Norepinephrine Promotes Microglia to Uptake and Degrade Amyloid β Peptide through Upregulation of Mouse Formyl Peptide Receptor 2 and Induction of Insulin-Degrading Enzyme

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Locus ceruleus (LC) is the main subcortical site of norepinephrine synthesis. In Alzheimer's disease (AD) patients and rodent models, degeneration of LC neurons and reduced levels of norepinephrine in LC projection areas are significantly correlated with the increase in amyloid plaques, neurofibrillary tangles, and severity of dementia. Activated microglia play a pivotal role in the progression of AD by either clearing amyloid β peptide (A β) deposits through uptake of A β or releasing cytotoxic substances and proinflammatory cytokines. Here, we investigated the effect of norepinephrine on A β uptake and clearance by murine microglia and explored the underlying mechanisms. We found that murine microglia cell line N9 and primary microglia expressed β_2 adrenergic receptor (AR) but not β_1 and β_3 AR. Norepinephrine and isoproterenol upregulated the expression of A β receptor mFPR2, a mouse homolog of human formyl peptide receptor FPR2, through activation of β_2 AR in microglia. Norepinephrine also induced mFPR2 expression in mouse brain. Activation of β_2 AR in microglia promoted A β_{42} uptake through upregulation of mFPR2 and enhanced spontaneous cell migration but had no effect on cell migration in response to mFPR2 agonists. Furthermore, activation of β_2 AR on microglia induced the expression of insulin-degrading enzyme and increased the degradation of A β_{42} . Mechanistic studies showed that isoproterenol induced mFPR2 expression through ERK1/2-NF- κ B and p38-NF- κ B signaling pathways. These findings suggest that noradrenergic innervation from LC is needed to maintain adequate A β uptake and clearance by microglia, and norepinephrine is a link between neuron and microglia to orchestrate the host response to A β in AD.

Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disease. It is the most frequent cause of dementia in the elderly. AD brain pathology is characterized by amyloid plaques, neurofibrillary tangles, and neuronal loss (Hardy and Higgins, 1992; Selkoe, 1999; Mudher and Lovestone, 2002). Activated microglia and astrocytes were usually found to be associated with the amyloid plaques. Activation of microglia leads to uptake and clearance of amyloid β peptide (A β) (Frautschy et al., 1998; Qiu et al., 1998; Simard et al., 2006; Hickman et al., 2008). However, excessive activation of microglia leads to production and release of inflammatory cytokines, nitric oxide, and reactive oxygen species,

which contribute to neuronal dysfunction and cell death (Griffin et al., 1998).

The locus ceruleus (LC) is a noradrenergic nucleus located in the pontine tegmentum and serves as the main subcortical site for the synthesis of norepinephrine (NE) and its precursor enzymes (Freedman et al., 1975). Ascending noradrenergic axons project to the hippocampus, the frontal and entorhinal cortices, and to a minor extent to various other brain regions. Degeneration of LC neurons and reduced levels of NE in LC projection areas are well known features of AD. In AD patients the central portion of the LC, which is considered to project predominantly to the hippocampus, frontal and temporal cortex areas that are usually severely affected by senile plaque and neurofibrillary tangle formation, shows the most extensive loss of cells (Marcyniuk et al., 1986). The decreased LC neuron numbers are significantly correlated with the increase of amyloid plaques, neurofibrillary tangles, and severity of dementia (Marcyniuk et al., 1986; Bondareff et al., 1987; Grudzien et al., 2007; Weinshenker, 2008). In rodent AD models, neurotoxin *N*-(2-chloroethyl)-*N*-ethyl-bromobenzylamine (DSP4)-induced LC neuron degeneration and NE deficiency exacerbate A β deposition, inflammation, neurodegeneration in brain, and cognitive impairment (Heneka et al., 2006; Kalinin et al., 2007). NE supplement can ameliorate the inflamma-

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Figure 1. Norepinephrine and isoproterenol induce mFPR2 gene expression in microglia and brain. N9 cells (**A, C**) or murine primary microglia (**B, D**) were incubated with different concentrations of NE (**A, B**) for 6 h or ISO (**C, D**) for 9 h, and then total RNA was extracted and examined for mFPR2 mRNA level by real-time PCR. Results represent the mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01 versus cells cultured with control medium.

Figure 2. Norepinephrine and isoproterenol upregulate mFPR2 expression through activation of β_2 AR. **A**, The expression of β adrenergic receptors in N9 cells and mouse primary microglia was examined by RT-PCR. **B, C**, N9 cells pretreated with 10 μ M propranolol (Pro) for 1 h were incubated with or without 1 μ M NE for 6 h (**B**) or 10 μ M ISO for 9 h (**C**), and total RNA was extracted and examined for mFPR2 mRNA level by real-time PCR. Results represent the mean $\pm$ SD of three independent experiments. * p < 0.05 versus cells cultured with control medium. # p < 0.05, ## p < 0.01, compared with cells treated with NE or ISO alone.

tion induced by LC degeneration in mouse AD models (Heneka et al., 2002). These results suggest that NE has protective effect against AD lesions. *In vitro* studies showed that NE could promote A β uptake by murine microglia (Kalinin et al., 2007; Heneka et al., 2010). However, the underlying mechanisms and the effect of NE on A β clearance and the expression of enzymes involved in A β degradation by microglia are not clear.

Our previous studies revealed that human G protein-coupled receptor formyl peptide receptor 2 (FPR2) and its mouse homolog mFPR2 are functional receptors for A β (Le et al., 2001; Tiffany et al., 2001; Yazawa et al., 2001). FPR2 is expressed at high levels by mononuclear (microglial) cells infiltrating senile plaques in brain tissues from AD patients (Le et al., 2001). FPR2/mFPR2 mediates the uptake of A β by microglia and the chemo-

tactic activity of A β_{42} (Le et al., 2001; Yazawa et al., 2001; Iribarren et al., 2005; Chen et al., 2006). In this study, we report that activation of the β_2 adrenergic receptor (AR) on microglial cells induces the expression of mFPR2 through ERK1/2-NF κ B and p38 NF- κ B-dependent signaling pathways (where ERK1/2 is extracellular signal-regulated kinase 1/2 and NF- κ B is nuclear factor κ B). Microglial cells activated by β AR agonist exhibited a markedly increased capacity to endocytose A β_{42} through mFPR2 and to degrade A β_{42} through upregulation of the A β -degrading enzyme, insulin-degrading enzyme (IDE).

Materials and Methods

Reagents. A β_{42} was purchased from California Peptide Research. A β_{42} was dissolved in DMSO at a concentration of 2 mM as stock solution and kept at -70°C . Norepinephrine, isoproterenol (ISO), and propranolol were purchased from Sigma. W peptide (WKYMVm) and MMK-1 (LESI-FRSLFRVM) were synthesized and purified by the Department of Biochemistry, Colorado State University (Fort Collins, CO). SB203580, PD098059, SP600125, and BAY117082 were obtained from Calbiochem. Antibodies against total or phosphorylated ERK1/2, p38, c-jun N-terminal kinase (JNK), and I κ B α were purchased from Cell Signaling Technology. Mouse anti-A β antibody and anti-actin monoclonal antibody were from Sigma. The 6E10 antibody against A β was from Covance. Antibodies against neprilysin and insulin-degrading enzyme were from Millipore. HRP-conjugated secondary antibodies were from Proteintech. DMEM and Iscove's modified Dulbecco's medium (IMDM) were purchased from Invitrogen. Fetal bovine serum (FBS) was from Hyclone.

Cell culture. Primary mouse microglia was isolated and purified from whole brain of newborn C57BL/6 mice as described previously (Xu and Drew, 2006). The experiments using animals were in accordance with the

National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Biological Research Ethics Committee, Institute for Nutritional Sciences, Chinese Academy of Sciences (Shanghai, China). Murine microglial cell line N9 was grown in IMDM supplemented with 5% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10 μ M 2-mercaptoethanol (2-ME). mFPR2/293 cells expressing myc-tagged mFPR2 (Ying et al., 2004) were maintained in DMEM containing 10% FCS, 800 μ g/ml G418, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Reverse transcription PCR and quantitative real-time PCR. Total RNA was extracted from cells with TRIzol reagent (Invitrogen) and depleted of contaminating DNA with RNase-free DNase. Reverse transcription (RT) of RNA was performed with Moloney murine leukemia virus reverse transcriptase (Fermentas) and oligo(dT) according to the manufacturer's protocol. The primers of PCR were: β_1 AR (sense: 5'-TCGCTACCAGAGTTTGTCTGACGC, antisense: 5'-AACAC-CCGACAGGTACACGAAGG); β_2 AR (sense: 5'-CGACTACAAACCGTCAC-CAA-3', antisense: 5'-ATAGCGATCCACTGCAATCA-3'); β_3 AR (sense: 5'-ATGACTAGACCACGAGGTGTAAGG-3', antisense: 5'-CAGGGTTAG-GAAAGCAGTAGGA-3'); mFPR1 (sense: 5'-CAGCCTGTACTTTC-GACTTCTCC-3', antisense: 5'-ATTGGTGCCTGTATCACTGGTCT-3'); mFPR2 (sense: 5'-CTTTATCTGCTGGTTTCCCTTTC-3', antisense: 5'-CTGGTGCTTGAATCACTGGTTTG-3'); β -actin (sense: 5'-TGTGAT-GGTGGGAATGGGTCAG-3', antisense: 5'-TTTGATGTACAGCA-CGATTTC-3'). PCR products were visualized by ethidium bromide staining in 1.5% agarose gel and quantified using Gel-Pro Analyzer software (Media Cybernetics). All experiments were replicated at least three times.

Relative quantification of gene expression was conducted with the ABI Prism 7500 sequence detector (Applied Biosystems Inc). PCR results

were normalized to the expression of β -actin. Real-time PCR primers for mFPR2 were as follows: 5'-CATGGGCCAGGACT-TTCGT-3' (sense) and 5'-TGGTGCTTGA-ATCACTGGTTTG-3' (antisense). Mouse β -actin primers were: 5'-CAACGAGCGGTTCCGAT-3' (sense); and 5'-GCCACAGGATTCCATAC-CCA-3' (antisense).

Intracerebroventricular injection. Male C57BL/6 mice (8 weeks old) were used in the study. Animals were anesthetized with chloral hydrate (0.18 ml of 6% chloral hydrate per mouse) and immobilized in a stereotaxic apparatus, and the skulls were exposed for trepanation. Five microliters of 1 μ M NE ($n = 6$) or the same volume of PBS ($n = 7$) was injected into the lateral ventricle. Coordinates were determined according to a mouse stereotaxic atlas as follows: -1 mm posterior, 1 mm lateral, 1.7 mm ventral to bregma. After 24 h, mice were killed by cervical dislocation. The brains were removed, washed in PBS, and homogenized in TRIzol reagent. RNA was extracted and examined for mFPR2 mRNA level by real-time PCR.

Calcium mobilization assay. Ca^{2+} mobilization was measured with FlexStation II³⁸⁴ system (Molecular Devices) as described previously (Zhu et al., 2009). Briefly, N9 cells were loaded with Fura-3 AM, washed, and resuspended in emission buffer at a density of 4×10^6 cells/L for 15 min. The fluorescence before and after the addition of different concentrations of W peptide was measured with the excitation wavelength at 485 nm and the emission wavelength at 525 nm and presented as relative fluorescence units (RFU).

mFPR2 short hairpin RNA constructs, transfection, and virus transduction. To construct mFPR2 short hairpin RNA (shRNA) expression plasmid, two complementary mFPR2 DNA oligos (see below) were synthesized, annealed, and inserted between BbsI and XhoI sites of the plasmid pBS-hU6-I. The fragment containing human U6-RNA Pol III promoter and mFPR2 shRNA then were subcloned into FG12 between the XbaI and XhoI sites. FG12 vector also expresses a human UbiC-driven green fluorescent protein (GFP) gene to provide a marker for tracking transduced cells. The resulting plasmids were confirmed by restriction enzyme digestion and DNA sequencing. mFPR2 target sequences are nucleotides 297–315 (ATTAGTTCACATTGTGGTA) (shRNA-a), 537–555 (CTTTGGATCCTGGGCTCAA) (shRNA-b), and 836–854 (TTCTTGACATGTTTGTTAA) (shRNA-c) of mouse FPR2 mRNA (GeneBank accession no. NM_008039). BLAST (Basic Local Alignment Search Tool) search against mouse genome sequences confirmed that only mFPR2 gene was targeted. Recombinant lentiviruses were produced by transient transfection of HEK293T cells with lentiviral mFPR2 shRNA vector, the lentiviral packaging constructs pRSV/REV and pMDLg/pRRE, and the vesicular stomatitis virus glycoprotein (VSVG)-expressing plasmid pCMVG using the calcium phosphate method, as described previously (Tiscornia et al., 2006). mFPR2/293 cells and N9 cells were transduced with concentrated lentivirus stocks at the same multiplicity of infection in the presence of 4 μ g/ml polybrene. After 3–7 days, GFP-positive cells were sorted by fluorescence-activated cell sorter and kept growing for future use.

Chemotaxis assay. Chemotaxis assays for N9 cells pretreated with 10 μ M ISO or 500 ng/ml lipopolysaccharide (LPS) for 24 h and mFPR2/293 cells transduced with control lentiviruses or mFPR2 shRNA lentiviruses

Figure 3. Isoproterenol treatment of microglia increases calcium mobilization but not cell migration in response to mFPR2 agonists. **A–C**, N9 cells were treated with or without 40 μ M ISO for 24 h and then loaded with Fura-3-AM and stimulated with different concentrations of W peptide and measured fluorescence (RFU) (**A**, **B**) or examined for mFPR1 and mFPR2 expression by RT-PCR (**C**). Data shown in **A** and **B** are representative of three independent experiments with similar results. Data in **C** represent the mean $\pm$ SD of three independent experiments. ****** $p < 0.01$, compared with cells cultured in medium alone. **D**, **E**, N9 cells were cultured in the presence or absence of 500 ng/ml LPS or 10 μ M ISO at 37°C for 24 h and then examined for migration in response to W peptide (**D**), and MMK-1 (**E**). The results are expressed as the mean $\pm$ SD of migrated cells in three high-power fields in triplicate samples, ****** $p < 0.01$, cell migration in response to W peptide or MMK-1 compared to medium control; **##** $p < 0.01$, comparison of spontaneous cell migration between N9 cells treated with ISO and LPS. **F**, Visualization of N9 cell migration in response to W peptide.

were performed with 48-well chemotaxis chambers (NeuroProbe) as described previously (Cui et al., 2002a). Polycarbonate filters (8 μ m pore size, incubated for 90 min at 37°C) were used for measurement of N9 cell migration. For mFPR2/293 cells, 10 μ m pore size, collagen type I-coated filters (incubated for 4 h) were used. The results are expressed as the mean $\pm$ SD of migrated cells in three high-power fields in triplicate samples or as chemotaxis index, which represents the fold increase in the number of migrated cells in response to chemoattractants over the spontaneous cell migration (to control medium).

Immunofluorescence confocal microscopy. N9 cells or primary microglia grown on slides in a 24-well plate were treated with 10 μ M ISO at 37°C for 24 h, followed by treatment with or without 10 μ M A β ₄₂ for 30 min, and then fixed in 4% paraformaldehyde for 10 min at room temperature. Slides were washed with PBS and incubated with 5% BSA in PBS with 0.05% Tween 20 at room temperature for 1 h to reduce nonspecific binding of antibodies to the cell surface and for cell permeabilization. A mouse anti-A β antibody was applied to the slides and incubated overnight at 4°C. The slides were washed and incubated with Alexa Fluor

Figure 4. Isoproterenol enhances A β_{42} uptake by microglia. **A, E.** Primary microglia (**A**), N9 cells (**E**) transduced with control lentivirus (Mock), or mFPR2 shRNA-c lentivirus (shRNA-c) were treated with or without 10 μ M ISO for 24 h followed by incubation with 10 μ M A β_{42} for 30 min and then examined for A β uptake with antibody against A β (red). Nuclei were stained with Hoechst (blue). Scale bars, 20 μ m. Data are expressed as mean $\pm$ SD ($n = 2$, with duplicate samples in each experiment); ** $p < 0.01$, compared with A β uptake by primary microglia or mock transduced N9 cells without ISO treatment; # $p < 0.01$, compared with A β uptake by mock transduced N9 cells pretreated with ISO. **B–D.** mFPR2/293 cells were transduced with control lentiviruses (Mock) or mFPR2 shRNA lentiviruses, mFPR2 mRNA level was examined by RT-PCR (**B**), and cell migration in response to mFPR2 agonists (**C**: MMK-1; **D**: A β_{42}) was examined by chemotaxis assay. Data are expressed as mean $\pm$ SD ($n = 3$); * $p < 0.05$, ** $p < 0.01$, chemotaxis index of mFPR2 shRNA lentivirus transduced cells versus control lentivirus transduced cells (Mock).

546-conjugated goat anti-mouse IgG (Invitrogen) for 60 min, washed in PBS, stained with Hoechst 33342, and mounted. Immunofluorescence labeling was observed under a laser scanning confocal microscope (LSM 510 META, Zeiss). Cells in three high-power fields (each includes ~ 40 cells) were quantified for amyloid uptake by using ImageJ program (<http://rsbweb.nih.gov/ij/>).

Immunoblot analysis. Western blotting was performed as described previously (Wang et al., 2008). The following primary antibodies were used: antibodies against total or phosphorylated ERK1/2, p38, JNK, I κ B α ; antibodies against neprilysin and IDE. Primary antibodies were recognized by HRP-conjugated secondary antibodies. Immunoblot results were quantified by using Gel-Pro Analyzer software (Media Cybernetics).

To examine A β_{42} uptake and degradation by N9 cells and murine primary microglia, A β_{42} was oligomerized as described previously (Tahara et al., 2006). After treatment of the cells with A β_{42} for 12 h, A β level in the supernatant and cell lysate was detected by Western blot as described previously (Schägger, 2006). Briefly, 20 μ l of supernatant or 50 μ g of cell lysate were boiled for 5 min and underwent Tris-Tricine SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membrane was blocked with 5% nonfat milk and then incubated with anti-A β antibody (6E10), or anti-actin monoclonal antibody overnight at 4°C. After incubation with HRP-

conjugated secondary antibody, the protein bands were detected with a SuperSignal West Pico chemiluminescent substrate (Pierce) and X-Omat BT film (Eastman Kodak).

Statistics. The significance of the difference between test and control groups was analyzed with Student's *t* test, and *p* values ≤ 0.05 were considered statistically significant.

Results

Norepinephrine and isoproterenol upregulate mFPR2 expression through activation of β_2 AR

Human G protein-coupled receptor FPR2 and its mouse homolog mFPR2 have been reported to be the receptor for A β_{42} . FPR2/mFPR2 mediates the internalization of A β_{42} by microglia (Yazawa et al., 2001; Iribarren et al., 2005; Chen et al., 2006). We first checked the effect of NE and β AR agonist ISO on mFPR2 mRNA expression in N9 cells by real-time PCR. As shown in Figure 1, NE (Fig. 1A) and ISO (Fig. 1C) both upregulated mFPR2 mRNA level in a dose-dependent manner. The inductive effect of these two compounds on mFPR2 expression was also confirmed in mouse primary microglia (Fig. 1B,D). In C57BL/6 mice injected with 1 μ M NE in the lateral ventricle (5 μ l/mouse, $n = 6$), the mFPR2 mRNA level in brain at 24 h after injection was elevated to 1.40 ± 0.29 -fold ($p = 0.02$) of control mice injected with same volume of PBS ($n = 7$), demonstrating the upregulation of mFPR2 expression by NE *in vivo*.

To investigate the involvement of β AR in the upregulation of mFPR2 by NE and ISO, we examined the expression of β AR in N9 cells and primary microglia by RT-PCR. The results showed that both N9 cells and primary microglia express β_2 AR but not β_1 and β_3 ARs (Fig. 2A).

Pretreatment of N9 cells with the $\beta_{1,2}$ AR antagonist propranolol significantly inhibited the inductive effect of NE and ISO on mFPR2 expression (Fig. 2B and C), suggesting that NE and ISO upregulate mFPR2 through activation of β_2 AR.

We then checked whether the mFPR2 upregulated by isoproterenol is functional. Calcium mobilization is a signaling event of mFPR2 activation (Tiffany et al., 2001). N9 cells without ISO treatment had no response to W peptide, a ligand for mFPR2 (Fig. 3A). However, after treatment of N9 cells with ISO for 24 h, W peptide induced a robust and transient calcium flux (Fig. 3B). Although W peptide is also reported to be an agonist for mFPR1 (He et al., 2000), ISO had no significant effect on mFPR1 expression in N9 cells (Fig. 3C). These results indicate that mFPR2 induced by ISO could mediate cell response to FPR2 ligand. Together, the above results demonstrated that activation of β_2 AR on mouse microglia could increase the expression of mFPR2 and its response to agonist. As N9 cells and primary microglia both only express β_2 AR and respond to NE and ISO in a similar way, we used ISO to treat microglia in most of the following experiments.

As NE and ISO have been reported to enhance microglial cell migration (Heneka et al., 2010), we then examined whether these compounds promote cell migration through upregulation of mFPR2. We found that pretreatment of N9 cells with ISO significantly increased spontaneous cell migration but had no significant effect on cell migration in response to W peptide (Fig. 3D,F) and MMK-1 (Fig. 3E), a potent mFPR2 agonist derived from random peptide library (Hu et al., 2001). As positive control, pretreatment of N9 cells with LPS significantly augmented cell migration in response to W peptide and MMK-1, confirming our previous report that LPS increases microglial cell migration to mFPR2 agonists through upregulation of mFPR2 expression (Cui et al., 2002a). These results suggest that activation of β_2 AR on microglia enhance cell migration independently of mFPR2.

Isoproterenol enhances A β_{42} uptake by microglia through mFPR2

The effect of ISO on A β uptake by microglia was examined by immunofluorescence confocal microscopy. We found that ISO treatment could significantly increase the internalization of A β_{42} in primary murine microglia (Fig. 4A) and N9 cells (data not shown) after incubation with A β_{42} for 30 min. These results were consistent with previous reports (Kalinin et al., 2007; Heneka et al., 2010).

To examine whether β_2 AR activation enhances the capability of microglia to uptake A β through upregulation of FPR2, we used a RNA interference technique to inhibit mFPR2 expression. Three lentiviral mFPR2 shRNA expression vectors targeting the 297–315 (shRNA-a), 537–555 (shRNA-b), and 836–854 (shRNA-c) regions of mFPR2 coding sequence were constructed. mFPR2/293 cells transduced with various mFPR2 shRNA lentiviruses inhibited mFPR2 expression at mRNA and protein levels with different potency (Fig. 4B and data not shown). The inhibitory effect of mFPR2 shRNA-c is the most potent. To determine whether expression of mFPR2 shRNA could induce functional silencing of mFPR2, we examined the effect of mFPR2 shRNA on chemotactic response to MMK-1 (Fig. 4C) and A β_{42} , the major component of amyloid plaque in AD brain (Fig. 4D). mFPR2 shRNA-a and mFPR2 shRNA-b showed certain levels of attenuation of agonist-induced cell migration, albeit with lower efficacy compared with mFPR2 shRNA-c (Fig. 4C,D). Thus, mFPR2 shRNA-c is a selective and highly efficacious mFPR2 shRNA-expressing vector that attenuates both mFPR2 expression and its key function. It was used in the next experiments.

Similar to the results from primary microglia, treatment of control lentivirus-transduced N9 cells with 10 μ M ISO markedly increased the uptake of A β_{42} (Fig. 4E, top). However, transduc-

Figure 5. Activation of β_2 AR on microglia cells increases the protein level of IDE and enhances A β degradation. **A**, N9 cells were treated with 10 μ M ISO for 12 and 24 h and cell lysates were subjected to immunoblot analysis with antibodies against IDE, neprilysin (NEP), or β -actin. **B**, Primary microglia were treated with 10 μ M ISO or 1 μ M NE for 12 and 24 h and then examined for IDE expression by immunoblot analysis. Data are the mean $\pm$ SD ($n = 3$); * $p < 0.05$, ** $p < 0.01$, compared with cells cultured in medium alone. **C–F**, N9 cells (**C**, **D**) or primary microglia (**E**, **F**) pretreated with control medium, 10 μ M ISO, or 10 μ M ISO combined with 100 μ g/ml insulin for 24 h were incubated with 2 μ M A β_{42} for an additional 12 h and then examined for A β in cell lysate and supernatant with Western blot. Data are the mean $\pm$ SD ($n = 3$); ** $p < 0.01$, compared with A β in cells treated with A β_{42} alone; ## $p < 0.01$, compared with A β in cells treated with ISO followed by A β_{42} .

tion of N9 cells with mFPR2 shRNA-c lentivirus abrogated the uptake of A β_{42} by ISO-activated microglial cells (Fig. 4E, bottom). These results suggest that ISO enhances A β_{42} uptake by microglia through upregulation of mFPR2.

Activation of β_2 AR on microglial cells increases the protein level of IDE and enhances A β degradation

We then examined the effect of β_2 AR activation on the expression of enzymes involved in A β degradation, including IDE and neprilysin. Western blot analysis showed that pretreatment of N9 cells with 10 μ M ISO significantly induced increase of IDE expression at 12 and 24 h and enhanced neprilysin expression at 24 h (Fig. 5A). Treatment with 1 μ M NE or 10 μ M ISO also significantly induced IDE expression in primary microglia (Fig. 5B), suggesting that β_2 AR activation enhances IDE expression in microglia. However, neprilysin expression could hardly be detected in primary microglia with or without NE/ISO treatment (data not shown).

To further check the effect of β_2 AR activation on A β degradation, we incubated microglial cells with A β_{42} for a long time (12 h) and examined the ingested A β with Western blot analysis.

Figure 6. Isoproterenol induces mFPR2 mRNA expression in microglia through activation of MAP kinases. N9 cells were treated with 10 μ M ISO for different periods of time (**A**) or treated with or without different concentrations of PD98059 (PD) (**C**) for 1 h followed by 10 μ M ISO for 30 min and then examined for p38 or ERK1/2 phosphorylation by Western blot. N9 cells pretreated with different concentrations of SB203580 (SB) (**B**) or PD (**D**) for 1 h were stimulated with or without 10 μ M ISO for 9 h and then examined for mFPR2 mRNA level by RT-PCR. All data are mean $\pm$ SD ($n = 3$); ** $p < 0.01$, compared with cells without stimulation.

Intracellular aggregates of A β were readily detectable in the cell lysate from N9 cells (Fig. 5C,D) and primary microglia (Fig. 5E,F). Pretreatment of N9 cells and primary microglia with ISO for 24 h significantly decreased A β in supernatant and cell lysate (especially the low molecular weight aggregates). These results, together with data in Figure 4, A and E, demonstrated that activation of β_2 AR on microglia promoted A β uptake and degradation. We further evaluate the contribution of IDE and neprilysin to ISO-induced A β degradation. Treatment of N9 cells (Fig. 5D) or primary microglia (Fig. 5F) with insulin, a competitive inhibitor of IDE, significantly inhibited ISO-induced A β clearance, suggesting ISO enhances A β degradation through induction of IDE. However, neprilysin inhibitor phosphoramidon could not suppress ISO-induced A β clearance in both N9 cells and primary microglia (data not shown). Together, activation of β_2 AR on microglia promoted A β uptake and degradation by upregulating the expression and function of mFPR2 and IDE, respectively.

Isoproterenol induces FPR2 mRNA expression in microglia through MAP kinases and NF- κ B

We further explored the mechanisms underlying FPR2 upregulation by β_2 AR activation. Because the MAPK (mitogen-activated protein kinase) pathway has been implicated in the induction of mFPR2 in microglial cells via activation of TLR4 by LPS (Cui et al., 2002a) and MAP kinases are downstream signaling molecules

of β AR activation (Liu et al., 2006; Gong et al., 2008; Qian et al., 2009), we assessed the role of MAPKs in ISO induction of mFPR2 in microglial cells. As shown in Figure 6A, ISO induced a rapid and potent phosphorylation of p38 MAPK in N9 cells. In cells pretreated with SB203580, a p38 MAPK inhibitor, the level of mFPR2 mRNA induced by ISO was significantly reduced (Fig. 6B). ISO additionally induced increased phosphorylation of ERK1/2, and the MEK (MAPK kinase) inhibitor PD98059 dose-dependently inhibited ERK1/2 phosphorylation and mFPR2 expression induced by ISO (Fig. 6C,D). Although ISO also induced JNK phosphorylation in N9 cells, JNK inhibitor SP600125 had no effect on ISO-induced mFPR2 expression (data not shown). These results indicate that MAPKs p38 and ERK1/2 play an essential role in ISO induction of the mFPR2 gene in mouse microglial cells.

NF- κ B has been reported to mediate peptidoglycan-induced mFPR2 expression in microglial cells (Chen et al., 2006). NF- κ B is also a molecule of β AR signaling pathway (Takemoto et al., 1999; Chandrasekar et al., 2004). We analyzed the sequence of the promoter region of mFPR2 and found the existence of NF- κ B binding sites. Pretreatment of N9 cells with a highly selective I κ B α phosphorylation inhibitor, BAY117082, significantly inhibited ISO-induced mFPR2 expression (Fig. 7A). Consistently, stimulation of N9 cells and primary microglia with ISO markedly increased I κ B α phosphorylation (Fig. 7B,C) and I κ B α degradation (Fig. 7D). These results suggest that NF- κ B is an important transcription factor that mediated the upregulation of mFPR2 expression by ISO. Pretreatment of N9 cells with BAY117082 reversed ISO-induced I κ B α degradation (Fig. 7D). Pretreatment of N9 cells with PD98059 or SB203580 also reversed I κ B α degradation induced by ISO, suggesting that ERK1/2 and p38 are upstream signaling molecules mediating ISO-induced NF- κ B activation. Together, these results suggest that ISO induces mFPR2 expression through p38-NF- κ B and ERK1/2-NF- κ B signaling pathways.

7B,C) and I κ B α degradation (Fig. 7D). These results suggest that NF- κ B is an important transcription factor that mediated the upregulation of mFPR2 expression by ISO. Pretreatment of N9 cells with BAY117082 reversed ISO-induced I κ B α degradation (Fig. 7D). Pretreatment of N9 cells with PD98059 or SB203580 also reversed I κ B α degradation induced by ISO, suggesting that ERK1/2 and p38 are upstream signaling molecules mediating ISO-induced NF- κ B activation. Together, these results suggest that ISO induces mFPR2 expression through p38-NF- κ B and ERK1/2-NF- κ B signaling pathways.

Discussion

In this study, we demonstrated that NE and ISO could induce mFPR2 mRNA expression in microglial cells through β_2 AR-mediated activation of MAP kinases ERK1/2 and p38, as well as transcription factor NF- κ B. Upregulation of mFPR2 expression by β_2 AR activation in microglia markedly enhanced A β_{42} uptake. We also found that activation of β_2 AR in microglia increased the expression of A β -degrading enzyme IDE and enhanced the degradation of A β_{42} .

Microglia are resident brain mononuclear phagocytes and have functions similar to peripheral macrophages. They play a critical role as resident immunocompetent and phagocytic cells in the CNS and serve as scavenger cells in the event of infection, inflammation, trauma, ischemia, and neurodegeneration

Figure 7. Isoproterenol induces mFPR2 mRNA expression through ERK/p38-NF- κ B pathway. **A**, N9 cells pretreated with or without different concentrations of BAY117082 (BAY) for 1 h were stimulated with 10 μ M ISO for 9 h and then examined for mFPR2 expression by RT-PCR. **B**, **C**, N9 cells (**B**) or primary microglia (**C**) were stimulated with 10 μ M ISO for 30 min and then examined for I κ B α phosphorylation by Western blot. **D**, N9 cells pretreated with or without 50 μ M PD98059 (PD), 50 μ M SB203580 (SB), or 20 μ M BAY for 1 h were stimulated with 10 μ M ISO for 30 min and then examined for I κ B α level by Western blot. All data are mean $\pm$ SD ($n = 3$); * $p < 0.05$, ** $p < 0.01$, compared with cells cultured in medium alone; ### $p < 0.01$, compared with cells treated with ISO alone.

(Kreutzberg, 1996; González-Scarano and Baltuch, 1999; Aloisi et al., 2001). Although microglial activation has become increasingly regarded as a contributor to AD pathogenesis by producing neurotoxins including reactive oxygen species and proinflammatory cytokines, it also plays a neuroprotective role in AD by secreting proteolytic enzymes that degrade A β and express receptors that are involved in the endocytosis and clearance of A β . The notion that microglia actively uptake A β peptide is supported by experiments using *in vitro* culture (Frackowiak et al., 1992; Pocock et al., 2002) and animal AD models (Frautschy et al., 1998; Weldon et al., 1998). In mouse AD models, active immunization with A β peptide (Schenk et al., 1999; Lombardo et al., 2003) or passive immunization with antibodies to A β (Wilcock et al., 2003) resulted in substantial removal of existing A β deposits, apparently by microglia. A series of cell membrane receptors has been reported to be able to mediate binding and endocytosis of A β , including G protein-coupled receptor formyl peptide receptor 2, scavenger receptors, receptor of advanced glycation end products, Fc receptors, complement receptors, and CD14 (Paresce et al., 1996; Yan et al., 1996; Bard et al., 2000; Webster et al., 2000; Le et al., 2001; Liu et al., 2005). Human FPR2, also called formyl peptide receptor-like 1, is encoded by a gene that shares homology to the human formyl peptide receptor (FPR1) gene. FPR2 was initially known for its low-affinity interaction with formyl-methionyl-leucyl phenylalanine, the high-affinity agonist for FPR1. Subsequent studies have identified many agonists for this receptor, including microbe- and host-derived peptides, lipoxin A4, synthetic peptides, and nonpeptide agonists (Le et al., 2002; Ye, 2009; Ye et al., 2009). Although FPR2 was initially detected in phagocytic leukocytes, other cell types also express this receptor, such as microglia and astrocytes (Le et al., 2002). A β ₄₂, the key causative factor in AD, has been reported to activate microglia through human FPR2 or its mouse homolog mFPR2 (Le et al., 2001; Tiffany et al., 2001; Yazawa et al., 2001). The amount of FPR2 expressed on the cell surface is regulated through receptor internalization and mobilization from intracellular pools as well as at the transcriptional level (Ye, 2009). For example, the cell surface number of FPR2 is regulated by ligand-induced receptor internalization; stimulation of neutrophils with several agonists that induce degranulation upregulates the FPR2-mediated response to WKYMVm; differentiated HL-60 cells ex-

press more FPR2 transcript than undifferentiated HL-60 cells. IL-13 and interferon- γ stimulate the expression of FPR2 in enterocytes. We previously reported that murine microglial cells in the nonstimulated state express low levels of mFPR2 (Cui et al., 2002a) which are upregulated by pathogen-associated molecular patterns and proinflammatory cytokines, such as LPS, TNF- α , PGN, CpG, IFN- γ and CD40 (Cui et al., 2002a,b; Iribarren et al., 2005; Chen et al., 2006; 2007). The upregulation of mFPR2 was associated with a markedly increased microglial chemotaxis to A β ₄₂ and its endocytosis via mFPR2. These results suggest that mFPR2 in microglia not only mediates the proinflammatory activity of A β ₄₂ but also may actively participate in the clearance of the amyloid β peptide. Our present study reveals that NE, the endogenous neurotransmitter mainly produced by LC neurons, is able to induce the expression of mFPR2 and promote A β uptake by microglia. The inductive effect of NE on mFPR2 expression is mediated by MAPK-NF- κ B signaling pathways.

Neurons can influence microglial functions through direct cell-to-cell interactions as well as with the release of soluble mediators. Microglial processes have been reported to be associated with synapses anatomically (Pow et al., 1989), and microglia may also respond to “volume neurotransmission” (Thomas, 1992), in which transmitters may exist and act extrasynaptically. Among signals from neurons that may have an active role in controlling microglial activation are those from two major neurotransmitters: NE and acetylcholine (Carnevale et al., 2007). NE has been reported to be a potent regulator of microglial function. Activation of β AR in microglia has been shown to be capable of inhibiting inflammatory responses induced by various stimuli, including A β , IFN γ , and LPS (Loughlin et al., 1993; Mori et al., 2002; Dello Russo et al., 2004; Färber et al., 2005; Madrigal et al., 2005; Heneka et al., 2010). Meanwhile, both *in vitro* and *in vivo* studies have demonstrated that β AR activation induces proinflammatory cytokine expression in microglia (Tomozawa et al., 1995; Chandrasekar et al., 2004; Johnson et al., 2005; Blandino et al., 2006). NF- κ B has been reported to be involved in both of the anti-inflammatory and proinflammatory effects of β AR agonists (Farmer and Pugin, 2000; Chandrasekar et al., 2004). These results suggest that β AR activation may exert anti-inflammatory and proinflammatory effects under different circumstances. Rat primary microglia express β_1 and β_2 ARs but not β_3 AR (Tanaka et

al., 2002). Our results showed that only β_2 AR could be detected in both primary mouse microglia and N9 cells. Supporting our results, Hertz et al. (2010) examined the expression of β AR subtypes in acutely isolated murine microglia by microarray and found that microglia mainly express β_2 AR. Our results demonstrated that NE and ISO upregulated the expression of mFPR2 in murine microglia and enhanced the uptake and clearance of A β_{42} through the activation of β_2 AR, and ISO enhanced microglial cell migration independent of mFPR2. Thus, NE acts as a link between neuron and microglia to orchestrate the host responses to A β in the pathogenesis of AD.

A β is produced continuously in brain, and its concentration is determined in part by the activities of several degradative enzymes, including neprilysin, IDE, ECE-1 (endothelin-converting enzyme 1) and ECE-2, and probably plasmin (Leissring et al., 2003; Eckman and Eckman, 2005). IDE mRNA and protein levels in hippocampus were significantly decreased in apolipoprotein E $\epsilon 4^+$ AD patients (Cook et al., 2003). In AD mouse models, the expression levels of neprilysin and IDE in microglia were decreased in 14-month-old mice (Hickman et al., 2008). Recent studies demonstrated that IDE expression and activity were complexly regulated by various factors, including aging, Apo E, cytokines, and vitamin E (Eckman and Eckman, 2005; Jiang et al., 2008; Shimizu et al., 2008; Nishida et al., 2009). Therefore, the upregulation of IDE represents a promising strategy for therapy and prevention for AD. Our results showed that NE and ISO could induce IDE expression and promote A β degradation through activation of β_2 AR in primary microglia. To our knowledge, this is the first report demonstrating that the endogenous neurotransmitter NE plays an important role in maintaining expression and activity of IDE in microglia, which contribute to A β clearance in brain.

LC neuron degeneration-induced NE deficiency has been linked to more severe brain lesions in both AD patients and animal models (Bondareff et al., 1987; Heneka et al., 2002). In this study we found that by activation of β_2 AR in microglia, NE enhanced A β uptake through upregulation of mFPR2 and increased A β_{42} clearance through induction of IDE. These results suggest that noradrenergic innervation from LC is needed to maintain adequate A β uptake and clearance by microglia and that LC neuron degeneration in AD not only impairs neuron-neuron transmission but also may contribute to dysregulation of the normal neuron-glial interaction, abnormal glial reaction, defective A β clearance, and, eventually, neurodegeneration. In addition, our findings indicate that the roles of β_2 AR in regulating microglial functions may be used in developing new therapeutic strategies in the treatment of AD.

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