometer with 488 nm excitation using emission filters appropriate for Alexa Fluor 488 dye and R-phycoerythrin. A decrease in the ratio of red/green fluorescence intensity was interpreted as loss of $\Delta\Psi$ m, whereas an increase in the ratio was interpreted as gain in $\Delta\Psi$ m.

Western Blotting

Cells were collected and homogenized in 200 μ l of lysing buffer. After incubation for 20 min on ice, cell lysate was centrifuged and protein concentration in the extracts was determined by the Bradford assay. Proteins in cell extracts were denatured with sodium dodecyl sulfate (SDS) sample buffer and separated by 10% SDS–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes by using a Bio-Rad miniprotein-III wet transfer unit. The membranes were incubated with 5% BSA dissolved in Tris-buffered saline with Tween 20 (TBST) (pH 7.5, 10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20) at room temperature for 1 h. This was followed by incubating the membranes with different antibodies (JNK, phosphor-JNK; ERK, phosphor-ERK; p38, phosphor-p38; NF- κ B and phosphor-NF- κ B

B at 1:1000) overnight at 4°C. After adding the secondary antibody (1:1000) for 1 h, the protein bands on the membranes were detected with an enhanced chemiluminescence kit.

Statistical Analysis

All values shown are means $\pm$ SEM. The significance of the difference between control and samples treated with various drugs was determined by one-way ANOVA followed by the post-hoc least significant difference test. Differences were considered significant at $P < 0.05$.

Results

Tryptase induced microglia activation

Cell survival measured by 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) analysis revealed that incubation with tryptase (0.001, 0.01, 0.1 and 1 μ g/ml) for 24 h had no effect on microglia viability (data not shown).

The amoeboid morphological change was hallmark of microglia activation. Typically, ‘resting’ microglia are characterized by ramified and either bipolar or unipolar processes, whereas activated microglia become round with enlarged and amoeboid cell bodies. After incubation with tryptase (0.001, 0.01, 0.1 and 1 μ g/ml) for 24 h, most microglia was activated at a minimum effective dose of 0.01 μ g/ml, indicated by dramatic amoeboid morphological changes (Fig. 1). These results suggest that mast cell tryptase is able to activate microglia.

Tryptase induced TNF-alpha, IL-6 and ROS production in microglia in vitro

Since microglia-mediated neuroinflammation is mainly owing to oversecretion of proinflammatory and cytotoxic factors from activated microglia, the levels of proinflammatory factors in the supernatant were determined. The time course study showed that tryptase (0.1 μ g/ml)-induced release of TNF-alpha from microglia began at 0.5 h and lasted at least until 24 h (Fig. 2A). However, release of IL-6 induced by tryptase (0.1 μ g/ml) initiated at 2 h following incubation (Fig. 2B), and ROS production induced by tryptase (1 μ g/ml) started at 0.5 h following incubation (Fig. 2C).

As shown in Fig. 3, incubation with tryptase at 0.001, 0.01, 0.1 and 1 μ g/ml for 24 h produced a concentration dependent increase in TNF-alpha secretion from microglia with a minimum effective dose of 0.01 μ g/ml (Fig. 3A). Similarly, tryptase (0.001, 0.01, 0.1 and 1 μ g/ml) also increased the IL-6 production at a minimum effective dose of 0.01 μ g/ml (Fig. 3B). However, a minimum effective dose of tryptase for induction of ROS secretion

Fig. 1. Effects of tryptase (Tt) on the morphological changes of microglia. ‘Resting’ microglia are characterized by ramified and either bipolar or unipolar processes, whereas activated microglia become round with enlarged and amoeboid cell bodies. Tryptase (0.001, 0.01, 0.1, 1 $\mu\text{g/ml}$) was incubated with microglia at 37°C for 24 h. Con = control. Scale bar: 50 μm .

from the microglia was at 1 $\mu\text{g/ml}$ (Fig. 3C). These results indicate that tryptase is capable of inducing TNF- α , IL-6 and ROS secretion from microglia.

Inhibition of tryptase-induced TNF- α and IL-6 production by an antagonist of PAR-2

As shown in Fig. 3, PAR-2 inhibitor FSLLRY-NH₂ (400 μM) alone failed to affect the productions of TNF- α , IL-6 and ROS in microglia. However, FSLLRY-NH₂ (400 μM) was able to inhibit tryptase (0.1 and 1 $\mu\text{g/ml}$) induced TNF- α and IL-6 increase in microglia (Fig. 3A, B). FSLLRY-NH₂ had no effect on tryptase-induced ROS production in microglia (Fig. 3C). These results indicate that tryptase is able to induce TNF- α and IL-6 release from microglia via PAR-2.

Inhibition of tryptase-induced mitochondrial membrane potential loss in microglia by an antagonist of PAR-2

As mitochondrial membrane potential ($\Delta\Psi_m$) change has been demonstrated to be involved in microglia activation and production of proinflammatory factors, molecular probe JC-1 was used to detect the effect of tryptase on mitochondrial membrane potential variation in microglia. As shown in Fig. 4, after exposure to tryptase (0.1 $\mu\text{g/ml}$) for 30 min, most microglia displayed a loss or collapse of $\Delta\Psi_m$ indicated by fluorescence of JC-1 shifted from red-orange to greenish-yellow. The fluorescence ratio of red-orange to greenish-yellow was decreased by 42% of that in the control. PAR-2 inhibitor FSLLRY-NH₂ (400 μM) alone failed to affect the mitochondrial membrane potential. However, FSLLRY-NH₂ (400 μM) prevented tryptase-induced mitochondrial membrane potential loss in microglia. These data indicate that tryptase may induce mitochondrial membrane depolarization in microglia via PAR-2.

Fig. 2. Time courses of tryptase induced release of TNF- α (A), IL-6 (B) and reactive oxygen species (ROS) (C). Tryptase at 0.1 $\mu\text{g/ml}$ (for TNF- α and IL-6) and 1 $\mu\text{g/ml}$ (for ROS) was incubated with microglia at 37°C for 0.5, 2, 6 and 24 h, respectively. ROS was presented as changes of DCF fluorescence. * $P < 0.05$, ** $P < 0.01$ vs. the response to medium alone. Data are presented as the mean $\pm$ SEM of four independent experiments.

Fig. 3. Effects of tryptase (Tt) on release of TNF- α (A), IL-6 (B) and reactive oxygen species (ROS) (C) from microglia. Tryptase at 0.001, 0.01, 0.1 and 1 μ g/ml was incubated with microglia in the presence or absence of FSLRY-NH₂ (FS, 400 μ M) an antagonist of tryptase at 37°C for 24 h before culture supernatant being collected. ROS was presented as changes of DCF fluorescence. * P < 0.05, ** P < 0.01 vs. control groups (Con), # P < 0.05 vs. tryptase treatment groups. Data are presented as the mean $\pm$ SEM of four independent experiments.

Inhibition of tryptase-induced ERK and p38 MAPK activation in microglia by an antagonist of PAR-2

In order to further understand the actions of tryptase on microglia, we examined influence of tryptase on phosphorylation of cell signaling molecules. Tryptase at 0.1 μ g/ml activated ERK (p44/42) and p38 MAPK, but not JNK, which was confirmed by increased

Fig. 4. PAR-2 antagonist FSLRY-NH₂ (FS, 400 μ M) alleviates tryptase (Tt, 0.1 μ g/ml)-induced mitochondrial membrane potential loss in microglia. Quantification of mitochondrial membrane potential expressed as a ratio of J-aggregate to JC-1 monomer (red : green) fluorescence intensity. * P < 0.05, ** P < 0.01 vs. control groups (Con), # P < 0.05 vs. tryptase treatment groups. Data are presented as the mean $\pm$ SEM of four independent experiments.

phosphorylation of tyrosine residues of these kinases as determined by Western blot analysis. The time course experiments showed that treatment with tryptase (0.1 μ g/ml) led to a rapid and transient phosphorylation of ERK and p-38 MAPK with the peak levels of phospho-ERK occurring at 30 min, and phospho-p-38 occurring at 15 min, respectively. However, tryptase (0.1 μ g/ml) failed to affect the level of phospho-JNK (Fig. 5A). Microglia

Fig. 5. Western blot analysis of effects of PAR-2 antagonist and inhibitors of cell signaling pathways on tryptase-induced ERK/p38/JNK MAPK activation and TNF-alpha and IL-6 production in microglia. A: Tryptase activates ERK and p38, but not JNK, which is assessed by increased phosphorylation of tyrosine residues of these kinases. Microglia was treated with 0.1 µg/ml of tryptase for the indicated time periods. B: A PAR-2 antagonist FSLRY-NH₂ (Fs, 400 µM) suppressed tryptase (Tt)-induced ERK and p38 MAPK activation in microglia. C: PD98059 (PD, 10 µM), SB203580 (SB, 10 µM) and SP600125 (SP, 10 µM) alone failed to affect the productions of TNF-alpha and IL -6 in microglia. D: Pretreatment with PD98059 and SB203580 for 30 min inhibited the Tt-induced TNF-alpha and IL-6 production in microglia, but SP600125 failed to affect it. Activated ERK (p-ERK), p38 (p-p38), and JNK/SAPK (p-JNK) species were detected by immunoblot analysis with antibodies specific for the phosphorylated forms of each kinase. The amount of protein loaded in each lane was confirmed by measuring the amount of ERK, p38 and JNK reacted to the antibody against the unphosphorylated form of each kinase. **P* < 0.01, ***P* < 0.001 vs. control groups, ##*P* < 0.001 vs. Tt (0.1 µg/ml) treatment groups. Data are presented as the mean ± SEM of four independent experiments.

were pretreated with PAR-2 antagonist FSLRY-NH₂ (400 µM) for 30 min and then exposed to tryptase (0.1 µg/ml) for another 30 min or 15 min for detecting phosphorylation of ERK or p-38 MAPK, respectively. PAR-2 antagonist FSLRY-NH₂ alone had no effect on the phosphorylation of ERK and p-38, but it suppressed tryptase-induced ERK and p38 MPAC activation in microglia. (Fig. 5B). These results indicate that tryptase activates ERK and p-38 MAPK signaling pathways via PAR-2 in microglia.

Inhibition of tryptase-induced TNF-alpha and IL-6 release from microglia by inhibitors of ERK or p38 MAPK

To investigate the involvement of MAPK signaling pathways in the TNF-alpha and IL-6 production, we used

pharmacological inhibitors of ERK, p38, and JNK pathways. PD98059 a potent, cell-permeable, and selective inhibitor of MEK1, which results in the inhibition of the phosphorylation and activation of ERK1/2. SB203580 is a pyridinyl imidazole compound which acts as a competitive inhibitor of ATP binding on the p38 kinase, and thus serves as a specific inhibitor of p38 MAPKs. SP600126 is a potent, selective, reversible, and cell-permeable inhibitor of JNK. As shown in Fig. 5C, PD98059, SB203580 and SP600125 alone failed to affect the productions of TNF-alpha and IL -6 in microglia. PD98059 and SB203580, but not SP600126 significantly reduced TNF-alpha and IL-6 production induced by tryptase (Fig. 5D), indicating that ERK MAPK and p-38 MAPK signaling pathways are involved in the tryptase-induced TNF-alpha and IL-6 production.

Fig. 6. Western blot analysis of effects of PAR-2 antagonist and inhibitor of NF-kappa B on tryptase-induced NF-kappa B activation in microglia. A: Activation of NF-kappa B p65 by tryptase. Microglia was treated with tryptase at 0.1 μ g/ml for the indicated time periods. B: PAR-2 antagonist FSLLRY-NH₂ (FS, 400 μ M) suppressed tryptase (Tt, 0.1 μ g/ml)-induced NF-kappa B p65 activation in microglia. C: PD98059 and SB203580 reduced activation of NF-kB p65 induced by tryptase. D: Pretreatment with PDTC (20 μ M) for 30 min inhibited the Tt-induced TNF-alpha and IL-6 production in microglia. * P < 0.05, ** P < 0.01 vs. control groups, ## P < 0.01 vs. Tt treatment groups. Data are presented as the mean $\pm$ SEM of four independent experiments.

Inhibition of tryptase-induced TNF-alpha and IL-6 release from microglia by inhibitor of NF-kappa B

To investigate the involvement of NF-kappa B pathway in the TNF-alpha and IL-6 production, Western blot analysis of expression of NF-kappa B p65 were performed. Time course study showed that tryptase activated NF-kappa B p65 within 15 min, and was peaked at 30 min (Fig. 6A). PAR-2 antagonist FSLLRY-NH₂ (FS, 400 μ M) prevented tryptase-induced NF-kappa B p65 increase (Fig. 6B). To evaluate the causal link between MAPK and NF-kappa B pathway, we used PD98059 and SB203580 after tryptase treatment on the NF-kB p65 activation. We found that PD98059 and SB203580 used alone failed to affect the NF-kB p65 activation. However, they reduced activation of NF-kB p65 induced by tryptase (Fig. 6C). In order to further examine whether the activation of NF-kappa B was directly related to tryptase-induced TNF-alpha and IL-6 release, a known NF-kappa B inhibitor pyrrolidine dithiocarbamate (PDTC) was applied together with tryptase to microglia. PDTC at 20 μ M significantly reduced the tryptase-induced TNF-alpha and IL-6 production (Fig. 6D), suggesting that NF-kappa B pathway is involved in tryptase-induced TNF-alpha and IL-6 production in rat primary microglia.

Discussion

In this study, we found that PAR-2 was able to mediate tryptase-induced activation of microglia, and production of TNF-alpha and IL-6 from microglia. The most possible signaling pathway of tryptase-induced TNF-alpha and IL-6 production in rat primary microglia is PAR-2 – MAPK – NF-kappa B.

As low as 0.01 μ g/ml of recombinant mast cell tryptase induced an approximately 4.1-fold and 3.7-fold increase in TNF-alpha and IL-6 release, respectively, from microglia, indicating this protease is a potent stimulus. The time course study showed that tryptase-induced TNF-alpha release was earlier than IL-6 release, suggesting that tryptase was more likely to induce the release of TNF-alpha than IL-6. While the knowledge about the effect of tryptase on TNF-alpha and IL-6 release from microglia is limited, a previous study finding that mast cell tryptase can activate peripheral mononuclear cells to secrete TNF and IL-6 [4] may support our current observation. A key pro-inflammatory protein synthesized and released by activated microglia is TNF-alpha [24]. The presence of elevated levels of TNF-alpha has been documented in many forms of neurodegenerative disease of both chronic (e.g., AD, Parkinson's disease, and

amyotrophic lateral sclerosis) and acute types (e.g., stroke and head trauma) [25]. Recent evidence points out a crucial role of IL-6, one of pro-inflammatory proteins released by activated microglia, within CNS [26]. In CNS, IL-6 can trigger inflammation related cellular responses including neurogenesis, gliogenesis, cell growth, cell survival, myelination and demyelination [27-29]. Normally, IL-6 is expressed at relatively low levels in the brain, but it is elevated in the cerebral spinal fluid and brain homogenates in the presence of brain injury or inflammation [30]. In the present study, we also found that tryptase was able to induce enhanced ROS production. ROS is an important molecules secreted by microglia [31]. Considerable amount of evidence suggests oxidative stress induced by microglia-derived ROS is a major contributor to neurodegeneration [32, 33]. We found that the concentration of tryptase, which induced ROS release, was 100 times than that induced TNF-alpha and IL-6 release from microglia, suggesting that tryptase was more likely to cause microglia-mediated inflammatory response than oxidative stress. Since TNF-alpha and IL-6 can potentiate damage of CNS cells [34], our current observation implicates that mast cell tryptase can induce inflammation in CNS by stimulating TNF-alpha and IL-6 release from microglia.

Tryptase induced TNF-alpha and IL-6 release from microglia appeared to at least partially rely on activation of PAR-2 as a specific antagonist peptide of PAR-2 FSLLRY-NH₂ was able to block the action of tryptase on microglia. The report which demonstrated that microglia expresses PAR-2 [14] may provide molecule basis for tryptase working on. Moreover, tryptase has been shown to activate PAR-2 on epithelial as well as endothelial cells and neurons [35, 36], and contribute to inflammation and hypersensitivity reactions [37, 38], further supporting our observation that actions of tryptase on microglia are through activation of PAR-2. Taken the above information together with a report that the expression of PAR-2 was up-regulated during brain damage [39], it is not difficult to anticipate that PAR-2 may play an important regulatory role in the nervous system, such as neurogenic inflammation. The finding that tryptase-induced ROS production was independent on activation of PAR-2 implicates that actions of tryptase on microglia are unnecessarily via PAR-2, it may involve some other mechanisms.

Mitochondrial dysfunction in microglia has been observed in several animal models of neurodegeneration and aging, which may contribute to the detrimental effects of neuroinflammation seen in neurodegenerative diseases

[40]. Most mitochondrial dysfunctional signals are associated with activated microglia [41]. Loss of $\Delta\Psi_m$ indicated the dysfunction of mitochondria, which induced microglia activation and production of proinflammatory factors from microglia [42]. Our finding that FSLLRY-NH₂ abolished tryptase induced mitochondrial membrane depolarization in microglia indicated that mitochondrial dysfunction was involved in the tryptase-induced the microglia activation and pro-inflammatory release.

The downstream MAPKs have been demonstrated to regulate microglia activation and the production of proinflammatory factors from microglia [43-45]. Among them, ERK, p38 and JNK/SAPK are activated by thrombin, LPS, β -amyloid and gangliosides, respectively. However, there are still some discrepancies with regard to the effect of different inhibitors of MAPKs. Thus, LPS- and thrombin-induced nitrite production was significantly reduced by PD98059 and SB203580, whereas ganglioside-induced nitrite production was inhibited by PD98059, but not by SB203580 [45-47]. The intracellular signaling mechanisms that mediate the trypsin induced activation of microglia are ERK and JNK, but not p38 MAPK, as PD98059 and SP600126 significantly reduced nitrite production whereas SB203580 did not affect trypsin-induced NO production [14]. However, in the present study, ERK, p38 MAPK signaling pathways, but not JNK were involved in the tryptase-induced TNF-alpha and IL-6 production. The difference between tryptase and trypsin signaling pathways may be due to that tryptase only activates PAR-2, whereas trypsin activates both PAR-1 and PAR-2.

NF- kappa B is also a common mediator of microglia activation, which can be activated by β -amyloid, interferon-g, and gangliosides [48]. Inhibition of tryptase-induced NF-kappa B activation by FSLLRY-NH₂ indicates that the influence of tryptase on NF- kappa B activation is through activating PAR-2. We found that PD98059 and SB203580 reduced activation of NF- κ B p65 induced by tryptase, suggestion that tryptase activate NF-kappa B by MAPK pathways. In summary, the signaling pathways of tryptase-induced TNF-alpha and IL-6 production seem as followings: activation of PAR-2 – ERK or p38 MAPK phosphorylation – NF- kappa B phosphorylation.

In conclusion, to our knowledge, this is the first study to demonstrate the ability of mast cell tryptase in induction of mitochondrial dysfunction in microglia and activation of microglia via PAR-2. The intracellular signaling mechanisms that mediate the action of mast cell tryptase on microglia are PAR-2 – MAPK – NF-kappa B

pathway. These results implicate that tryptase is likely to act as a proinflammatory mediator in brain through activation of microglia.

Abbreviations

$\Delta\Psi_m$ (mitochondrial membrane potential); CNS (central nervous system); DCF (dichlorofluorescein); DCFH (dichlorodihydrofluorescein); DMEM (Dulbecco's modified Eagle's medium); ERK (extracellular regulated protein kinases); FS (FSLRY-NH₂); IL (interleukin); JNK (c-JUN N-terminal kinase); MAPK (mitogen-activated protein kinases); MS (multiple sclerosis); MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide); NF- κ B (nuclear factor κ B), NO (nitric oxide); PAR (protease-activated receptor), ROS (reactive oxygen species), PD (PD98059); PDTC (pyrrolidinedithiocarbamate); SB (SB203580); SP (SP600125), TBI (traumatic brain injury), TNF (tumor necrosis factor), Tt (tryptase).

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