SHORT COMMUNICATION

EP4 Receptor—Associated Protein in Microglia Promotes Inflammation in the Brain

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Accepted for publication
April 4, 2016.

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Microglial cells play a key role in neuronal damage in neurodegenerative disorders. Overactivated microglia induce detrimental neurotoxic effects through the excess production of proinflammatory cytokines. However, the mechanisms of microglial activation are poorly understood. We focused on prostaglandin E₂ type 4 receptor-associated protein (EPRAP), which suppresses macrophage activation. We demonstrated that EPRAP exists in microglia in the brain. Furthermore, EPRAP-deficient mice displayed less microglial accumulation, and intraperitoneal administration of lipopolysaccharide (LPS) led to reduced expression of tumor necrosis factor- α and monocyte chemoattractant protein-1 mRNA in the brains of EPRAP-deficient mice. Consistently, EPRAP-deficient microglia showed a marked decrease in the production of tumor necrosis factor- α and monocyte chemoattractant protein-1 induced by LPS treatment compared with wild-type controls. In addition, EPRAP deficiency decreased microglial activation and neuronal cell death induced by intraventricular injection of kainic acid. EPRAP deficiency impaired the LPS-induced phosphorylation of c-jun N-terminal kinase and p38 mitogen-activated protein kinase in microglia. The phosphorylation levels of mitogen-activated protein kinase kinase 4—which phosphorylates c-jun N-terminal kinase and p38 mitogen-activated protein kinase—were also decreased in EPRAP-deficient microglia after LPS stimulation. Although EPRAP in macrophages plays a role in the attenuation of inflammation, EPRAP promotes proinflammatory activation of microglia through mitogen-activated protein kinase kinase 4-mediated signaling and may be key to the deteriorating neuronal damage brought on by brain inflammation. (*Am J Pathol* 2016, 186: 1982–1988; <http://dx.doi.org/10.1016/j.ajpath.2016.04.002>)

Microglial cells are resident innate immune cells in the brain. Microglial activation is necessary for neuron survival, but overactivated microglia can cause damage to the central nervous system by releasing excess proinflammatory cytokines. Activation of microglia plays an important role in neurodegenerative disorders such as Alzheimer disease or stroke, with microglial activation reported to increase with disease progression. Although these concepts have been widely accepted in past years,^{1–3} the detailed molecular mechanisms that govern microglial activation remain poorly understood.

Prostaglandin E₂ (PGE₂) type 4 (EP4) receptor-associated protein (EPRAP) was isolated as a novel, cytoplasmic signaling partner of EP4.⁴ In mice, EPRAP is also known as

Fem1a—an ortholog of *Caenorhabditis elegans* FEM-1, which participates in sex determination.⁵ Our group has generated EPRAP-deficient [EPRAP-knockout (KO)] mice and has used an animal model of chronic inflammation, whereas others have demonstrated that EPRAP attenuates macrophage activation in the periphery.⁶ However, the role of EPRAP in the brain is still unknown. Herein, we examine the localization of EPRAP in the brain and the role of EPRAP in brain inflammation.

Supported in part by the Japan Society for the Promotion of Science (JSPS) grants KAKENHI 26460338 (M.M.) and 15K08230 (M.M.) and grant-in-aid for JSPS Fellows 820140600019 (R.F.).

Disclosures: Ono Pharmaceutical supplied ONO-AE1-329.

Materials and Methods

Experimental Animals

EPRAP-KO mice were generated as previously described.⁶ C57BL/6 mice were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Seven- to 9-week-old male mice were used for these experiments. For the systemic lipopolysaccharide (LPS) administration model, LPS from *Escherichia coli* O55 (Wako Pure Chemical Industries, Osaka, Japan) was administered intraperitoneally at a dose of 100 mg/kg both in wild-type (WT) and EPRAP-KO mice. For the kainic acid injection model, mice were injected with kainic acid (0.2 µg in 4.0 µL of phosphate-buffered saline; Sigma-Aldrich, St. Louis, MO) at a rate of 0.5 µL/min into the right ventricle (anterior-posterior, −2.0 mm; medial-lateral, −2.9 mm; dorsal-ventral, −3.8 mm) with the use of a 26-gauge needle, as described previously.⁷ Hippocampal neuronal cell death was shown by cresyl violet staining. Live neurons with normal visible nuclei were counted. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto University.

Primary Microglia

Mouse microglial cells were isolated from mixed cultures of cerebrocortical cells from postnatal, 0- to 2-day-old WT or EPRAP-KO mice, as described previously.⁸

Western Blot Analysis

Proteins were separated by SDS-PAGE. The blots were blocked with 2% albumin from bovine serum and were incubated with antibodies against FEM1A/EPRAP (Abcam, Cambridge, United Kingdom), β -actin, phospho-p44/42 mitogen-activated protein kinase (MAPK; Thr202/Thr204), p44/42 MAPK, phospho-p38 MAPK (Thr180/Thr182), p38 MAPK, phospho-stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK; Thr183/Thr185), SAPK/JNK, phospho-SAPK/Erk (extracellular signal-related kinase) kinase/mitogen-activated protein kinase kinase 4 (SEK1/MKK4; Ser257), and SEK1/MKK4 (Cell Signaling Technology, Beverly, MA). The level of phosphorylation of MKK4 was quantified by scanning the blots and performing densitometry analyses with the use of ImageJ software version 1.6.0_24 (NIH, Bethesda, MD; <https://imagej.nih.gov/ij/download.html>). The blot intensity of phosphorylated MKK4 was normalized to that of MKK4.

Immunohistologic Assessment

Murine brains were sliced and stained with antibodies against EPRAP (Abcam), ionized calcium-binding adapter molecule 1 (Iba-1) (Wako Pure Chemical Industries), glial fibrillary acidic protein (Merck Millipore, Darmstadt, Germany), neuronal nuclei (NeuN) (Merck Millipore), and

oligodendrocyte transcription factor 2 (Oligo-2) (Merck Millipore). The ImageJ software (NIH) was used to measure the optical density of Iba-1 immunoreactivity.

Measurement of Cytokine Release

Quantitative determinations of tumor necrosis factor (TNF) α and monocyte chemoattractant protein (MCP)-1 concentrations in supernatant fluids were performed with the BD CBA assay (BD Bioscience, San Jose, CA). For inhibition of JNK or mixed-lineage kinase 1 (MLK1), cells were preincubated with SP600125 or HG-10-102-01 for 30 minutes and then treated with 10 ng/mL LPS for 24 hours.

Total RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse-transcribed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The mRNA levels of proinflammatory cytokines in the cerebral cortices were measured 6 hours after LPS administration. The following primers were used for mouse β -actin forward, 5'-CCTG-AGCGCAAGTACTCTGTGT-3', and reverse, 5'-GCTG-ATCCACATCTGCTGGAA-3'; for CD68 forward, 5'-CT-TCCCACAGGCAGCACAG-3', and reverse, 5'-AATG-ATGAGAGGCAGCAAGAGG-3'; for TNF α forward, 5'-CATCTTCTCAAAATTCGAGTGACAA-3', and reverse, 5'-TGGGAGTAGACAAGGTACAACCC-3'; and for MCP-1 forward, 5'-GCTGGAGCATCCACGTGTT-3', and reverse, 5'-ATCTTGCTGGTGAATGAGTAGCA-3'. All experiments were performed in duplicate, and results were normalized with β -actin as a reference gene.

Statistical Analysis

Results are expressed as means $\pm$ SEM. Student's *t*-tests and Tukey-Kramer tests were used to determine statistically significant differences. *P* < 0.05 was considered statistically significant.

Results

EPRAP Exists in Microglia in the Brain

Western blot analysis of brain homogenates from WT mice showed that EPRAP exists in various regions of the brain such as the olfactory bulb, the hippocampus, and the cortex (Figure 1A). Immunohistochemical analysis showed that EPRAP colocalized to Iba1⁺ microglia and glial fibrillary acidic protein-positive astrocytes but not to Oligo-2⁺ oligodendrocytes or NeuN⁺ neurons (Figure 1B). Through these findings, we demonstrate that EPRAP is expressed in the brain and exists in both microglia and astrocytes.

Figure 1 Expression of EPRAP in the brain. **A:** Western blot analysis shows EPRAP expression in the brain of 8-week-old WT mice. **B:** Double immunolabeling demonstrates that EPRAP colocalizes with Iba-1⁺ microglia and GFAP⁺ astrocytes in the cortex of WT mice. EPRAP was not expressed in Oligo-2⁺ oligodendrocytes and NeuN⁺ neurons in the cortex. EPRAP, EP4 (prostaglandin E₂ type 4 receptor)-associated protein; Iba-1, Ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei; Oligo-2, oligodendrocyte transcription factor 2; WT, wild-type.

EPRAP Deficiency Inhibits Microglial Activation in the Brain after Systemic LPS Administration

Previous studies suggested that EPRAP in macrophages suppressed inflammation.^{6,9} We focused on elucidating the function of EPRAP in brain inflammation. LPS, a component of the Gram-negative bacterial cell wall, is one of the most potent activators of inflammation. A well-studied model of innate immunity in the brain involved systemic administration of LPS.¹⁰ LPS disrupted the blood-brain barrier, and the peripheral immune response to LPS can be transmitted to the brain parenchyma such as the cortex.¹¹ Although no significant difference was found between WT and EPRAP-KO mice in Iba1⁺ microglia before LPS treatment, LPS-treated EPRAP-KO mice showed less Iba1⁺ microglia accumulation in the cortex than LPS-treated WT mice (Figure 2, A and B). Quantitative RT-PCR analysis showed that mRNA expression of CD68, TNF α , and

MCP-1 were also decreased in the cortex of EPRAP-KO mice (Figure 2C). Thus, unlike the anti-inflammatory effects of EPRAP in macrophages, these results suggested that EPRAP may, in fact, promote inflammation in the brain.

EPRAP Deficiency Decreases Neuronal Cell Death and Microglial Activation in the Brain after Kainic Acid Injection

Previous studies indicated that glutamate excitotoxicity can induce microglial activation, thereby augmenting brain damage in neurodegenerative disorders.^{7,12,13} To clarify the role of EPRAP in inflammation induced by an endogenous substance, we used intracerebroventricular injection of kainic acid, which induces neuronal cell death by stimulation of the glutamate receptor.¹² Compared with WT mice, EPRAP-KO mice showed less microglial activation in the hippocampus after kainic acid administration (Figure 2D).

Accordingly, EPRAP-KO mice kept more live neuronal cells than WT mice 3 days after kainic acid injection (Figure 2, E and F). Further, we confirmed that no significant difference was found in live neuronal cells between WT and EPRAP-KO mice before kainic acid injection (Supplemental Figure S1A); EPRAP-KO mice kept live neuronal cells 5 days after kainic acid injection (Supplemental Figure S1B). Thus, EPRAP deficiency may decrease neuronal cell death by suppressing microglial activation.

EPRAP Deficiency Decreases Proinflammatory Cytokine Expression in Microglia after LPS Stimulation

Our *in vivo* studies suggested that EPRAP is involved in microglial activation. Hence, we isolated primary microglia from WT and EPRAP-KO mice to elucidate the role of EPRAP in microglial activation *in vitro*. Although EPRAP in macrophages attenuated inflammatory activation of cells,⁹ EPRAP deficiency markedly decreased production of TNF α and MCP-1 in primary microglia after administration of LPS (Figure 3), suggesting that EPRAP might contribute to proinflammatory signaling in microglia.

EPRAP Deficiency Reduces Phosphorylation of MKK4 after LPS Stimulation

It was reported that MAPK pathways are involved in the regulation of proinflammatory cytokines in microglia.^{14,15} Thus, we evaluated activation of the MAPK pathways in microglia after LPS stimulation. EPRAP deficiency did not affect the level of phosphorylation of p44/42 MAPK, but it decreased the phosphorylation of p38 MAPK and JNK in microglia (Figure 4A). Consistent with this finding, activation of MKK4, a key upstream activator of p38 and JNKs,¹⁶ was significantly suppressed in EPRAP-KO microglia after LPS stimulation (Figure 4, A and B). When cells were

Figure 2 EPRAP deficiency decreases microglial activation. **A:** The upper panels show Iba-1⁺ microglial cells in the cerebral cortex of WT and EPRAP-KO mice before LPS treatment (LPS⁻). The lower panels show that EPRAP deficiency reduces Iba1⁺ microglia in the cerebral cortex 6 hours after 100 mg/kg intraperitoneal LPS administration (LPS⁺). **B:** The expression levels of Iba-1 in the cortex before and after LPS administration. **C:** Expression levels of mRNA of CD68, TNF α , and MCP-1 is induced by intraperitoneal LPS administration in the cortex of WT and EPRAP-KO mice. **D:** Iba-1 staining shows that EPRAP deficiency reduces microglial activation in the hippocampus 3 days after intracerebroventricular injection of 0.2 μg of KA (KA⁺). **E:** Cresyl violet staining in the CA1 region of the hippocampus. **Inset:** Arrows indicate dying cells and arrowheads show live cells. **F:** EPRAP-KO mice harbor more live neuronal cells in the CA1 region than WT mice 3 days after KA injection. Results are expressed as means $\pm$ SEM. $n = 6$ for each group (B); $n = 4$ to 6 for each group (C); $n = 4$ for each group (D). * $P < 0.05$, ** $P < 0.01$. Scale bars: 50 μm (A, D, E, main image); 10 μm (E, insets). EPRAP, EP4 (prostaglandin E₂ type 4 receptor)-associated protein; Iba-1, Ionized calcium-binding adapter molecule 1; IR, immunoreactivity; KA, kainic acid; KO, knockout; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; WT, wild-type.

Figure 3 EPRAP deficiency decreases proinflammatory cytokine expression in primary microglia. Production of TNF α and MCP-1 in primary microglia from WT and EPRAP-KO mice. The cytokine levels in cell-conditioned media were measured after 24-hour incubation with 10 ng/mL LPS. Results are expressed as means $\pm$ SEM. $n = 3$ for each group. ** $P < 0.01$. EPRAP, EP4 (prostaglandin E₂ type 4 receptor)-associated protein; KO, knockout; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; WT, wild-type.

pretreated with a selective JNK inhibitor, SP600125, productions of TNF α and MCP-1 showed no significant difference between WT and EPRAP-KO microglia (Figure 4C). MLK1 is an upstream molecule of MKK4.¹⁷ Notably, selective MLK1 inhibitor, HG10-102-01,¹⁸ also abrogated the protective effect of EPRAP deficiency in LPS-induced microglial activation (Figure 4C). Taken together, this suggested that EPRAP may play a key role in MKK4-related signaling in microglia.

Discussion

Microglial activation is closely linked to the pathophysiology and disease progression in numerous neurodegenerative disorders.¹ A more detailed understanding of the mechanisms of microglial activation is important for the development of new therapies for these diseases.

Here, we first showed that EPRAP exists in microglia in the brain. Next, with the use of models of brain inflammation and primary microglia, we explored the role of EPRAP in microglia as a novel molecular regulator of microglial activation. EPRAP deficiency suppressed brain inflammation in both classic immune and endogenous substance-induced inflammatory responses in microglia. Moreover, our results indicate that EPRAP deficiency decreases the level of phosphorylation of JNK and p38 MAPK by suppressing an up-stream signaling molecule, MKK4, in microglia. JNKs in microglia are reported to be involved in the release of TNF α and MCP-1.¹⁴ It was also reported that p38 MAPK plays an important role in microglial activation by TNF α signaling in microglia.¹⁹ JNKs and p38 are activated by kinases such as MKK4 in response to stress, such as cytokines and LPS.^{20,21} Previous studies that used MKK4-KO mice suggested that MKK4-mediated signaling plays a key role in

promoting inflammation in several types of cells.¹⁶ MKK4 mRNA is expressed widely in mouse and human tissues, but it is most abundant in the brain.^{22,23} It was reported that the MKK4-JNK signaling pathway in microglia regulates TNF α production by glycogen synthase kinase-3 β .²⁴

The protein kinases that directly regulate MKK4 *in vivo* remain unknown. However, several MAPK kinase kinases (MKKKs) activate MKK4 in *in vitro* experiments.²⁵ In addition, some interacting proteins regulate the MKKKs-MKK4 pathway. For example, AKRL1 and AKRL2 (Akr1p-like 1 and 2), two nonkinase proteins, were shown to activate MKK4 and to interfere at the MKKK level through a yet-unclear mechanism.²⁶ Both AKRL1 and AKRL2 possess ankyrin repeats that function in protein-protein interactions. Notably, EPRAP contains multiple ankyrin repeats through which direct protein-protein interactions occur.⁹ Thus, it is possible that EPRAP might interact directly with kinases that activate MKK4 and thereby regulate inflammatory activation of microglial cells in the brain.

Mkk4-floxed mice crossed with nestin-Cre mice lack MKK4 in the nervous system and display severe growth retardation manifested in reduced body weight, and generally die by 21 days postpartum.²⁷ These mice show abnormal alignment of Purkinje cells in the cerebellum and delayed radial migration in the cortex. However, EPRAP-KO mice displayed normal growth in stature and weight,⁶ and their brains appeared normal. These facts suggest that, although EPRAP performs a role in microglial activation in the brain, EPRAP may not be involved in MKK4-mediated signaling associated with neuronal development.

In contrast to the proinflammatory effects of EPRAP in microglia, previous studies proved that EPRAP exerts anti-inflammatory effects in macrophages by suppressing the MEK-ERK pathway by PGE₂-EP4 signaling.^{4,6,9} It was reported that PGE₂ exerts diverse effects on inflammation, immune surveillance, and homeostasis.²⁸ Studies have shown that PGE₂-EP4 signaling has an anti-inflammatory role in microglia and in macrophages.^{29,30} We found that an EP4-specific agonist decreased LPS-induced proinflammatory cytokine expression, not only in WT microglia but also in EPRAP-KO microglia (data not shown). This evidence suggests that the proinflammatory effects of EPRAP in microglia are independent from PGE₂-EP4 signaling. Thus, the differences observed in the role of EPRAP must originate from intrinsic differences between microglia and macrophages. Microglia share many features with other myeloid cells; however, studies indicate that microglia show particular differences in protein expression compared with their myeloid cousins, such as macrophages.^{31,32} In fact, transcriptional expression of tumor progression locus 2, a key player involved in mediating the anti-inflammatory effects of EPRAP in macrophages,⁹ is lower in microglia than in macrophages

Figure 4 EPRAP deficiency decreases the activation of MKK4, JNK, and p38 MAPK in microglia after LPS stimulation. **A:** The phosphorylation levels of MKK4, JNK, and p38 MAPK 1 hour after 10 ng/mL LPS stimulation in primary microglial cells isolated from WT and EPRAP-KO mice. **B:** Densitometric quantification of phospho-MKK4 in WT and EPRAP-KO microglia. Integrated band densities were normalized to those of MKK4. **C:** The effects of MKK4-related signaling inhibitors on the production of TNF α and MCP-1. Primary microglial cells were pretreated with 10 μ mol/L JNK inhibitor SP600125 or 10 μ mol/L MLK1 inhibitor HG-10-102-01 for 30 minutes and then incubated with 10 ng/mL LPS for 24 hours. Results are expressed as means $\pm$ SEM. $n = 3$ for each group (**B**); $n = 4$ to 6 for each group (**C**). * $P < 0.05$, ** $P < 0.01$. EPRAP, EP4 (prostaglandin E_2 type 4 receptor)-associated protein; JNK, c-jun N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MKK4, mitogen-activated protein kinase kinase 4; MLK1, mixed-lineage kinase 1; LPS, lipopolysaccharide; P-, phosphorylated; TNF, tumor necrosis factor; WT, wild-type.

(data not shown). Furthermore, evidence has demonstrated differential responsiveness of microglia and macrophages to the same stimuli. For example, stimulation of sphingosine-1-phosphate receptor 1 induced distinct intracellular signaling responses in microglia and macrophages, despite the apparent similarity in receptor expression.³³ EPRAP and proteins such as these that play opposing roles in microglia and macrophages may characterize the phenotypic differences between these cell types.

Conclusions

EPRAP promotes inflammation by regulation of MKK4-related pathways in microglia. EPRAP is, thus, thought to play a key role in microglial activation during the progression of neurodegenerative disorders. However, further investigation of EPRAP will be required to reveal the mechanism of its role in MKK4-mediated signaling pathways in microglia. Finally, EPRAP may be a key

factor in determining the phenotypic and functional differences between microglia and macrophages. We hope that the results of this study find application in furthering our current understanding of brain inflammation and, ultimately, in the treatment of neurodegenerative disorders.

Acknowledgments

We thank Ono Pharmaceutical for supplying ONO-AE1-329, Erina Tajima and Yoshiko Fujiwara for their skilled technical assistance, and Yuki Fujiyoshi-Mori and Yuta Yamashita for their cooperation in isolating primary microglia.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.04.002>.

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